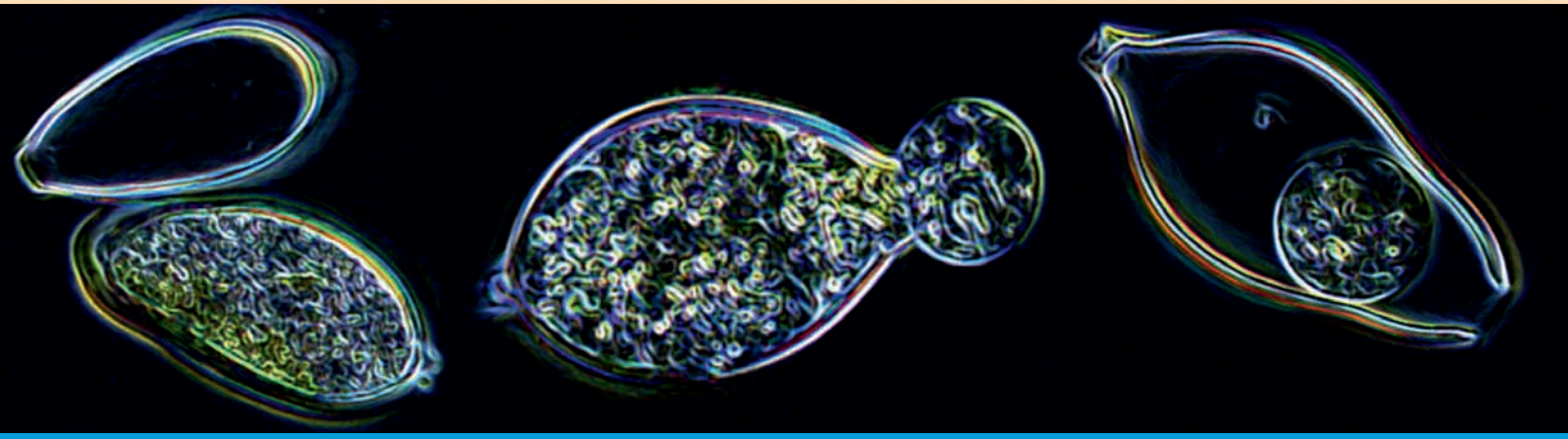
DPG | Spectrum Phytomedizin

H.B. Deising, B. Fraaije, A. Mehl,
E.C. Oerke, H. Sierotzki, G. Stammler

Modern Fungicides and Antifungal Compounds IX

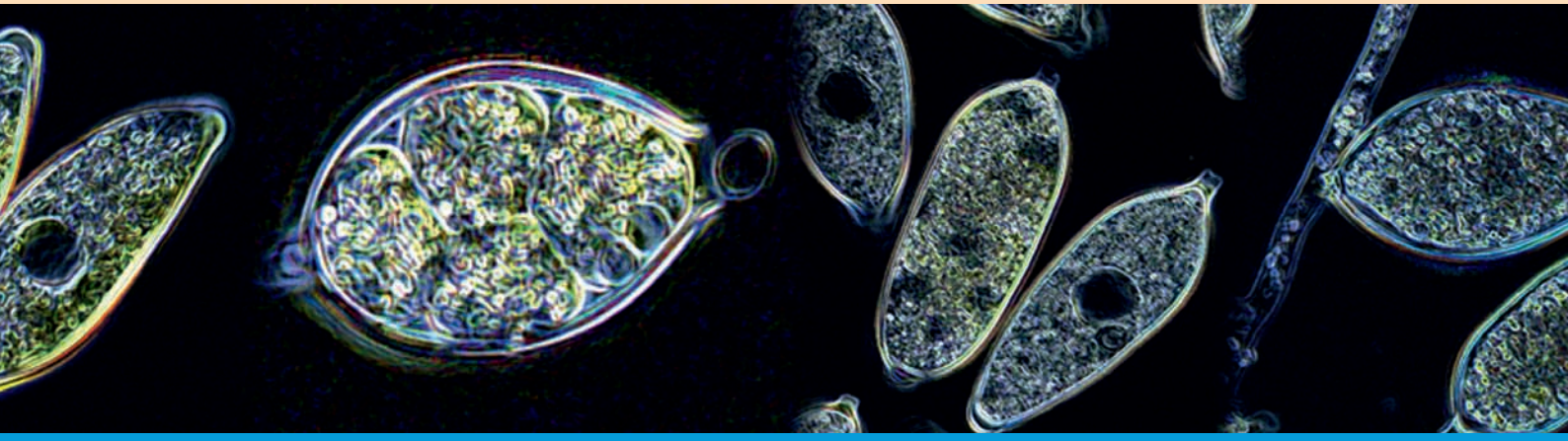


Proceedings of the 19th International Reinhardsbrunn Symposium
April 7 – 11, 2019 Friedrichroda, Germany

DPG | Spectrum Phytomedizin

Proceedings of the 19th International Reinhardsbrunn Symposium on Modern Fungicides and Antifungal Compounds 2019

The tri-annual Reinhardsbrunn Symposia have a longstanding tradition and are the most important international meetings focusing on fungicide science today. Participants from twenty-four different countries around the globe presented more than one hundred outstanding contributions, covering topics like different modes of fungicide resistance, resistance monitoring and management in different areas around the world, new applications and technologies, biorational fungicides and biocontrol, and regulatory aspects. Highlighting these exciting scientific topics, the outstanding contributions of all presenters at the symposium demonstrated the excellence not only of experienced but also of young scientists in an increasingly important field of plant protection.



ISBN: 978-3-941261-16-7 urn:nbn:de:0294-sp-2020-reinh-8

DPG Spectrum Phytomedizin

Deising H.B.; Fraaije B.; Mehl A.; Oerke E.C.; Sierotzki H.; Stammler G.

Modern Fungicides and Antifungal Compounds IX

Proceedings of the 19th International Reinhardsbrunn Symposium

April 07-11, 2019, Friedrichroda, Germany

Publisher

Bibliografische Information der Deutschen Bibliothek

Die Deutsche Bibliothek verzeichnet diese Publikation in der Deutschen Nationalbibliografie Detaillierte bibliografische Daten sind im Internet über http://dnb.ddb.de abrufbar.

ISBN: 978-3-941261-16-7 Persistent Identifier: urn:nbn:de:0294-sp-2020-reinh-8

Das Werk einschließlich aller Teile ist urheberrechtlich geschützt.

Jede kommerzielle Verwertung außerhalb der engen Grenzen des Urheberrechtsgesetzes ist ohne Zustimmung der Deutschen Phytomedizinischen Gesellschaft e.V. unzulässig und strafbar. Das gilt insbesondere für Vervielfältigungen, Übersetzungen, Mikroverfilmungen und die Einspeicherung und Verarbeitung in elektronischen Systemen. Die DPG gestattet die Vervielfältigung zum Zwecke der Ausbildung an Schulen und Universitäten.

All rights reserved. No part of this publication may be reproduced for commercial purpose, stored in a retrieval system, or transmitted, in any form or by any means, electronic, mechanical, photocopying, recording or otherwise, without the prior permission of the copyright owner. DPG allows the reproduction for education purpose at schools and universities.

© 2020 DPG Verlag

Messeweg 11-12, 38104 Braunschweig

Email: geschaeftsstelle@phytomedizin.org

Internet: www.phytomedizin.org

Lectorate: Christian Carstensen, Edenkoben

Design and production (cover): Corinna Senftleben, Braunschweig

Fotos (cover): *Phytophthora infestan*s: sporangia and zoospore release, Ulrike Steiner, Bonn

Printed in Germany by Lebenshilfe Braunschweig gemeinnützige GmbH

The Reinhardsbrunn Symposium has been made
possible by the financial support of the
German Science Foundation (DFG).

Deutsche Forschungsgemeinschaft

Project No. DE 403/23-1

In addition the proceedings volume
of this symposium is financially supported by
the Fungicide Resistance Action Committee (FRAC).

FRAC
FUNGICIDE RESISTANCE ACTION COMMITTEE

We thank both organizations for their generous support.

CONTENTS

Participants of the 19th International Reinhardsbrunn Symposium on Modern Fungicides and Antifungal Compounds, April 07-11, 2019, Friedrichroda, Germany

Preface

The Reinhardsbrunn Symposium, as initiated in 1966 by Prof. Dr. Horst Lyr, Kleinmachnow, GDR, has a tradition standing for more than 50 years. We are proud to say that, on the world-wide scale, the Reinhardsbrunn Symposium is the most important international meeting with focus on fungicide science today.

The 19th Reinhardsbrunn Symposium was held at the H+Hotel close to Friedrichroda in Thuringia, Germany, from April 07 – 11, 2019. The increasing importance of the Reinhardsbrunn Symposium becomes evident by comparing the number of attending scientists in 2019 with that of the attendees in 2016. While the 18th Reinhardsbrunn Symposium in 2016 hosted 132 scientists, more than 170 scientists from 24 countries participated in the 2019 meeting, including Australia, Belgium, Brazil, Canada, China, Czech Republic, Denmark, France, Germany, Greece, Ireland, Italy, Japan, Lithuania, Poland, Quatar, Russia, Serbia, Singapore, Spain, Sweden, Switzerland, United Kingdom, and the USA. The long list of nations participating can be taken as a clear indication of the attractiveness of this international scientific meeting focusing on fungicide research.

In total, over the five days of the meeting, 57 oral presentations were given, with six key-note talks. In addition, 39 posters have been exhibited.

The six key-note lectures gave an excellent overview on the latest developments in the field of fungicide research. Susan Knight of Syngenta Asia Pacific, Singapore, gave an excellent overview on how fungicide resistance management is performed in Asia, with a focus on small farms and options to improve understanding of the rational of fungicide use. This talk paved the way for subsequent talks focusing on different monitoring techniques and fungicide sensitivities in fungal populations. On the world-wide scale, one of the major threats of today is the soybean rust fungus. The second key-note lecture, given by Claudia Godoy, Empresa Brasileira de Pesquisa Agropecuária – Embrapa, Brazil, discussed ways of controlling this drastic soybean disease. The third key-note lecture, as given by Ewa Matyjaszczyk of the Institute of Plant Protection of the National Research Institute of Poland, addressed legislative aspects of fungicide applications, and discussed advantages and disadvantages of seed dressing versus foliar fungicide applications. This lecture particularly attracted fungicide scientists with interests in applied plant protection. The vast majority of plant pathogenic fungi are aerially dispersed. Thus, monitoring airborne plant pathogens is of prime interest, as it allows predicting disease severities. This aspect was extensively covered by Jon West of Rothamsted Research, UK, in key-note lecture 4. In key-note 5, Holger B. Deising addressed the question whether or not consumers' risks may be associated with biological disease control employing antagonistic micro-organisms. This issue is of particular relevance, as in antagonistic interactions several silent secondary metabolism gene clusters are activated, giving rise to putatively toxic or even carcinogenic compounds. As in the previous meetings, the session focusing on new technologies and applications gained significant interest. Uwe Conrath gave a key-note lecture on

bifunctional fusion peptides and microgel-based release of antifungal compounds and showed how novel techniques developed in basic science may lead to the establishment of commercial plant protection products.

The oral presentations of regular participants were organized into 11 sessions including topics such as fungicide resistance monitoring and management, molecular mechanisms and diagnosis of fungicide resistance, regulatory aspects, resistance modeling, digital pathogen sensing, and several others.

The excursion of the Reinhardsbrunn meeting introduced the participants to the Adventure Potash Mines at the town of Merkers-Kieselbach and the history of potash mining in Thuringia. The fascinating tour at several hundred meters below the surface included highlights such as visiting the stunning crystal grotto, the historical gold room, the "world's deepest concert hall", and a unique underground bucket-wheel excavator. I am absolutely convinced that most if not all scientists experienced both the scientific program and the underground visit of the Potash Mines as highlights of the Reinhardsbrunn Symposium.

A conference can only be as successful as the participants and the organizing committee make it. Here, I would like to highlight the outstanding organizing skills of Christian Carstensen, (DPG; Deutsche Phytomedizinische Gesellschaft; German Society of Plant Diseases and Plant Health). Christian's input and help made this conference an outstanding success, which I would like to acknowledge very much. In addition, I thank the program committee: Bart Fraaije, Andreas Mehl, Helge Sierotzki, Gerd Stammler and Erich Oerke. I am proud and happy that I was allowed to chair this committee. Last but not least I would like to acknowledge the financial support provided by the German Research Foundation (DFG), which allowed the 19th Reinhardsbrunn Symposium to take place at Friedrichroda in Thuringia, Germany, in 2019, and by the Fungicide Resistance Action Committee (FRAC), which helped to finance this proceedings volume generously.

For the organizing committee

Halle (Saale), March 20, 2020

FUNGICIDE RESISTANCE MONITORING

Knight SC, Stammler G, Torriani SFF, Review of Fungicide Resistance Management in Asia. In: Deising HB; Fraaije B; Mehl A; Oerke EC; Sierotzki H; Stammler G (Eds), "Modern Fungicides and Antifungal Compounds", Vol. IX, pp. 3-12. © 2020. Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-16-7

Review of Fungicide Resistance Management in Asia

Knight SC[1], Stammler G[2], Torriani SFF[3]

[1]Syngenta Asia Pacific Pte. Ltd.; No. 1 Harbour Front Avenue, #03-03 Keppel Bay Tower; 098632; Singapore

[2]BASF SE, Agricultural Research Station, 67117 Limburgerhof, Germany,

[3]Syngenta Crop Protection AG, Syngenta Research Center Stein, 4332 Stein; Switzerland

Corresponding Author Email: susan.knight@syngenta.com

ABSTRACT

In Asia, resistance to crop protection products is increasingly recognized as a threat to agricultural productivity. There is greater awareness of the need to assess and mitigate the risk of fungicide resistance. The tropical or subtropical climate favours multiple cropping in many Asian countries, and continuous cropping with little crop rotation is commonplace. The risk of fungicide resistance is particularly high in fruit and vegetable crops, since they receive numerous sprays in a single season.

Rice is the most important staple crop in Asia, grown on over 160 million hectares. Rice is vulnerable to attack by numerous plant pathogens, and disease pressure may be intense, particularly in high yielding areas. Globally, rice sheath blight and rice blast have the greatest impact on rice production. However, other locally important rice foliar and grain pathogens cause significant loss of yield or quality. Under high disease pressure, farmers make three or more fungicide applications, and with two or three successive crops in one year, there is clearly a risk that resistance will evolve to fungicides, especially in the absence of an appropriate anti-resistance strategy. The broad spectrum of rice pathogens creates a challenge for resistance management, because disease control programmes need to address the resistance risk for all prevalent pathogens. Sensitivity monitoring studies have been conducted for rice fungicides in several Asian countries, and the results highlight the need to strengthen resistance management efforts in this crop.

Resistance to fungicides cannot be managed effectively by a single company, and requires the cooperation of all fungicide manufacturers, as well as the distribution channel, governments, academics and other stakeholders. Before 2018, in most Asian countries, there were no active Fungicide Resistance Action Committees (FRAC), nor any other cross-industry group, to tackle fungicide resistance management (with exception of China and Japan). To address this gap, the Asia-FRAC was formed in 2017, supported by CropLife Asia. Its role is to drive better resistance management throughout Asia, principally by supporting the establishment of national FRACs and through driving educational and communication initiatives.

The challenge of managing resistance in Asia is formidable. In many countries, the growers lack a basic understanding of resistance management. The conclusions and recommendations from laboratory and field studies need to be converted into simple and compelling guidelines, and these need to be communicated to millions of smallholder farmers. However, a strong foundation for resistance management is being built in Asia by the FRAC network. This includes a campaign to raise awareness of fungicide mode of action, and to drive the inclusion of this information on product labels. The authors are optimistic that these efforts will support farmers in adopting sustainable disease control programmes.

INTRODUCTION

The fungicide resistance risk triangle is a useful tool for evaluating risk of resistance (Kuck & Russell 2006). The risk of resistance developing to a fungicide is driven by three components. The first is the inherent pathogen risk. Pathogens are classified as high risk if they have a history of developing fungicide resistance, or if they would be expected to develop resistance rapidly, based on their biological characteristics. These are generally pathogens with short lifecycles, that have multiple generations in a single crop, and that produce spores in huge numbers. The second consideration is the risk associated with the mode of action (MoA) of the active ingredient. For example, multisite fungicides, which interfere with multiple biochemical pathways, are considered as low risk, and resistance to these active ingredients is less likely to evolve. The final consideration is the agronomic risk, which encompasses the environment in which the fungicide is used, including farmer practices for managing disease.

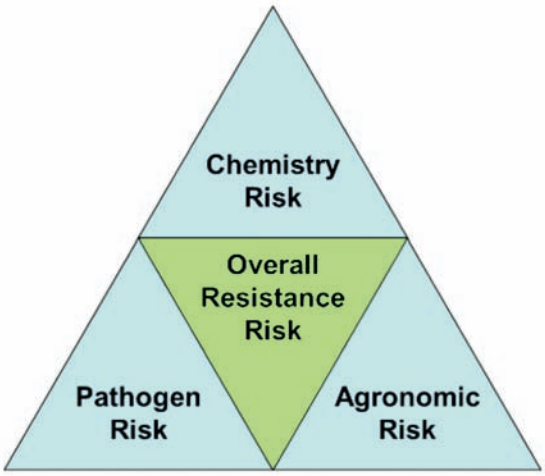


Figure 1. Fungicide resistance risk triangle

The agronomic risk is disproportionately high in many Asian countries. In tropical and subtropical climates, the warm and humid environment is conducive to pathogen development, and farmers may exacerbate this favourable environment through practices that allow the pathogen to survive continuously, such as planting the same crop in consecutive seasons, with limited crop rotation. The total sales of fungicides in the Asia-Pacific region exceeds four billion

US dollars (Anonymous 2017a). The intensity of fungicide use varies across countries and crops, but may be extremely intensive, particularly in vegetable production. Furthermore, in much of Asia, farm size is small (often one hectare or less), with an estimated half billion farmers across more than 15 countries. These smallholder farmers may be unfamiliar with resistance management principles, resulting in poor adherence to stewardship guidelines that are designed to delay the onset of resistance.

The global Fungicide Resistance Action Committee (FRAC) was established in 1982 (www.frac.info) and encompasses a network of global, regional and national working groups. However, until recently, national FRACs were not active in Asian countries, except for China and Japan (the Japan FRAC was formed in 2007 and FRAC-China was created in 2016). In 2017, the Asia-FRAC was launched, with a remit to support Asian countries in the implementation of fungicide resistance management (Knight et al. 2017).

Rice is the most important staple crop in Asia, with an area of 160 million hectares (representing over 90% of global production and consumption) (Anonymous 2018). Rice is vulnerable to attack by numerous plant pathogens, and worldwide yield loss due to fungal and bacterial pathogens is estimated at 10%. Losses would be much greater without fungicides (Oerke 2006). Rice sheath blight (*Rhizoctonia solani*) and rice blast (*Pyricularia oryzae*) are the pathogens with greatest impact on rice production globally. However, many locally important foliar and grain pathogens may cause significant loss of yield or quality. In the intensively managed crops of the irrigated lowlands, disease pressure is high, and farmers may make three or more fungicide applications. In these locations, there may be two, or sometimes three, successive crops grown in one year, underlining the need to pay attention to fungicide resistance management in Asian rice.

Rice blast is the single most damaging disease in temperate rice, particularly in Northeast China, Japan, Korea and parts of Vietnam. It has been classified by the FRAC as a high risk pathogen, because of its short lifecycle (one week under favourable conditions), and abundant production of conidia (thousands per lesion), which are dispersed by wind over large areas. Consequently, it is the main target pathogen for resistance monitoring studies in rice. Resistance to quinone outside inhibitors (QoI) in blast is caused by a point mutation at position 143 in the cytochrome *b* target site gene. The mutation is associated with a high resistance factor (RF), and QoIs would be expected to give reduced disease control if the pathogen population has a high frequency of the mutation.

This paper summarizes the current status of fungicide resistance in Asia, with focus on rice. Results from fungicide sensitivity monitoring in *P. oryzae* are given, which exemplify the need for resistance management in this region.

MATERIALS AND METHODS

Fungicide sensitivity monitoring was conducted in *P. oryzae* to detect the frequency of the target site mutation G143A. Each sample consisted of 20 pieces of leaf tissue with visible symptoms

collected from a commercial field. Samples were homogenized, DNA extracted and the G143A mutation was quantitatively analyzed by pyrosequencing as previously described (Stammler et al. 2007; www.frac.info).

Sensitivity monitoring programme in China, India, Indonesia, Philippines and Vietnam (conducted by BASF)

A total of 82 populations of *P. oryzae* were collected from 2015-2017 (34 in 2015; 23 in 2016; 25 in 2017).

Sensitivity monitoring programme in Vietnam (conducted by Syngenta)

A total of 122 populations of *P. oryzae* were collected from 2015 to 2018 in Vietnam (32 in 2015, 29 in 2016, 31 in 2017 and 30 in 2018).

RESULTS

The target site mutation G143A was not detected in any of the *P. oryzae* populations collected from China, India, Indonesia and the Philippines. However, variable frequencies of the mutation were detected in populations collected from Vietnam (Tables 1 and 2).

Table 1. Allele frequency of G143A in *P. oryzae* populations (BASF data)

Country	Number of samples in each sensitivity class (based on % G143A)					
	Total	< 2%	2-10%	11-30%	30-75%	>75%
China	8	8	0	0	0	0
India	26	26	0	0	0	0
Indonesia	15	15	0	0	0	0
Philippines	1	1	0	0	0	0
Vietnam	32	6	5	5	9	7

Table 2. Allele frequency of G143A in *P. oryzae* populations from Vietnam (Syngenta data)

Year	Number of samples in each sensitivity class (based on % G143A)					
	Total	< 2%	2-10%	11-30%	30-75%	>75%
2015	32	8	4	4	10	6
2016	29	16	0	2	9	2
2017	31	6	0	6	13	6
2018	30	4	1	6	18	1
Total	122	34	5	18	50	15

DISCUSSION

Resistance to fungicides has been confirmed in numerous pathogens across Asia. The majority of reports have been published in Northeast Asia (China, Japan, Republic of Korea), reflecting the greater focus on fungicide resistance investigation in these countries. However, there are also confirmed findings of fungicide resistance in other parts of Asia, highlighting the need for resistance management throughout the region.

In several economically important plant pathogens, resistance to a particular MoA is a worldwide problem. For example, populations of potato and tomato late blight (*Phytophthora infestans*) with resistance to the PhenylAmides (PA) fungicide class have been reported globally, impacting some countries in Asia (www.frac.info). Vegetable pathogens such as grey mould (*Botrytis cinerea*) have evolved resistance to multiple fungicides classes worldwide, including Northeast Asia, particularly in covered crops (www.frac.info). Downy mildew of grape (*Plasmopara viticola*) is notorious for evolving fungicide resistance, and resistance has been reported to QoIs and the Carboxylic Acid Amides (CAA) in parts of Maharashtra, Western India, where grape is intensively grown (Sawant et al. 2016; 2017). In Japan, apple scab (*Venturia inaequalis*) has developed resistance to the QoIs and DeMethylation Inhibitors (DMI) (Hirayama et al. 2017). In most of these pathogens, resistance to a new MoA was reported first in Europe or the Americas. This has provided Asia with an early warning of the risk that resistance may evolve in a particular pathogen.

However, some crops are grown very intensively in Asia, and fungicide resistance issues may be more serious or widespread, compared with other regions. For example, cucurbit downy mildew (*Pseudoperonospora cubensis*) has evolved resistance to PAs, QoIs and CAAs in several Asian countries (www.frac.info). Moreover, in *P. cubensis*, resistance to OxySterol Binding Protein Inhibitors (OSBPIs) was reported in 2018 in South Korea. Populations of cucurbit powdery mildews (*Sphaerotheca fuliginea*, *Podosphaera xanthii*) have been reported with resistance to DMIs, QoIs and Succinate DeHydrogenase Inhibitors (SDHI) (www.frac.info). Fusarium head blight (*Fusarium graminearum*) is a serious pathogen of wheat in Northeast Asia, and resistance has been reported to Methyl Benzimidazole Carbamates (MBC) following a national sensitivity monitoring programme for more than a decade in China (Chen & Zhou 2009). Corn is grown widely in South and Southeast Asia, and there is very little use of foliar fungicides. However, for more than two decades, there has been reliance on seed treatments with PAs to control downy mildew (*Peronosclerospora maydis*). This intense selection pressure has resulted in the evolution of resistance to PAs, which has accelerated in recent years due to the intensification of corn production, with continuous cultivation and no rotation in many areas (Danaatmadja 2019, *pers. comm.*).

In contrast to the smallholder farms that predominate in most of Asia, banana is produced in highly mechanized commercial plantations on the Philippine island of Mindanao. A cultivar with high susceptibility to black sigatoka (*Mycosphaerella fijiensis*) is grown almost exclusively, and conditions are highly favourable for disease development. Rigorous disease management is practiced, based on monitoring, sanitation and the application of synthetic and

biological fungicides (up to 50 applications per year). Over the past decades, resistance has evolved to MBCs, DMIs and QoIs (www.frac.info). Fungicide resistance represents a serious threat to the economic viability of banana production, and a FRAC Banana Working Group was created in 1987, with membership from the agrochemical industry and banana producing companies. Resistance monitoring has been conducted for 25 years in black sigatoka in the Philippines and these data have helped to shape the FRAC Banana Working Group guidelines. Sensitivity to DMIs and QoIs has stabilized in recent years (www.frac.info), despite the high intensity of fungicide programmes. This is testimony to the success of a unique global collaboration, with exchange of information and ideas among diverse organizations and across continents, which must be sustained in this monoculture.

None of the above examples of fungicide resistance in Asia are unexpected, considering the elements of the fungicide resistance risk triangle. Many of these pathogens have been classified by the FRAC as high risk (e.g. *P. viticola*, *B. cinerea*, cucurbit powdery mildews, *P. cubensis*, *V. inaequalis*, *M. fijiensis*). In other cases, the inherent pathogen resistance risk is not high (e.g. *P. infestans, F. graminearum, P. maydis)*, but there was over-reliance on one (or few) MoA with high inherent risk, exerting high selection pressure for the evolution of resistant genotypes. An important task for the Asia-FRAC network is to identify high risk situations and make recommendations to avoid the over-reliance on limited MoAs and delay the onset of resistance. Sensitivity monitoring is ongoing for a number of important fungal pathogens, but this needs to be expanded, particularly in Southeast and South Asia, to help with addressing issues and improving recommendations.

As rice is the staple crop of Asia, a valid concern is whether fungicide resistance in this crop could represent a threat to food security. In *P. oryzae*, resistance has been reported to several MoAs, including the QoIs, phosphorothiolates and dithiolanes, as well as Dehydratase-type Melanin Biosynthesis Inhibitors (MBI-D) (www.frac.info; Uesugi 1981; Yamaguchi et al. 2002).

In Japan, QoIs were launched during the late 1990s for the control of rice blast, sheath blight and other pathogens. QoI-resistant populations of *P. oryzae* were first reported in 2012 (www.jcpa.or.jp), and all cases of resistance were associated with the G143A mutation. By 2014, QoI resistant populations had been reported from 15 of 47 prefectures, which prompted prefectural governments to prohibit the use of QoIs for rice in locations with high frequencies of resistant genotypes. Sensitivity monitoring studies from 2018 suggest that the frequency of resistant populations has stabilized in those prefectures in which QoIs have been withdrawn. Results also indicate that resistance has not evolved in those locations in which farmers applied QoIs in alternation with fungicides having a different MoA (Anonymous 2017b). Sensitivity monitoring is ongoing, and the results will be used to guide recommendations for rice fungicides in Japan. Currently, there is insufficient evidence to determine whether a fitness penalty is associated with G143A in *P. oryzae*, although reduced virulence of *P. oryzae* with the G143A mutation has been observed in laboratory studies (Ma & Uddin 2009). In Japan, fungicides are commonly applied as granule treatments in seedling boxes, giving up to 90 days of disease

control. This is a boon for growers in a country with an ageing farmer population and high labour cost. However, the pathogen population is exposed to a single MoA for an extended period, exerting a high selection pressure for resistance.

Increasing adoption of QoIs in rice has prompted *P. oryzae* sensitivity monitoring studies in a number of Asian countries. Results from QoI sensitivity monitoring in 2015-2017 indicated that G143A was not present in any of the populations sampled from China, India, Indonesia and Philippines (www.frac.info). Nearly eight million hectares of rice is grown in Vietnam, and production is extremely intensive in the Mekong Delta region, with up to five crops grown in a two-year period. Sensitivity monitoring studies confirmed the presence of G143A, with wide variation in resistance frequencies among the locations tested. Farmers will need to deploy fungicides from other MoAs to manage rice blast in locations with high G143A frequencies. There is no evidence of an overall increase of QoI resistance from 2015 to 2018. However, if the pathogen population is exposed to continued selection pressure from QoIs, resistance frequency would be expected to rise.

Another rice pathogen that has evolved resistance to fungicides is *Gibberella fujikuroi*, which causes bakanae disease, in which the rice plant becomes spindly and non-productive. The pathogen is seed-borne, and can be controlled with a fungicidal seed treatment. This is extremely convenient for farmers, but seed treatments are delivered as a single application, which increases the likelihood that farmers will rely on one or few MoA. Resistance has been reported in *G. fujikuroi* to MBC and DMI fungicides in China, Japan and the Republic of Korea (Shin et al. 2008; Yang HongFu et al. 2013; Tateishi et al. 2019).

Besides blast and bakanae, there are no publications of fungicide resistance in other rice pathogens in Asia. However, numerous pathogens infect rice, and disease control programmes need to address the resistance risk for all prevalent pathogens. Sheath blight is classified as a low risk pathogen; it only has one infection cycle per crop, spreading via sclerotia produced at the end of the season, and rarely produces spores. Thus far, no fungicide-resistant populations have been reported from Asia. However, a QoI-resistant population was reported from rice sheath blight in 2011 in the southern states of USA (www.frac.info). In this area, the rice is commonly rotated with soybean, and farmers were using QoIs to control rice and soybean diseases. *R. solani* strain AG1-IA infects rice and soybean, resulting in exposure of the pathogen to QoIs throughout the year. This underlines the need for vigilance in the irrigated tropical lowlands of Asia, where two (or even three) rice crops are grown per year.

The most important factor influencing the risk of fungicide resistance in rice is the availability of diverse fungicidal MoAs, allowing the implementation of a resistance management programme. Rice blast may infect rice throughout the crop cycle, and in most Asian countries, farmers typically make two to four foliar fungicide applications to control blast. In Japan and South Korea, the practice of using water-dispersible granules, with a long duration of control, has reduced the number of fungicide applications to one or two. Under both scenarios, effective fungicides for blast are needed from at least two MoAs to enable compliance with the FRAC resistance management guidelines. There are nine MoAs that include fungicides with efficacy

against blast. However, fungicides from all of these MoAs are not registered in every country, and farmers often rely on a limited number of MoAs. In Japan, farmers predominantly use fungicides from Reductase-type Melanin Biosynthesis Inhibitors (MBI-R) and plant defence inducers. MBI-Rs have been used for 40 years, with no reports of resistance, and the plant defence inducers are associated with low resistance risk. In other Asian countries, most of the fungicides commonly used for blast belong to the DMIs, QoIs and MBI-Rs. Therefore, in most Asian countries, farmers have the tools to manage resistance to rice blast, although resistance to QoIs limits the range of effective fungicides available to farmers in the affected areas of Japan and Vietnam. We can conclude that fungicide resistance does not currently represent a grave threat to rice production in Asia. Nevertheless, the reported cases of fungicide resistance demonstrate that we cannot afford to be complacent, particularly in view of the need to feed a growing Asian population. Fungicides with novel MoAs are needed, to reduce the selection pressure for resistance to current MoAs, and to replace products that are no longer effective.

In the short term, the most urgent requirement is to support farmers with the implementation of fungicide resistance management strategies. Since 2017, the CropLife Asia & Asia-FRAC network has led workshops to facilitate dialogue with all those who provide expertise and advice to farmers (government, local industry associations, farmer associations and academia). During these workshops, national priorities have been agreed for resistance management efforts, and activities have been initiated to create and disseminate resistance management guidelines to growers. These guidelines need to be simple and practical, and must also include non-chemical practices, particularly crop rotation in locations where year-round cropping is practiced.

A serious obstacle to the adoption of resistance management guidelines is that farmers, retailers and advisors in many Asian countries are often unaware of the MoA of the fungicides that they use. To address this, CropLife International (CLI) member companies have made a commitment to include MoA codes on product labels by 2023. This labelling will provide clear information on the type of pesticide and its MoA, and will help farmers to implement fungicide resistance management programmes. However, CropLife member companies represent less than half of the fungicide market in Asia. To maximize the benefit of this initiative for farmers, all pesticide manufacturers should include MoA information on their labels. CLI is encouraging regulatory authorities to introduce mandatory MoA labelling, which has already been implemented by many Asian country governments.

Figure 2. Example of CropLife International guidelines for Mode of Action labeling

Most farmers seek farming advice from retailers or other farmers, which means that communications must include retailers, trade associations and farmer networks. The reliance on retailer advice may decrease, as farmers are increasingly using the internet and social media to seek information. Labour shortages are fuelling an increase in professional spray applicators that are contracted by farmers, and advice on resistance management will need to target this

group in future. It is vital for farmers, retailers, advisors, scientists and all stakeholders in Asian countries to acknowledge that resistance management is a shared responsibility. There needs to be greater awareness of the escalating cost and complexity of developing new fungicides, and the importance of sustaining the effectiveness of existing products.

An additional challenge in Asia is the prevalence of incorrectly labelled and illegal crop protection products. Farmers may inadvertently make an excessive number of applications from the same MoA group. Support from governments to eliminate non-compliant products, together with mandatory MoA labelling, will help Asian farmers to manage resistance to all crop protection products.

In summary, there is increasing recognition of the importance of managing fungicide resistance throughout Asia. This is a challenging task, but we are entering a new era, with strengthened cooperation among fungicide manufacturers and other stakeholders, and unprecedented opportunities to connect with farmers through digital technology.

ACKNOWLEDGEMENTS

The authors thank the members of CropLife Asia and the Asia-FRAC for their role in establishing the Asia-FRAC (Victor Alpuerto, Luis Camacho, Dietrich Hermann, JinSuk Hong, Horace Hu, Sudhakar Kandru, Susan Knight, Janghoon Lee, Srinivas Parimi, David Penna, Dorin Pop, Andrew Roberts, Makoto Shiraishi, Wenshyaun Fong, Sianghee Tan, Alisa Yu and De-you Zhou). Valuable information was also provided by Yanuar Danaatmadja, Hideo Ishii, Yuejian Lu, Yoshihide Nakajima and Rungroj Uthut.

REFERENCES

Anonymous (2017a). In: *Phillips McDougall, 2017 Market, Industry Overview*.

Anonymous (2017b). In: *National Agriculture and Food Research Organization Report*, March 2017.

Anonymous (2018). In *FAO Rice Market Monitor* April 2018, XXI(1).

Chen Y; Zhou M-G (2009). Characterization of *Fusarium graminearum* isolates resistant to both carbendazim and a new fungicide JS399-19. *Phytopathology* 99, 441-446.

Hirayama K; Hanaoka T; Araya J; Tsushima Y; Akahira T (2017). DMIs-resistant Strains of *Venturia inaequalis* Causing Apple Scab in Aomori Prefecture, Japan. *Annual Report of the Society of Plant Protection of North Japan* 2017, 108-114.

Knight SC; Hu H; Kandru S; Penna D; Pop D; Roberts A; Shiraishi M; Fong WS; Tan S; Yu A Yu (2017). Establishment of the Asia Fungicide Resistance Management Action Committee (AFRAC). In *Proceedings of the 2017 Asian Conference on Plant Pathology* (poster presentation), p131.

Kuck K-H; Russell PE (2006). FRAC: combined resistance risk assessment. *Aspects of Applied Biology* 78, 3–10.

Ma B; Uddin W (2009). Fitness and Competitive Ability of an Azoxystrobin-Resistant G143A Mutant of *Magnaporthe oryzae* from Perennial Ryegrass. *Plant Disease* 93, 1044-1049.

Oerke EC (2006). Crop losses to pests. *Journal of Agricultural Science* 144, 31–43.

Sawant SD; Ghule MR; Sawant IS (2016). First Report of QoI Resistance in *Plasmopara viticola* from Vineyards of Maharashtra, India. *Plant Disease* (Disease Note), 100 229.

Sawant SD; Ghule MR; Sawant IS (2017). Occurrence of CAA Fungicide Resistance and Detection of G1105S Mutation in *Plasmopara viticola* Isolates From Vineyards in Sangli, Maharashtra, India. *Plant Disease* (Disease Note) 101, 259.

Shin MU, Kang HJ, Lee YH, Kim HT (2008). Detection for the resistance of *Fusarium* spp. Isolated from rice seeds to prochloraz and cross-resistance to other fungicides inhibiting sterol biosynthesis. *Korean Journal of Pesticide Science.* 12, 277–282.

Stammler G; Itoh M; Hino I; Watanabe A; Kojima K, Motoyoshi M; Koch A; Haden E (2007). Efficacy of orysastrobin against blast and sheath blight in transplanted rice. *Journal of Pesticide Science* 32, 10-15.

Tateishi H; Miyake T; Suga H (2019). Polymorphism and expression level of *CYP51* (cytochrome P450) and sensitivity to ipconazole in *Fusarium fujikuroi* isolates. *Journal of Pesticide Science* 44, 25–32.

Uesugi Y (1981). Resistance to fungicides in *Pyricularia oryzae*. *Journal of Pesticide Science* 6, 239-246

Yamaguchi J; Kuchiki F; Hirayae K; So K (2002). Decreased effect of carpropamid for rice blast control in the west north area of Saga prefecture in 2001. *Japanese Journal of Phytopathology* 68, 261.

Yang HongFu; Ji MuXiang; Yao KeBing; Shu ZhaoLin; Yang, H. F.; Ji, M. X.; Yao, K. B.; Shu, Z. L. (2013). Study on resistance of *Fusarium moniliforme* to prochloraz and its management. *Acta Agriculturae Jiangxi* 25, 94-96.

Olaya G et al., Fluazinam sensitivity studies on the turf pathogen ***Clarireedia jacksonii*** (=*Sclerotinia homoeocarpa*) in the United States. In: Deising HB; Fraaije B; Mehl A; Oerke EC; Sierotzki H; Stammler G (Eds), "Modern Fungicides and Antifungal Compounds", Vol. IX, pp. 13-20. © 2020. Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-16-7

Fluazinam sensitivity studies on the turf pathogen *Clarireedia jacksonii* (= *Sclerotinia homoeocarpa*) in the United States

Olaya G[1], Linley R[1], Edlebeck K[1], Coffelt M[2], Agnew M[2].

[1]*Syngenta Crop Protection, Vero Beach Research Center, Vero Beach, Florida, USA*

[2] *Syngenta Crop Protection, Greensboro, North Carolina, USA*

Corresponding Author Email: gilberto.olaya@syngenta.com

ABSTRACT

Fluazinam (Secure®) was introduced in the United States in 2012 for the control of dollar spot disease caused by *Clarireedia jacksonii* (=*Sclerotinia homoeocarpa*). Fluazinam (FRAC 29) is a respirator inhibitor with a strong uncoupler activity of the oxidative phosphorilation, with a multi-site contact fungicide and a low resistance risk development. Since its introduction in the turf market fluazinam has been a key product for the control of dollar spot and the management of *C. jacksonii* isolates with resistance to DMI, MBC, dicarboxamides and more recently SDHI fungicides. Baseline sensitivity studies were conducted before the product market introduction and annual sensitivity monitorings have been conducted after its commercialization. The fluazinam sensitivity of *C. jacksonii* isolates have been determined by comparing the colony radial growth on ½ strength potato dextrose agar plates either amended or un-amended with fluazinam. Sensitivities (EC_{50} values) of 62 isolates never exposed to SDHI fungicides ranged from 0.001496 to 0.017051 (geometric mean: 0.005107) mg/L, whereas sensitivities of 267 isolates exposed to SDHI fungicides ranged from 0.000248 to 0.006930 (geometric mean: 0.003026) mg/L. After 6 years of commercial introduction *C. jacksonii* populations exposed remain sensitive to fluazinam.

INTRODUCTION

Dollar spot is a common and persistent disease that occurs on most turfgrass species throughout the world. Disease symptoms are characterized by small, circular, sunken patches that could coalesce into larger, irregular patches when disease is severe (Smiley et al. 2005). The fungus incites a foliar blight during periods of extended leaf wetness and temperatures of 15 to 30°C. Infection results in white to straw-colored lesions that coalesce on several tillers to produce spots 1 to 5 cm in diameter in turfgrass stands (Burpee 1997).

Dollar spot is caused by fungal pathogens in the genus *Clarireedia*. The pathogen was previously described as the ascomycete *Sclerotinia homoeocarpa* in 1937, but recent studies

have reclassified this fungal turf pathogen. The family of the fungus was determined to be in the Ruststroemiaceae instead of the Sclerotiniaceae. *Clarireedia* was described as a new genus in 2018 comprising four different species - *Clarireedia homoeocarpa, C. bennettii, C. monteithiana and C. jacksonii*. *Clarireedia homoeocarpa* seems to be limited geographically to the United Kingdom attacking mainly *Festuca rubra*, while *C. bennettii* occurs primarily on cool season grasses in the United Kingdom, The Netherlands and the United States. *C. jacksonii* and *C. monteithiana* have been reported on a variety of cool and warm season grass hosts, respectively, and seem to be globally distributed (Salgado et al. 2018).

In 2015, there were about 15,372 golf courses in the United States and about 18,639 golf course in the rest of the world (National Golf Foundation 2015).The fungicide market in the United States for golf courses could be estimated in around $212 million. The market is made by 49 brands, each selling over $1 million. The top 10 brands made up around $104 million. Fluazinam is sold as a solo active ingredient formulation or in combination with acibenzolar-S-methyl, and is a leading product in the turf market (Syngenta internal data).

Seven FRAC mode action groups are commonly used to control dollar spot in the United States. Fungicide groups, common names, resistance risk and mechanism are described on Table 1. Fluazinam is a protectant fungicide that attack fungal pathogens at multiple sites to provide effective disease control. Fluazinam inhibits fungal respiration by uncoupling mitocondrial oxidative phosphorylation. Besides dollar spot, Fluazinam is also labelled to control other diseases including anthracnose (*Colletotrichum cereale*), brown patch (*Rhizoctonia solani*) and other minor diseases.

Table 1. Fungicides commonly used in the United States to control dollar spot.

FRAC code	Fungicide group	Common name	Resistance risk	Resistance mechanism
1	MBC	Thiophanate methyl	H	F200Y (MR) E198K (HR) E198A (HR)
2	Dicarboxamides	Iprodione, Vinclozolin	M	Polymorphism in *Shos1,* Overexpression of efflux transporter *ShPDR1*
3	DMI	Propiconazole, Tebuconazole, Myclobutanil, Triadimefon, Triticonazole, Metconazole, Fenarimol	M	Induced overexpression of *CYP51* gene, ABC transporters
7	SDHI	Flutolanil, Boscalid, Fluxa-pyroxad, Penthiopyrad, Isofe-tamid, Fluopyram, Adepidyn	M to H	In the US: C-G91R, C-G150R In Japan: B-H267Y, B-181C>T silent, C-G159W
29	uncouplers of oxidative phosphorylation	Fluazinam	Low	
M5	Chloronitriles	Chlorothalonil	Low	
M3	Dithiocarbamates	Mancozeb	Low	

In this study, we report the establishment of a baseline sensitivity distribution of *C. jacksonii* isolates obtained before the commercial introduction of fluazinam and a sensitivity monitoring conducted from 2013 to 2018 with *C. jacksonii* isolates exposed to fluazinam after its commercial introduction.

MATERIALS AND METHODS

1. Isolates collection

Clarireedia jacksonii isolates are retrieved from infected *Agrostis stolonifera* and/or *Poa annua* turf samples collected every year that are part of a resistance monitoring efforts of Syngenta to provide resistance information to the golf course industry. Resistance monitoring for *C. jacksonii* isolates are conducted for the following fungicides: propiconazole, thiophanate-methyl, iprodione, fluazinam, boscalid, penthiopyrad and most recently adepidyn

Baseline sensitivity: Sixty-two *C. jacksonii* isolates never exposed to fluazinam and that were collected before 2013 were used to establish the fluazinam baseline sensitivity.

Sensitivity monitoring: Fluazinam sensitivity monitoring was conducted from 2013 to 2018. A total of 267 *C. jacksonii* isolates were collected from 77 golf courses (2013: 14 golf courses, 37 isolates; 2014: samples were not evaluated for fluazinam sensitivity; 2015:13 golf courses, 48 isolates; 2016: 6 golf courses, 19 isolates; 2017: 23 golf courses, 80 isolates; 2018: 21 golf courses, 83 isolates). Pure cultures of *C. jacksonii* were obtained by hyphal tipping of active growing colonies on potato dextrose agar. Cultures were grown at 20 C in an incubators set at 20 C and with 12 hours photoperiod. All isolates were stored in 15% glycerol or colonized rye grain at -80 °C until required.

2. Sensitivity test:

Sensitivity of each *C. jacksonii* isolate was determined *in vitro* by comparing the colony radial growth on ½ strength potato dextrose agar plates amended or not with fluazinam. The following concentrations of fluazinam were used: 0, 0.0001, 0.001, 0.01, 0.1 and 1 mg ai/L (Fig. 2). The plates were incubated at room temperature (about 22 °C) and the diameter of each colony was measured after 3 days. The sensitivity tests were set in a completely randomized design with two replications for each concentration of fluazinam.

Data obtained for each isolate were transformed using an arcsine transformation. EC_{50} values were calculated by regressing the transformed radial growth data against the log of the fungicide concentration. To determine if changes in the fluazinam sensitivity were occurring, the fungicide sensitivity distributions ($logEC_{50}$ values) of the isolates never exposed or exposed to fluazinam were compared using a Student *t* test.

RESULTS

The fluazinam baseline sensitivity distribution of 62 *C. jacksonii* isolates collected never exposed to fluazinam and before 2013, ranged from 0.0011496 to 0.017051 with a mean ED_{50} value of 0.005107 mg/L and a range of 11.4X (Fig. 1).

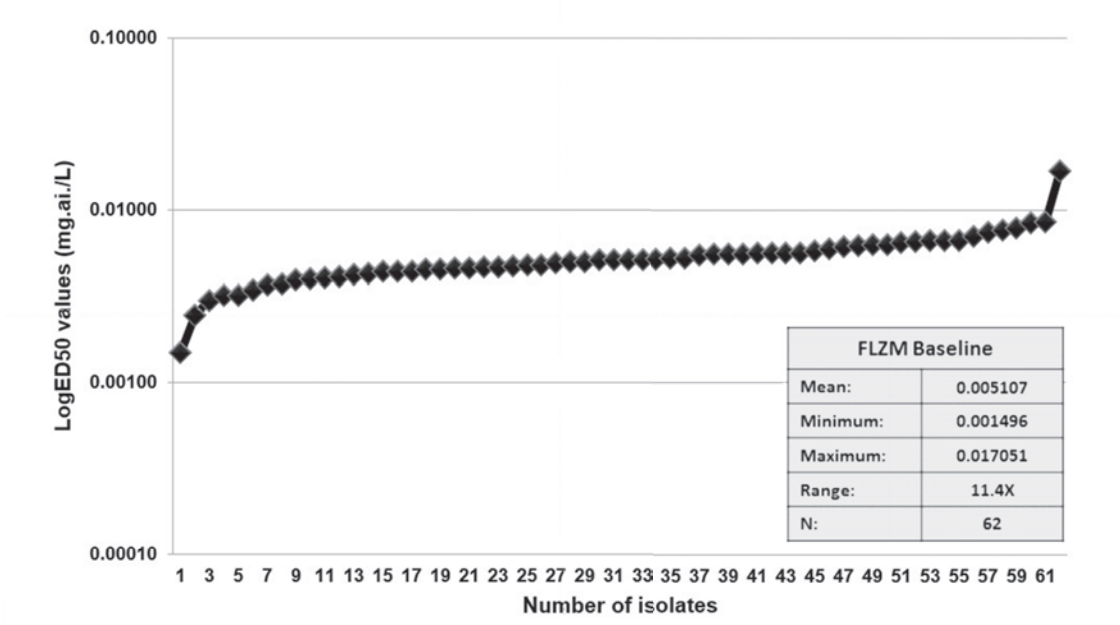


Fig 1A. Baseline isolates (fluazinam not exposed, collected before 2013)

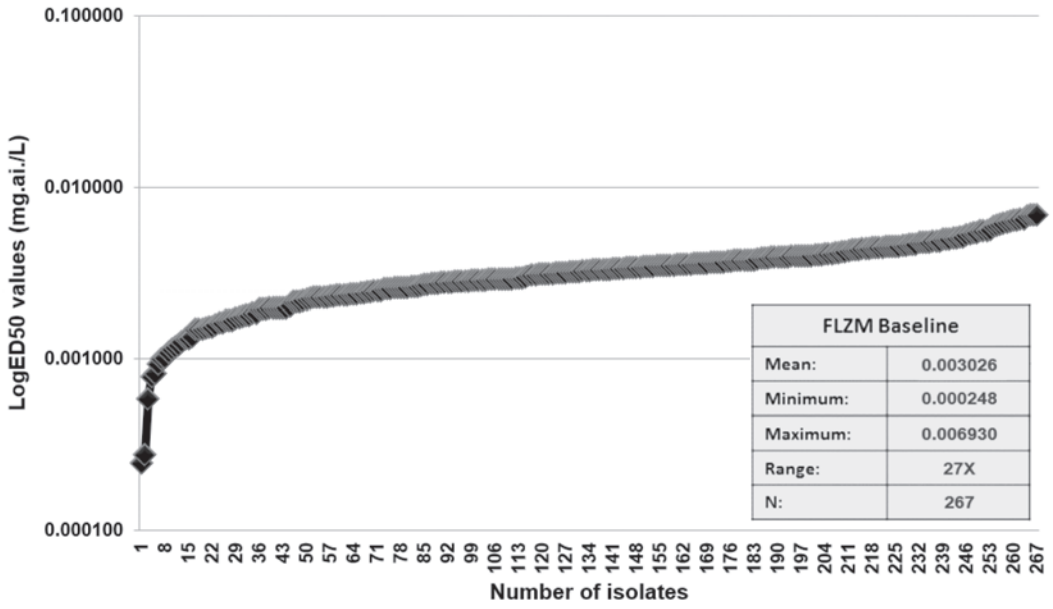


Fig 1.B. Monitoring isolates (fluazinam exposed, collected from 2013 to 2018).

Figure 1. Fluazinam EC_{50} values (the concentration that effectively reduces germination by 50% relative to the untreated control) from the *in vitro* mycelial growth inhibition sensitivity studies. Fluazinam EC values are given in milligram per liter.

The fluazinam sensitivity of the 267 *C. jacksonii* isolates exposed to fluazinam and collected from 2013 to 2018, ranged from 0.000248 to 0.006930 with a mean of 0.003026 mg/L and a range of 27X (Fig. 2).

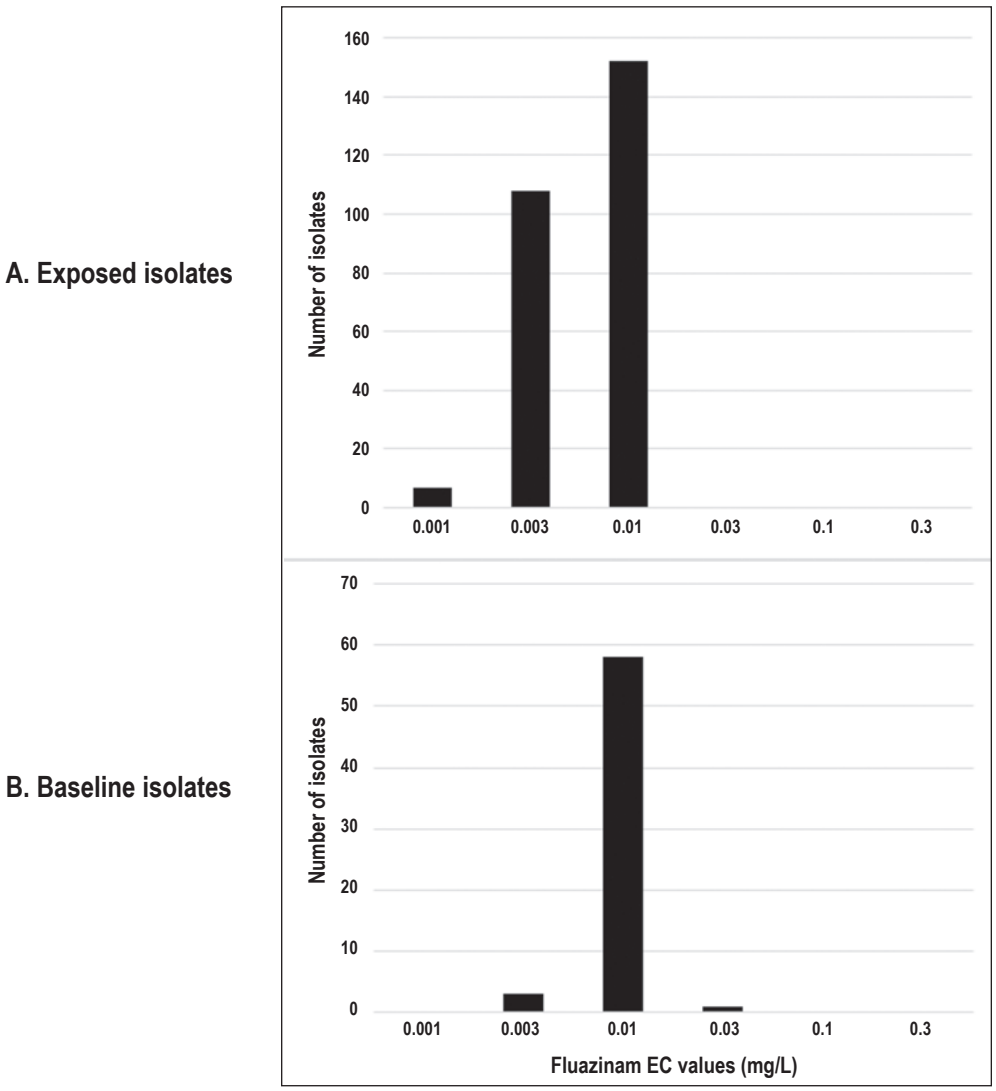


Figure 2. Frequency of fluazinam sensitivities (EC_{50} values in mg/L) in *Clarireedia jacksonii:* **A.** Monitoring isolates (fluazinam exposed, collected from 2013 to 2018); **B.** Baseline isolates (fluazinam not exposed, collected before 2013). The distribution of sensitivities of baseline and exposed isolates have different means ($P<0.001$) Being the exposed population more sensitive than the baseline.

Table 2. Sensitivity response to propiconazole, thiophanate methyl, iprodione, fluazinam, boscalid and penthiopyrad of 51 isolates of *C. jacksonni* collected in 2017.

	DMI[1] Propiconazole	MBC[2] T-methyl	Dicarbox.[3] Iprodione	Uncoupler[4] Fluazinam	SDHI[5] Boscalid	SDHI[5] Penthiopyrad
Sensitive	5	14	22	51	34	51
Reduced sensitive	33	-	22	0	8	0
Resistant	13	37	7	0	9	0
S	*<0.04*	*<10*	*<1*	-	*1-75*	*1-75*
RS	*0.04-0.2*	-	*1-2*	-	*75-100*	*75-100*
R	*>0.2*	*>10*	*>2*	-	*>100*	*>100*

[1]DMI: Demethylation Inhibitors (propiconazole)
[2]MBC: Methyl Benzimidazole Carbamates (thiophanate methyl)
[3]Dicardoxamides (iprodione)
[4]Uncoupler of oxidative phosphorilation (fluazinam)
[5]SDHI: Succinate dehydrogenase inhibitors (boscalid, penthiopyrad)

Comparing the fluazinam baseline and monitoring population sensitivities it was determined that the distribution of sensitivities of baseline non-exposed and fluazinam exposed isolates have different means ($P<0.001$). This indicates that the fluazinam exposed populations were more sensitive than the baseline (about 1.7X).

All isolates of *C. jacksonii* included in this study, showed different sensitivity patterns to propiconazole, thiophanate methyl, iprodione, boscalid and penthiopyrad. Isolates with multiple resistance were detected to one, two, three and four fungicides in different mode of action groups. Table 2, summarized the sensitivity data obtained for isolates collected in 2017.

DISCUSSION

After 6 years of commercial introduction of fluazinam, exposed *C. jacksonii* isolates remained sensitive to fluazinam. At the moment, no product failures have been reported for fluazinam. Fluazinam formulated alone or in combination with other fungicides has kept a high disease control standard. No known resistance has developed to fluazinam, making it an excellent partner for products that require the use of a protectant fungicide with a different mode of action including DMIs, MBCs, SDHIs and dicarboxamides.

Fluazinam has demonstrated excellent control of *C. jacksonii* isolates that are insensitive to several classes of fungicides. Fluazinam fungicide could be used to prevent or delay resistance development to other single-site mode of action fungicides keeping resistance frequencies to a level that allow a good dollar spot control.

In the United States, the common recommendations for rotation or tank-mixing of fungicides to control dollar spot are conducted using the respective resistance risk assessment for the fungicide and the pathogen. *C. jacksonii* is considered a medium to high risk fungal pathogen to developed resistance to fungicides. For high risk fungicides when apply alone

(benzimidazoles and SDHIs) it is recommended to rotate to a different class of fungicide after every application to tank mix with low risk fungicides for every application (fluazinam or chlorothalonil). For medium risk fungicides when apply alone (dicarboxamides and DMIs) it is recommended to rotate to a different class of fungicides after 1 or 2 applications or tank mixing with a low risk fungicide. For low risk fungicides (fluazinam and chlorothalonil), it is recommended to rotate after 3 or 4 applications, tank mixing is not necessary for resistance management, but may be necessary for optimal disease control.

The risk for resistance to fluazinam considered by FRAC to be low (FRAC, 2019). Resistance has been claimed in a few cases including a report in *Botrytis cinerea* from beans in Japan (FRAC, 2019) and in *Phytophthora infestans* from potato in The Netherlands (Schepers et al. 2018). In *B. cinerea*, two levels of sensitivity were detected, a low resistant factor (10X) and a high resistance factor (~10,000X). *In P. infestans*, under field conditions fluazinam-reduced sensitivity isolates were not effectively controlled in fungicide programs using 3 applications of fluazinam (Schepers et al. 2018). The molecular mechanism of *B. cinerea* and *P. infestans* with reduced sensitivity to fluazinam is unknown.

The major component of a fungicide program to control dollar spot of turf and to manage resistance is the alternation of fungicides with different modes of actions. Successful fungicide programs depends on the availability of proper fungicides alternatives. Cross-resistance between fluazinan and fungicides from a different mode of action group has not been documented. Multiple fungicide resistance has been documented in isolates of *C. jacksonii*. Resistance to one, two, three of four fungicide classes has been described (Sang et al. 2019). There is a high potential of losing control of the diseases if low risk fungicides are not include in the fungicide spray programs. Since 2013, the year of introduction of fluazinam in the turf market in the United States, fluazinam has become a key component for fungicide programs aiming at the control and management of resistant isolates (single or multiple resistance) developed to other fungicides (MBCs, DMIs, SDHIs or dicarboxamides).

REFERENCES

Burpee, LL (1997). Control of dollar spot of creeping bentgrass caused by an isolate of *Sclerotinia homoeocarpa* resistant to benzimidazole and demethylation-inhibitor fungicides. *Plant Dis.* 81:1259-1263.

Fungicide resistance Action Committee (FRAC). 2019. FRAC code list 2019. www.frac.info.

Salgado-Salazar C; Beirn LA; Ismaiel L; Boehm MJ; Carbone I; Putman AI; Tredway LP; Clarke BB; Crouch JA (2018). Clarireedia: A new fungal genus comprising four pathogenic species responsible for dollar spot disease of turfgrass. *Fungal Biol.* 122:761-773.

Sang H; Popko JT, Jung G (2019). Evaluation of a Sclerotinia homoeocarpa population with multiple fungicide resistance genotypes under different selection pressure. Plant Dis. 103:685-690.

Schepers HTMA; Kessel GJT; Lucca F; Forch MG; van den Bosch GBM; Topper CG; Evenhuis A. (2018). Reduced efficacy of fluazinam against *Phytophthora infestans* in the Netherlands. *Eur J Plant Pathol*. 151:947-960.

Smiley RW; Dernoeden PH; Clark PP (2005). Compedium of turfgrass diseases. American Phytopathological Society. St. Paul

Augusti GR et al., Fungicide sensitivity in South American *Zymoseptoria tritici* field populations. In: Deising HB; Fraaije B; Mehl A; Oerke EC; Sierotzki H; Stammler G (Eds), "Modern Fungicides and Antifungal Compounds", Vol. IX, pp. 21-26. © 2020. Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-16-7

Fungicide sensitivity in South American *Zymoseptoria tritici* field populations

Augusti GR, Hawkins NJ, Shaw MW, Fraaije BA

Rothamsted Research, Harpenden, Hertfordshire, AL5 2JQ, United Kingdom
Email: graugusti@yahoo.com.br

INTRODUCTION

Different classes of fungicides have been used to control *Zymoseptoria tritici*, the causal agent of Septoria tritici blotch (STB). Many mutations in *CYP51* resulting in protein alterations have been described contributing towards Demethylation Inhibitor (DMI) field resistance in *Z. tritici* (Lucas et al., 2015). The combination of different *CYP51* mutations is the result of a selection process driven by the application of different DMIs, used solo and in mixtures, over time. DMI insensitivity has also been associated with *CYP51* overexpression due to a 120 bp insert in the promoter region (Cools et al., 2012). More recently, the importance of MFS1, a Major Facilitator Superfamily transporter, was demonstrated for field strains where DMI and Succinate Dehydrogenase Inhibitor (SDHI) insensitivity was linked to overexpression due to different promoter inserts (Omrane et al., 2017). Although the status and mechanisms of fungicide resistance are well known in European populations of *Z. tritici*, this subject has not been explored with South American populations, where STB is an important wheat foliar disease in some regions. The southern cone of South America, the region that comprises Chile, Argentina and Uruguay, is one of the most critical areas in the world for development of wheat diseases (Kohli & Ackermann, 1998). As a result, fungicide use has intensified over recent decades and consequently can have selected for *Z. tritici* fungicide resistance.

MATERIAL AND METHODS

Isolates of *Z. tritici* were sampled at fungicide untreated commercial fields in Chile (Araucanía Region, n = 71), Argentina (Province of Entre Rios, n = 67) and Uruguay (Colonia Department, Ruta 50 (n = 53) and La Estanzuela (n = 42)) during the start of the 2016/17 crop season. For further comparisons isolates sampled in the UK (Rothamsted, (n = 50)) were included in the study as a representative of a modern European *Z. tritici* population. Sensitivity tests were carried out in liquid media using microtiter plates according to the methodology described by Cools et al. (2012) with fungicides from the following classes: DMIs (epoxiconazole, tebuconazole and prochloraz), SDHIs (bixafen and fluopyram) and the multisite fungicide chlorothalonil. In addition, both tolnaftate and fentin chloride acting as a fungicide and an efflux pump substrate were used to identify strains with a Multiple Drug Resistance (MDR) phenotype that is associated with enhanced efflux pump activity. Differences in fungicide sensitivity

between populations, as measured by mean EC_{50} values, were analysed using ANOVA. Isolates with a range of fungicide sensitivity levels to DMIs and SDHIs were further characterised by PCR amplification and sequencing of the corresponding target site encoding genes.

RESULTS AND DISCUSSION

Tebuconazole sensitivities (determined using EC_{50} values) were determined for all *Z. tritici* field isolates belonging to the 5 populations tested. In general, all four South American populations had mean $\log_{10}$ EC_{50} values greater than six DMI sensitive CYP51 wild-type reference strains ($\log_{10}$ EC_{50} = -1.75) (Figure 1). Isolates from Chile had the widest spectrum of tebuconazole sensitivity compared to the other three populations from South America. Although the Chile population contained the most insensitive strains found in South America, it showed the lowest minor mean of $\log_{10}$ EC_{50} values for tebuconazole ($p < 0.001$). The two Uruguayan populations were sampled only 6 km apart, however, they had different means in tebuconazole sensitivity ($p < 0.001$). The La Estanzuela distribution of tebuconazole sensitivity was skewed for lower EC_{50} values, but only Ruta 50 showed a similar mean to Rothamsted ($p < 0.001$). Rothamsted was the most insensitive population tested, with 25% of strains showing EC_{50} values greater than 10 ppm ($\log_{10}$ $EC_{50} > 1$).

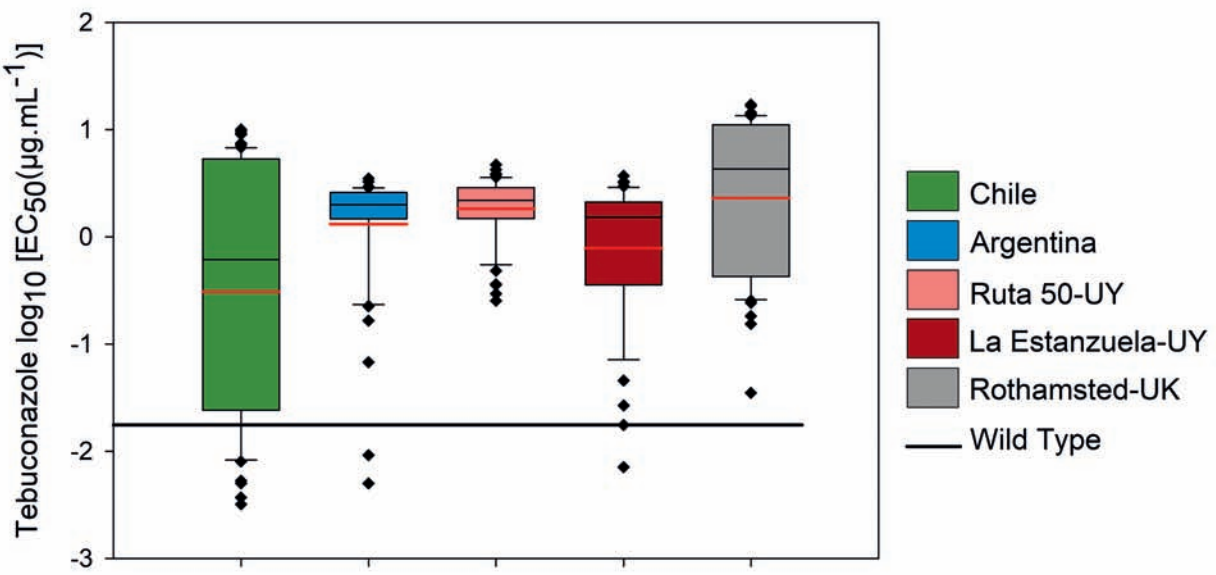


Figure 1 Sensitivity, measured as $\log_{10}$ [EC_{50} (µg mL^{-1})], to tebuconazole in *Z. tritici* field populations from Chile (Green), Argentina (Blue), Ruta 50-UY (Pink), La Estanzuela-UY (Dark Red) and Rothamsted-UK (Grey). The red bars are the mean EC_{50} values of each population and the black line represents the average EC_{50} value of six DMI sensitive CYP51 wild-type strains.

The CYP51 variant with the highest $\log_{10}$ EC_{50} value of tebuconazole found in all four South American populations was [L50S-S188N-A379G-I381V-Δ459/460-N513K]. Strains carrying this variant were further tested for sensitivity to epoxiconazole and prochloraz (Table 1). This

CYP51 variant is known for its reduction in tebuconazole and epoxiconazole sensitivity but increased sensitivity to prochloraz (Mullins et al., 2011).

Table 1 DMI sensitivities [EC_{50} (μg mL^{-1})] of CYP51 variant [L50S-S188N-A379G-I381V-Δ459/460-N513K] strains sampled in Chile, Argentina, Uruguay (Ruta 50 and La Estanzuela) and the UK (Rothamsted).

Population	n	CYP51 Promoter insert (bp)	MDR Phenotype[1]	Tebuconazole[2]	Epoxiconazole[2]	Prochloraz[2]
Chile	1	None	Sensitive	1.573	0.076	0.0002
				90	**17**	**0.08**
Chile	3	862	Sensitive	7.800±1.001	0.330±0.105	0.008±0.003
				444	**74**	**4**
Argentina	2	None	Sensitive	2.723±0.781	0.081±0.003	0.0002±0.0002
				155	**18**	**0.1**
Uruguay	2	None	Sensitive	3.230±1.503	0.091±0.067	0.001±0.0006
Ruta 50				**184**	**20**	**0.4**
Uruguay	1	None	Sensitive	3.720	0.117	0.002
La Estanzuela				**212**	**26**	**0.75**
UK	1	862	Resistant	13.40	1.090	0.087
				763	**244**	**43**

[1]Fungal growth at 10 μg mL^{-1} of tolnaftate.

[2]EC_{50}±SE values are presented together with resistance factor (RF) in bold. RFs were calculated by dividing the EC_{50} value of the CYP51 variant with the average EC_{50} value of six DMI sensitive CYP51 wild-type strains.

The presence of inserts in the CYP51 promoter region was determined for these isolates as described by Cools et al. (2012). An 862 bp insert was detected in three strains carrying the variant [L50S-S188N-A379G-I381V-Δ459/460-N513K] in Chile, whilst the same variant, when found in the other populations from South America, carried no inserts. The 120 bp insert described by Cools et al. (2012), initially only been associated with the same CYP51 haplotype [L50S-S188N-I381V-Δ459/460-N513K] but later also in a few [L50S-S188N-A379G-I381V-Δ459/460-N513K] strains (Fraaije, unpublished), was not found in South America. A noteworthy reduction in sensitivity, up to 4-fold, to the three DMI fungicides was observed in those strains from Chile with the 862 bp insert compared to the strain without an insert from this same population or from the other three South American populations (Table 1). This same CYP51 variant was found in Ireland with and without an insert of ~800 bp and the presence of

the insert was associated with a significant reduction of azole sensitivity (Dooley, 2015). In a recent study, Kildea et al. (2019) showed that this ~800 bp insert, when associated with haplotypes with Δ459/460, was in fact 862 bp long. In this same study, they demonstrated that the 862 bp insert did not confer significant levels of *CYP51* overexpression in comparison with 120 bp insert described by Cools et al. (2012). The same CYP51 variant with the 862 bp insert was also found in one strain from the Rothamsted population (Table 1). This strain showed the multiple drug resistance (MDR) phenotype by growing in the presence of 10 μg mL^{-1} of tolnaftate and showed an EC_{50} value of 0.3 μg mL^{-1} for fentin chloride. It carried the type II insert (339 bp) in the promoter region of the MFS1 encoding gene, which had previously been associated with enhanced efflux activity (Omrane et al., 2017). Increased drug efflux due to constitutively overexpression of the *MFS1* gene seems to be the main mechanism for the greater reduction in sensitivity for the three DMI fungicides, with prochloraz particularly affected (Table 1).

All DMIs have the same target site, but CYP51 alterations do not affect equally all compounds within this class. Thus, mixtures and alternations of DMIs could help slow down further shifts in sensitivity. However, the higher number and complexity of variants makes it difficult to predict the efficacy of DMIs mixtures/alternations. Therefore, it is suggested to avoid spraying DMIs as solo products and use mixtures of DMIs with other fungicides classes with high efficacy to control STB, like SDHIs and/or chlorothalonil.

Prior to the introduction of a new fungicide mode of action, the baseline sensitivity of a population is expected to be continuous and without evidence of bimodal shift (Lucas et al., 2015). This was expected to be seen in the *Z. tritici* populations from South America that have not been exposed to SDHI fungicides. The mean of $\log_{10} EC_{50}$ values for each population was similar to the reference strain IPO323 mean (bixafen $\log_{10} EC_{50}$ = -1.405, fluopyram $\log_{10} EC_{50}$ = -1.003) and the distribution of $\log_{10} EC_{50}$ for bixafen and fluopyram was unimodal across all populations from South America but not the UK population (Figure 2). Resistance to SDHI fungicides is known to be caused by non-synonymous mutations in the Sdh subunit B, C, and D encoding genes. It is noticeable that there were no shifts in bixafen sensitivity between South American populations (Figure 2). Nevertheless, two strains with the highest bixafen EC_{50} values in each population had the encoding gene of each Sdh subunit sequenced. As expected, no amino acid alterations linked to SDHI resistance were found in South America. One isolate from Rothamsted carried the mutations C-I29V and C-T79N. In contrast, the cumulative distributions for fluopyram showed few isolates with reduced sensitivity ($\log_{10} EC_{50} > 0.0$) in all populations but Chile (Figure 2). Resistance towards fluopyram and isofetamid has already been reported in *Z. tritici* European field populations as standing genetic variation (Yamashita & Fraaije 2018). A dispensable paralog of SdhC was associated with resistance to fluopyram and isofetamid in natural populations of *Z. tritici* (Steinhauer et al., 2019). A selection of less sensitive isolates with resistance factor > 10 to fluopyram was tested with isofetamid. Two strains from South America showed high levels of resistance to isofetamid, with resistance factors > 300 compared to the reference sensitive strain IPO323, even though no resistance (RF < 3) was detected for bixafen. Furthermore, these strains were not able to grow in the presence of 10 ppm of tolnaftate

neither showed reduced sensitivity to fentin chloride ($EC_{50} \leq 0.06$ µg mL^{-1}; RF ≤ 1.25), ruling out an efflux pump associated MDR phenotype. In the absence of Sdh alterations, it is likely that fluopyram insensitivity in these strains is due to the presence of the extra SdhC paralog.

The cumulative frequency distributions of log_{10} EC_{50} values of chlorothalonil were unimodal for all populations. Even though there were minor differences between populations, larger shifts in sensitivity were not observed (Figure 2).

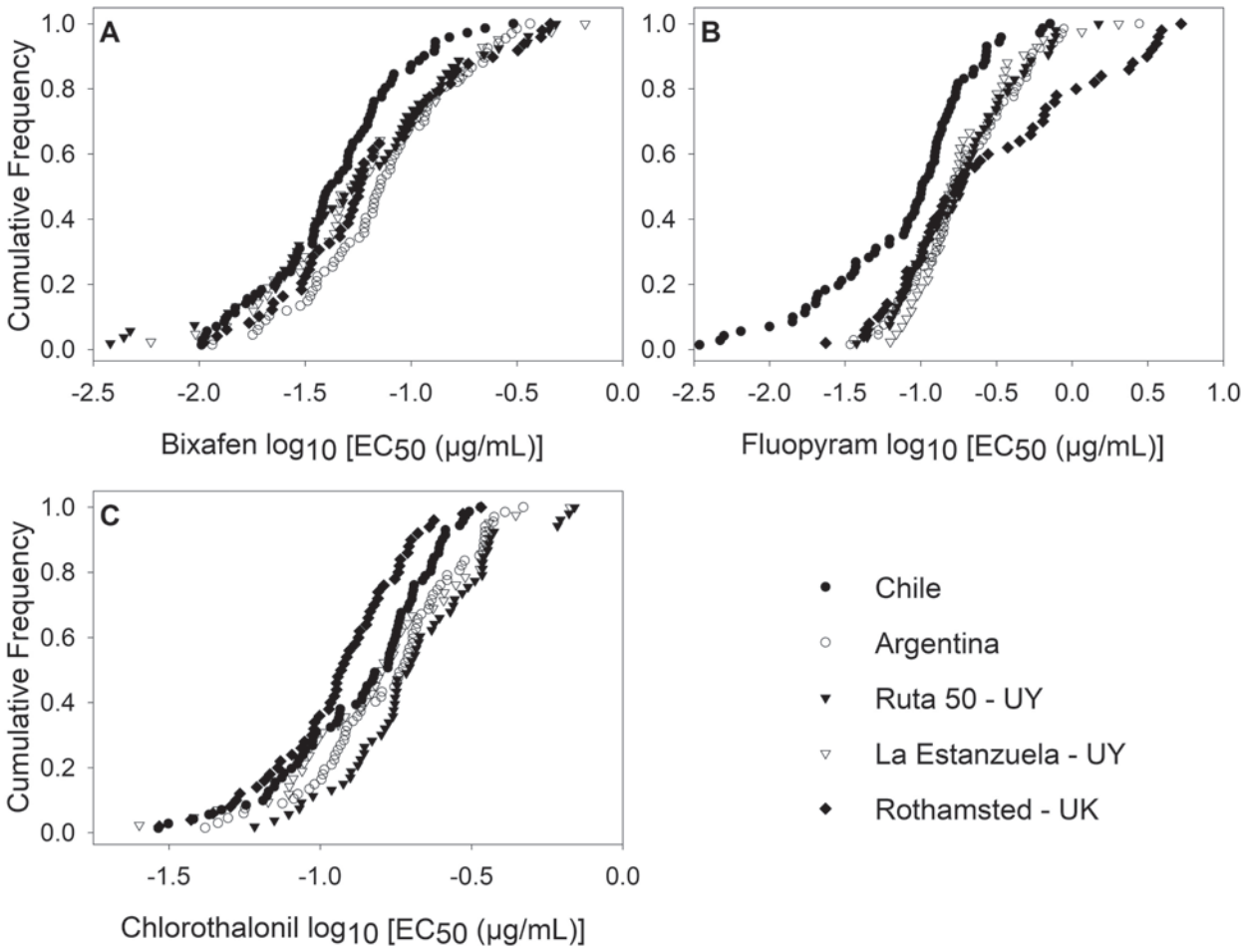


Figure 2 Cumulative frequency distribution of bixafen (A), fluopyram (B) and chlorothalonil (C) EC_{50} values of *Z. tritici* field populations from Chile (n=71), Argentina (n=67), Ruta 50-Uruguay (n=53), La Estanzuela-Uruguay (n=42) and Rothamsted-UK (50).

These results provide evidence of a significant shift towards reduced sensitivity to DMIs in *Z. tritici* field populations in South America, where fungicide field performance has already shown signs of decline (Cordo et al., 2017). Fungicide treatments are essential for maintaining healthy crops and so it is vital for wheat growers in South America to maintain the activity of current compounds, particularly the recently introduced SDHIs, and protect new molecules still in the registration process. To slow the development of fungicide resistance, learning how to best manage crops to minimise the selection pressure on fungicides is fundamental.

ACKNOWLEDGEMENTS

The PhD studies of GA was funded by the Brazilian Federal Foundation for Support and Evaluation of Graduate Education (CAPES). NH and BF are supported by the Rothamsted Research Smart Crop Protection (SCP) strategic programme (BBS/OS/CP/000001) funded through the BBSRC Industrial Strategy Challenge Fund.

REFERENCES

Cools HJ; Bayon C; Atkins S; Lucas JA; Fraaije BA (2012). Overexpression of the sterol 14α-demethylase gene (*MgCYP51*) in *Mycosphaerella graminicola* isolates confers a novel azole fungicide sensitivity phenotype. *Pest Management Science* 68(7), pp. 1034-1040.

Cordo CA; Trofino C; Stocco MC; Monaco CI; Kripelz NI; Balatti P (2017). Change in sensitivity to fungicide tebuconazole in populations of *Zymoseptoria tritici*, the causal agent of Septoria tritici blotch. In: *Cuarto Congreso International Cientifico Y Tecnologico De La Provincia De Buenos Aires*, p. 14. Buenos Aires.

Dooley H (2015). *Fungicide resistance management tactics: impacts on Zymoseptoria tritici populations*. University of Reading.

Kildea S; Marten-Heick T; Grant J; Mehenni-Ciz J; Dooley H (2019). A combination of target-site alterations, overexpression and enhanced efflux activity contribute to reduced azole sensitivity present in the Irish *Zymoseptoria tritici* population. *European Journal of Plant Pathology* 154(3), pp. 529-540.

Kohli MM; Ackermann MD de (1998). Evaluating southern cone wheat germplasm for spot blotch and tan spot. In: *Helminthosporium Blights of Wheat: Spot Blotch and Tan Spot*, pp. 230–240. CIMMYT.

Lucas JA; Hawkins NJ; Fraaije BA (2015). The Evolution of Fungicide Resistance. In: *Advances in Applied Microbiology*, 90, pp. 29–92.

Mullins JGL; Parker JE; Cools HJ; Togawa RC; Lucas JA; Fraaije BA; Kelly DE; Kelly SL (2011). Molecular modelling of the emergence of azole resistance in *Mycosphaerella graminicola*. *PLoS ONE* 6(6).

Omrane S; Audéon C; Ignace A; Duplaix C; Aouini L; Kema G; Walker A-S, Fillinger S (2017). Plasticity of the MFS1 promoter leads to multidrug resistance in the wheat pathogen *Zymoseptoria tritici*. *mSphere* 2(5).

Steinhauer D; Salat M; Frey R; Mosbach A; Luksch T; Balmer D; Hansen R; Widdison S; Logan G; Dietrich RA; Kema GHJ; Bieri S; Sierotzki H; Torriani SFF; Scalliet G (2019). A dispensable paralog of succinate dehydrogenase subunit C mediates standing resistance towards a subclass of SDHI fungicides in *Zymoseptoria tritici*. *bioRxiv*. doi: 10.1101/616904.

Yamashita M; Fraaije B (2018). Non-target site SDHI resistance is present as standing genetic variation in field populations of *Zymoseptoria tritici*. *Pest Management Science* 74(3), pp. 672–681.

Jørgensen LN et al, Efficacy of common azoles and mefentrifluconazole against septoria, brown rust and yellow rust in wheat across Europe. In: Deising HB; Fraaije B; Mehl A; Oerke EC; Sierotzki H; Stammler G (Eds), "Modern Fungicides and Antifungal Compounds", Vol. IX, pp. 27-34. © 2020. Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-16-7

Efficacy of common azoles and mefentrifluconazole against septoria, brown rust and yellow rust in wheat across Europe

Jørgensen LN[1], Matzen N[1], Havis N[2], Holdgate S[3], Clark B[3], Blake J[4], Glazek M[5], Korbas M[6], Danielewicz J[6], Maumene C[7], Rodemann B[8], Weigand S[9], Kildea S[10], Bataille C[11], Brauna-Morževska E[12], Gulbis K[12], Ban R[13], Berg, G[14]

[1]Aarhus University, Flakkebjerg, Slagelse, Denmark, [2] SRUC, Edinburgh, UK, [3]NIAB, Cambridge, UK, [4]ADAS Rosemaund, Preston Wynne, Hereford, UK, [5]Institute of Plant Protection, Sosnicowice, Poland, [6]Institute of Plant Protection – National Research Institute, Poznań, Poland, [7]Arvalis Institut du végétal, Station Expérimentale, Boigneville, France, [8]JKI, Braunschweig, Germany, [9]Institut für Pflanzenschutz, Bayerische Landesanstalt für Landwirtschaft, Freising-Weihenstephan, Germany,[10]Teagasc, Oak Park, Carlow, Ireland, [11]CRA-W, Protection des plantes et écotoxicologie, Gembloux, Belgium, [12]Latvian Plant Protection Research Centre, Riga, Latvia,[13]Plant Protection Institute, Plasmoprotect, St. István University, Gödöllö, Hungary, [14]Swedish Board of Agriculture, Alnarp, Sweden
Email: Lisen.jorgensen@agro.au.dk

ABSTRACT

Septoria tritici blotch – STB (*Zymoseptoria tritici)* is the most serious leaf disease of wheat in Northern Europe. Problems with fungicide resistance in the populations of *Z. tritici* have caused concerns for future control options. During two seasons (2017 and 2018), a common protocol was applied for testing the field performance of six azoles across Europe, including a new azole, mefentrifluconazole. In total, 26 trials were carried out in 11 countries with a focus on the control of STB. As has previously been reported, the control level of STB from currently registered azoles in different countries has been reducing but is also highly variable across Europe. This variation is linked to intensity of use and the specific azole resistance situation in the *Z. tritici* population. The new azole mefentrifluconazole performed superior compared to currently authorised azoles for control of STB.

INTROCUCTION

The primary aim of the current study was to generate updated efficacy profiles of older azoles commonly used for STB control in wheat across Europe. In addition, the study also aimed to assess the efficacy of mefentrifluconazole in comparison with the older azoles. The efficacy was investigated in areas having recognised problems with reduced performances of older azoles. The project is seen as a follow-up to a previous collaboration in the EuroWheat group – initiated

by activities in the European Network of excellence - ENDURE (Jørgensen et al. 2014) and the network for comparison of azoles (Jørgensen et al. 2018).

The changes seen in field control of STB using azoles have to some extent been shown to be influenced by specific CYP51 mutations. The patterns of decreasing field performances have been confirmed by rising EC_{50} values for several azoles (Blake et al. 2017; Huf et al. 2018). The level of resistance is found to be highly influenced by the local risk of STB, which affects the intensity and strategy for fungicide use, and the level of control (Heick et al. 2017; Jørgensen et al. 2017). Although epoxiconazole and prothioconazole showed robust field performance during initial sensitivity shift phase, the efficacy of these products has been declining in recent years (Cools & Fraaije 2013; Kildea 2016; AHDB 2018; Jørgensen et al. 2017).

MATERIAL AND METHODS

The current study was carried out over the growing seasons of 2017 and 2018 at 11 and 15 locations, respectively, across Europe including a variety of different climate zones and agricultural practices. The trials were carried out in Denmark, Scotland, England, Poland, France, Germany, Ireland, Sweden, Belgium, Latvia and Hungary by local scientific organisations. All experiments were carried out using standard procedures and a randomised plot design with a minimum plot size of 10 m^2 and 3-4 replicates. Varieties with moderate susceptibility to STB were chosen for all trials. Equipment used for fungicide applications varied from knapsack sprayers to self-propelled sprayers. Pressure of 1.8-4 bar and water volumes of 196-300 l/ha were used. Application timing was aimed at the time of flag leaf emergence at growth stage (GS) 37-39 (BBCH). In several cases, the trials were treated with cover sprays to keep early attacks of powdery mildew, yellow rust and STB under control as required.

Table 1 Treatments tested at all locations in both growing seasons. Fungicide doses (l/ha), amount of active ingredient (g/ha) and per cent of full rate (N) are stated in brackets.

Trt. No.	GS 37-39	l/ha	Active ingredient	g/ha (% N)	Year
1	Untreated	-	-		2017, 2018
2	Revysol	1.5	Mefentrifluconazole (MFA)	150 (100%)	2017, 2018
		0.75		75 (50%)	2018
3	Proline EC 250	0.8	Prothioconazole (PTH)	200 (100%)	2017, 2018
		0.4		100 (50%)	2018
4	Caramba 60	1.5	Metconazole (MCA)	90 (100%)	2017, 2018
5	Folicur EW 250	1.0	Tebuconazole (TCA)	250 (100%)	2017, 2018
6	Opus Max	1.5	Epoxiconazole (ECA)	150 (100%)	2017, 2018
7	Score 250 EC	0.5	Difenoconazole (DCA)	125 (100%)	2017, 2018
8	Revysol + Proline EC 250	0.75 + 0.4	Mefentrifluconazole (MFA) + Prothioconazole (PTH)	75 + 100 (50%+50%)	2018

Per cent leaf area with symptoms was assessed at regular intervals in accordance with EPPO guideline (1/26 (4)). Assessments carried out around 30-45 days after application (DAA), GS

73-75 were emphasised. Only one Swedish trial was not carried through to harvest due to drought and lack of disease. For every plot, grain yields were measured and adjusted to 85% dry matter. All fungicides were supplied by BASF, and applied at full or half recommended doses (Table 1). Most treatments were repeated in both years, while some treatments were altered from one year to the next. Both seasons included treatments with commonly used azoles and the new a.i. mefentrifluconazole (MFA).

RESULTS

Field effects: The disease severities and treatment effects varied widely across Europe. Nevertheless, distinct patterns of disease control were measurable across 25 trials and 10 countries. STB dominated most trials across the two years and developed sufficiently for treatment evaluation in 9-10 trials. Attacks were generally higher in 2017 compared to the 2018 season, which was extremely dry and hot. Across the two years, the older azoles on average provided 50-60% control. Overall the new a.i. MFA gave superior control at nearly all locations in both seasons, with an average control of >80%, when applied alone or in mixture with PTH (Table 2 and Fig. 1). Across the two years, preventive and curative treatments gave similar effects, measured on flag leaf and F-1 respectively (Fig. 1).

As evidence of the variable control from azoles, Poland, France and Belgium were exceptions among the group of trial locations in 2018 showing mostly high levels of control (>70%, leaf 1) from all azoles (Table 2). Similarly, one English trial carried out in 2018 in Caythorpe showed high levels of control from PTH, MCA and ECA. This pattern was not seen in the other English trial carried out that year, also located in Herefordshire at Rosemaund with relative low infestation, where all treatments except MFA and MFA+PTH gave poor control (below 40%). One trial from the West-Central Poland revealed quite different patterns of control effects. At that site, PTH, MCA and TCA along with MFA+PTH gave superior control effects assessed on leaf 2 (data not shown), whereas ECA was inferior and MFA alone merely gave moderate control. At several locations, shifts in efficacy were notable from 2017 to 2018, which was pronounced for the azoles PTH, MCA, TCA and ECA. In a few cases negative levels of control was seen, as per cent attack was higher than the disease level in untreated indicating no control effects at all.

Yellow rust attacks above 5% were seen in three trials in 2017 and two trials in 2018 (Fig. 2). All azoles gave nearly full protection against this disease, except in one Hungarian trial where TCA and ECA stood out as superior. Overall, DCA stood out with the lowest efficacy.

The extreme warm weather, which characterised the growing season of 2018, facilitated an unusually wide distribution of brown rust. Six trials had brown rust attacks above 5% in 2018, whereas only one trial had any significant brown rust attacks in 2017 (Fig. 2). A clear pattern of control effects was seen across the two seasons. MFA, MCA, TCA, ECA and MFA+PTH provided superior control, whereas efficacies of PTH and DCA were moderate and inferior, respectively.

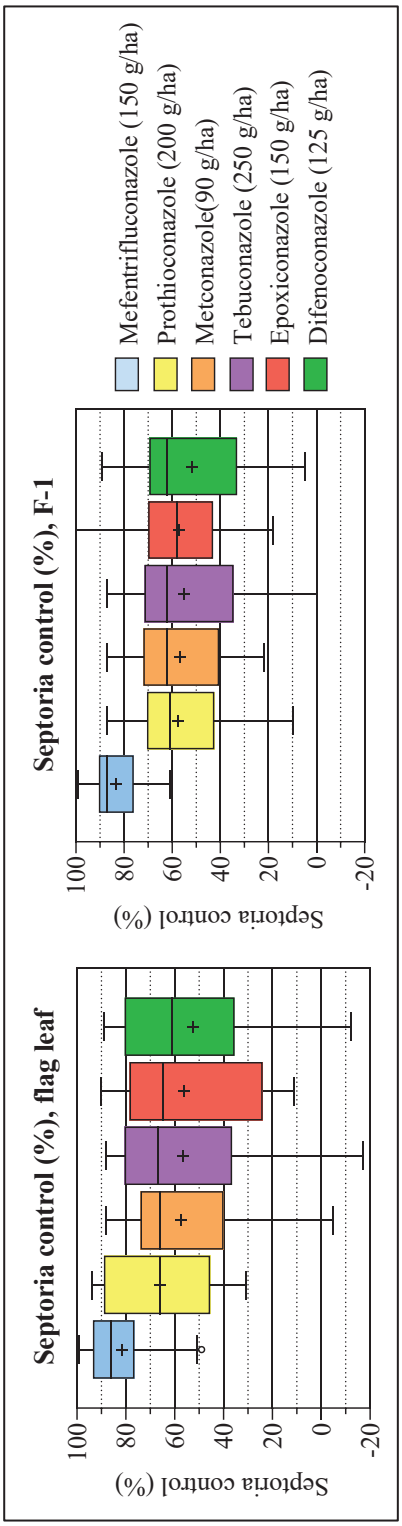


Figure 1 Control of Septoria tritici blotch (%), flag leaf (left) and F-1 (right). Eight and nine trials were included in 2017 and 2018 respectively. Boxplots are based on one average value per trial. Figures show the median (line dividing box), average (+), upper and lower quartiles (box) and maximum and minimum (whiskers).

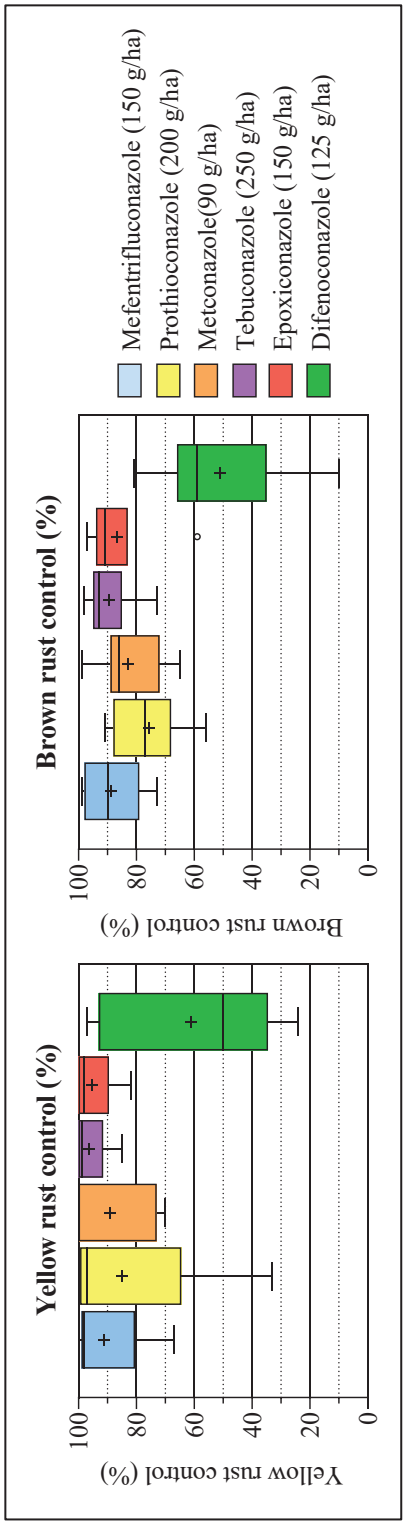


Figure 2 Yellow rust (YR) and brown rust (BR) control (%) on flag leaves. Three and two YR trials were included in 2017 and 2018, respectively. One and six BR trials in 2017 and 2018, respectively. Boxplots are based on one average value per trial. Figures show the Median (line dividing box), average (+), upper and lower quartiles (box) and maximum and minimum (whiskers).

England, Germany and Latvia were exceptions. In England, only DCA stood out with lower control effects than the other azoles. In one German trial, PTH gave good control, but MCA and ECA gave only moderate control. In Latvia, PTH gave good control, but curiously, only low control was seen from MFA+PTH.

Across all diseases and as an average across all locations MFA and MFA+PTH gave superior disease control. Only small improvements were seen from treating with MFA+PTH compared to MFA alone.

Table 2 Summary of STB control (%) assessed on flag leaves in 5 trials in 2017 and 8 trials in 2018. The treatments are regarded as preventive. The column 'Untr.' shows attacks (%) in untreated plots. Green colour highlights the best-ranked treatments in each trial. DAA, days after application; GS, (BBCH).

% Control, *Z. tritici*, Leaf 1				Untr.	MFA	MFA	PTH	PTH	MCA	TCA	ECA	DCA	MFA+PTH
				-	150	75	200	100	90	250	150	125	75 + 100
Trial	Country	GS	DAA		g/ha	g/ha	g/ha	g/ha	g/ha	g/ha	g/ha	g/ha	g/ha
17-1	Denmark	73	37	31.3	86	-	66	-	60	82	79	62	-
17-4	England	73	43	4.9	81	-	34	-	13	-17	11	38	-
17-9	Germany	85	44	6.9	49	-	35	-	-5	15	26	-12	-
17-10	Germany	83	42	59.3	79	-	70	-	66	63	68	63	-
17-11	Ireland	75	31	25.1	88	-	56	-	66	65	58	61	-
18-3	England	77	42	7.8	96	92	89	94	74	77	90	15	85
18-4	England	72	39	5.6	51	42	31	11	27	6	18	39	61
18-5	Poland	75	30	36.3	98	85	89	74	84	84	86	79	94
18-7	France	85	44	10.0	91	91	82	-	73	68	22	89	92
18-8	France	75	41	24.1	99	97	92	-	88	79	75	83	100
18-10	Germany	75	39	32.1	74	68	61	42	53	58	65	33	77
18-11	Ireland	73-77	31	3.0	86	77	59	45	73	67	57	50	81
18-13	Belgium	83	34	14.8	81	93	94	79	74	88	78	82	92
Average in 2017				23.1	77	-	52	-	40	42	49	42	-
Average in 2018				16.7	85	81	75	57	68	66	62	59	85.3

Table 3 Summary of yield responses and efficacy (%) of azoles against yellow rust (YR), brown rust (BR) and septoria (STB) on flag leaves and second leaves (F-1) from 2017-18. The column 'Untr.' shows attacks (%) in untreated plots.

			Untr.	MFA	PTH	MCA	TCA	ECA	DCA
Disease	Leaf	Trials	-	150 g/ha	200 g/ha	90 g/ha	250 g/ha	150 g/ha	125 g/ha
STB	F	13	20.1	81	66	57	57	56	52
	F-1	17	34.4	83	58	57	55	57	52
YR	F	5	9.9	91	85	89	96	95	61
BR	F	7	15.2	89	76	83	90	87	51
Yield increase (dt/ha)		17	80.6	+11.7	+ 8.8	+ 7.3	+ 9.1	+ 9.3	+ 6.6
Relative yield (%)				114.5	110.9	109.0	111.3	111.6	108.2

Yields: Significant (p=0.05) yield increases were seen in 17 out of the 25 harvested trials, whereas significant differences between yield responses were found in only six trials. The highest yield increases came from treatment with MFA in 2017 and MFA+PTH followed by

MFA alone in 2018. In STB-dominated trials, MFA and MFA+PTH gave the highest yield increases (+12.0 to 15.4 dt/ha), whereas the azoles PTH, TCA and ECA all affected yields similarly (+ 7.1 to 9.3 dt/ha). MCA and DCA fell slightly behind in these trials, increasing yields by 6.5-7.1 dt/ha. However, in yellow and brown rust dominated trials, the highest yield increases came from ECA. Regardless of disease, DCA gave the lowest yield increases.

Overall, yield increases from the different azoles corresponded to the field effects. A few locations had yield responses different from the responses expected based on the control effects. For example, PTH gave higher yield increases in 2017 than expected from the efficacy obtained. Also in one French trial at Bergerac in 2017, the yield increases were quite high in spite of low control effects. Another mismatch was seen in Poland, where control effects were generally high but yield responses small or insignificant, due to severe draught and premature ripening.

DISCUSSION

The *Z. tritici* populations of Western Europe shows significant differences in sensitivity to azoles. This is a result of differences in epedimic patterns and use history of fungicides. Intensive use of azoles has caused a gradual accumulation of several resistance mechanisms (Leroux & Walker 2011; Cools & Fraaije 2013; Omrane et al. 2015).

Overall, data from field trials carried out in 2017 and 2018 confirmed previous findings that the efficacy of older azoles against STB has been declining in recent years. Furthermore, previous findings showing that the efficacy of individual azoles varies significantly across Europe were confirmed (Jørgensen et al. 2018). DCA was new to this testing batch. This azole showed the greatest variation in STB control and overall an inferior effect compared with the other common azoles ECA, PTH, TCA and MCA, which on average gave very similar control against STB.

Despite the reduction in efficacy, the older azoles still add substantially to the control of STB having on average provided 52% to 66% control. Common for all locations was the fact that the new azole MFA gave clearly better STB control than the older azoles. MFA gave a consistently high level of control in spite of the fact that CYP51 mutations have accumulated in the European *Z. tritici* population and EC_{50} values to older azoles have increased (Huf et al. 2018). The high level of control from MFA across Europe indicates that this azole represents a different sensitivity pattern compared to PTH and ECA. The field performance of MFA does not look to be influenced by the currently dominating CYP51 mutations.

The efficacy of certain commonly used azoles such as ECA and PTH, against STB has been in decline during the past 5-10 years (Blake et al. 2017). Meanwhile, TCA, which was until recently deemed a lost cause, has regained strength in some areas like Belgium and Denmark and to a lesser extent in some parts of France, while the efficacy of MCA has increased in parts of France and Ireland, and DCA has shown good control in France and Belgium. These changes reflect the adaptations of *Z. tritici* populations, corresponding to the changes in European fungicide application patterns, and confirm previous studies showing differential correlation among azoles (Brunner et al. 2008, Leroux & Walker, 2011).

For control of yellow and brown rust, TCA and EPX provided between 87% and 97% control, and these azoles are still seen as the most effective actives for control of rust diseases.

ACKNOWLEDGEMENT

Special thanks to all the technicians, who have been involved in the trial work and to BASF A/S who has financed the project

REFERENCES

AHDB (2018) https://cereals.ahdb.org.uk/crop-management/disease-management/fungicide-performance.aspx

Blake J; Gosling P; Fraaije BA; Burnett FJ; Knight SM; Kildea S; Paveley ND (2017). Changes in field dose-response curves for DMI and QoI fungicides against *Zymoseptoria tritici*,

related to laboratory sensitivity phenotyping and genotyping assays. *Pest Management Science* 74, 302-313.

Brunner PC; Stefanato FL; McDonald BA (2008). Evolution of the CYP51 gene in *Mycosphaerella graminicola*: evidence for intragenic recombination and selective replacement. *Molecular Plant Pathology* 9, 305-316.

Cools HJ; Fraaije BA (2013). Update on mechanisms of azole resistance in Mycosphaerella graminicola and implications for future control. *Pest Management Science* 69, 150-5.

Heick TM; Justesen AF; Jørgensen LN (2017). Resistance of winter wheat pathogen *Zymoseptoria tritici* to DMI and QoI fungicides in the Nordic-Baltic region – a status. *European Journal of plant pathology* 149, 669-682.

Huf A; Rehfus A; Lorenz KH; Bryson R; Voegele RT; Stammler G (2018). Proposal for a new nomenclature for CYP51 haplotypes in Zymoseptoria tritici and analysis of their distribution in Europe. Plant Pathology 67, 1706-1712.

Jørgensen LN; Hovmøller MS; Hansen JG; Lassen P; Clark B; Bayles R; Rodemann B; Jahn M; Flath K; Goral T; Czembor J; Cheyron P; Maumene C; Pope C; Nielsen GC; Berg, G (2014). IPM strategies and their dilemmas including an introduction to www.Eurowheat.org. *Journal of Integrative Agriculture* 13, 265-281.

Jørgensen LN; Oliver R; Van den Bosch F; Paveley NP (2017). Targeting fungicides inputs according to need. *Annual Review of Phytopathology* 55, 181-203.

Jørgensen LN; Matzen N; Hansen JG; Semaskiene R; Korbas M; Danielewicz J; Glazek M; Maumene C; Rodemann B; Weigand S; Hess M; Blake J; Clark B; Kildea S; Batailles C; Ban R; Havis N; Treikale O (2018). Four azoles' profile in the control of Septoria, yellow rust and brown rust in wheat across Europe. *Crop Protection* 105, 16-27.

Kildea S (2016). Wheat disease control and resistance isssues. In. *National Tillage Conference 2016.* Oak Park, Crop Research, Carlow: Teagasc, 39.

Leroux P; Walker AS (2011). Multiple mechanisms account for resistance to sterol 14α-demethylation inhibitors in field isolates of *Mycosphaerella graminicola*. *Pest Management Science* 67, 44-59.

Omrane S; Sghyer H; Audéon C; Lanen C; Duplaix C; Walker AS; Fillinger S (2015). Fungicide efflux and the MgMFS1 transporter contribute to the multidrug resistance phenotype in *Zymoseptoria tritici* field isolates. *Environmental Microbiology* 17, 2805-2823.

Santos et al., Sensitivity of Asian Grapevine Leaf Rust Pathogen (*Phakopsora meliosmae-myrianthae*) to Pyraclostrobin in Brazil. In: Deising HB; Fraaije B; Mehl A; Oerke EC; Sierotzki H; Stammler G (Eds), "Modern Fungicides and Antifungal Compounds", Vol. IX, pp. 35-36. © 2020. Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-16-7

Sensitivity of Asian Grapevine Leaf Rust Pathogen (*Phakopsora meliosmae-myrianthae*) to Pyraclostrobin in Brazil

Santos RF, Visioli LH, Amorim L

Escola Superior de Agricultura "Luiz de Queiroz", Universidade de São Paulo, 13418-900 Piracicaba, SP, Brazil

Corresponding Author Email: ricardofeliciano@usp.br

INTRODUCTION

Asian grapevine leaf rust (AGLR), caused by *Phakopsora meliosmae-myrianthae* (syn. = *P. euvitis*), is one of the most severe fungal diseases affecting vineyards in Brazil. The disease was detected for the first time in Southern Brazil in 2001 (Tessmann et al. 2004) and subsequently spread to all grape-growing regions in the country (Primiano et al. 2017). Disease symptoms appear predominantly on mature leaves being characterised by yellow-orange pustules, which significantly reduce the photosynthetic efficiency of grapevine leaves. Early defoliation occurs when disease severity is high (Nogueira-Junior et al. 2017). In Brazil, AGLR has been controlled by single-site fungicide applications programmed at fixed intervals; however, the sensitivity of the pathogen to these fungicides has not been determined. Thus, our objective was to evaluate the sensitivity of *P. meliosmae-myrianthae* isolates to the QoI fungicide pyraclostrobin.

MATERIAL AND METHODS

In total, 58 monouredinial isolates were obtained from vineyards of seven Brazilian States (Ceará, Mato Grosso do Sul, Minas Gerais, Paraná, Pernambuco, Santa Catarina and São Paulo) in 2018 and used for pyraclostrobin sensitivity tests. The commercial formulation of pyraclostrobin (Comet, BASF) was used at the following concentrations: 0; 0.001; 0.01; 0.1; 1 and 10 µg mL^{-1}. Leaf disc of *Vitis labrusca* cv. Niagara Rosada inoculated with *P. meliosmae-myrianthae* and urediniospore germination assays were used to determine the effective concentration to inhibit disease severity or urediniospore germination by 50% (EC_{50}), respectively.

RESULTS

In our study, all isolates were sensitive to pyraclostrobin. EC_{50} values ranged from 0.006 to 0.100 µg mL^{-1} in the leaf disc assay (Fig. 1a) with a mean value of 0.040 ± 0.003 µg mL^{-1}. Similarly, the EC_{50} values for the urediniospore germination assay ranged from 0.010 to 0.084 µg mL^{-1} (Fig. 1b) and the mean EC_{50} value was 0.034 ± 0.005 µg mL^{-1}. All isolates were

completely inhibited at 10 µg mL^{-1} in both assays. Our results showed that pyraclostrobin is effective against *P. meliosmae-myrianthae* and can be used for control of AGLR. Molecular analysis is underway to characterize the *cytb* gene and to determine the presence of an intron located directly downstream of position 143.

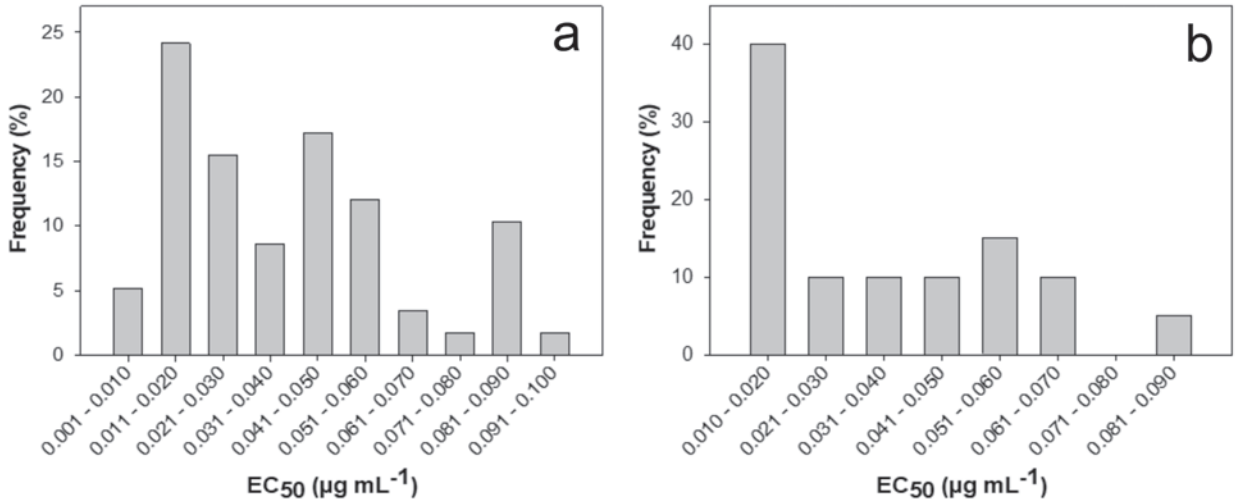


Figure 1 Sensitivity distribution of *Phakopsora meliosmae-myrianthae* isolates to pyraclostrobin based on effective concentration values needed to inhibit disease severity (**a**) and urediniospore germination (**b**) by 50% (EC_{50}).

ACKNOWLEDGEMENTS

This research was financially supported by São Paulo Research Foundation (grant numbers 2013/24003-9 and 2017/21412-6).

REFERENCES

Nogueira Júnior AF; Ribeiro RV; Appezzato-da-Glória B; Soares MKM; Rasera JB; Amorim L (2017). *Phakopsora euvitis* causes unusual damage to leaves and modifies carbohydrate metabolism in grapevine. *Frontiers in Plant Science* 8, 1675.

Primiano IV; Loehrer M; Amorim L; Schaffrath U (2017). Asian grapevine leaf rust caused by *Phakopsora euvitis*: an important disease in Brazil. *Plant Pathology* 66, 691–701.

Tessmann DJ; Dianese JC; Genta W; Vida JB; May-de Mio LL (2004). Grape rust caused by *Phakopsora euvitis*, a new disease for Brazil. *Fitopatologia Brasileira* 29, 338.

Carraro et al., Assessing the sensitivity levels of *Colletotrichum* spp. to multiple groups with potential use to manage the persimmon anthracnose in Brazil. In: Deising HB; Fraaije B; Mehl A; Oerke EC; Sierotzki H; Stammler G (Eds), "Modern Fungicides and Antifungal Compounds", Vol. IX, pp. 37-38. © 2020. Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-16-7

Assessing the sensitivity levels of *Colletotrichum* spp. to multiple chemical fungicide groups with potential use to manage persimmon anthracnose disease in Brazil

Carraro TAC[1], Lichtemberg PSF[2], Michailides TJ[2], May De Mio LL[1]
1Universidade Federal do Paraná, Rua d. Funcionários 1540, 80035-050 Curitiba-PR, Brazil
2University of California – Davis/Kearney Agricultural Research and Extension Center, 9240 South Riverbend Avenue, 93648 Parlier-CA, USA
Email: plichtemberg@ucanr.edu

INTRODUCTION

Brazil is the fifth largest producer of persimmon (*Diospyros kaki* L.) worldwide. Anthracnose, caused by *Colletotrichum* spp. can infect flowers, leaves, twigs, and fruit, resulting in significant crop losses due to immature fruit drop (Blood et al. 2015). In Brazil, the disease is mostly controlled by cultural practices and fungicide applications. Currently, Demethylation Inhibitors (DMI) and Quinone outside Inhibitors (QoI) are the main registered chemical group for this crop. These two groups present medium (DMI) to high (QoI) risk of developing resistance in the field, making the anti-resistance management difficult. Considering that, monitoring of fungicide sensitivity is necessary to suggest appropriate disease management and preserve fungicide efficacy. This study aimed to assess the sensitivity of *Colletotrichum* spp. to six active ingredients and to evaluate levels of cross-sensitivity among three fungicide groups.

MATERIAL AND METHODS

In total, 60 isolates of *Colletotrichum* spp. were collected and exposed to discriminatory doses to distinguish the sensitivity levels to azoxystrobin (1 µg ml^{-1}, May De Mio et al. 2011) and tebuconazole (0.3 µg ml^{-1}, Cox et al. 2007). Percentage of growth inhibition (PGI) was calculated according to Lichtemberg et al. (2018), with modifications, and isolates were classified as sensitive (PGI> 70%), intermediate (30% > PGI < 70%) and resistant (PGI < 30%). EC_{50} values were determined for 16 isolates using an EddyJet2 spiral plater. For that, DMIs were plated on PDA, while SDHIs and QoIs were plated on YBA, with the latest chemical group amended with the alternative oxidase inhibitor salicylhydroxamic acid (SHAM).

RESULTS AND DISCUSSION

The discriminatory dose assay revealed that most isolates (n=60) showed intermediate resistance to azoxystrobin (75%) and tebuconazole (50%). For QoI, similar sensitivity mean values were observed, i.e. 0.77 µg L^{-1} for azoxystrobin and 0.75 µg L^{-1} for trifloxystrobin, with highly significant cross-sensitivity levels (r^2 = 0.82) (Fig. 1A). Only wild-type genotypes were

observed, despite the presence of one isolate showing EC_{50} values with 6- and 13-fold increased resistance to azoxystrobin and trifloxystrobin (data not shown). The DMIs difenoconazole (0.15 µg L^{-1}) and tebuconazole (0.21 µg L^{-1}) revealed similar EC_{50} mean values, with moderate cross-sensitivity (r^2 = 0.62) (Fig. 1B). The SDHI benzivindiflupyr was more efficient (0.01 µg L^{-1}) than penthiopyrad (0.17 µg L^{-1}), with no cross-sensitivity observed (Fig. 1C). Variation among DMI correlation coefficients and unknown molecular basis in Colletotrichum spp. were also reported by Chen et al. (2016). The high and moderate cross-sensitivity levels observed for QoI and DMI, respectively, demonstrate the importance to create efficient spray programs including not only these fungicide groups, but also anilino-pyrimidines and copper fungicides.

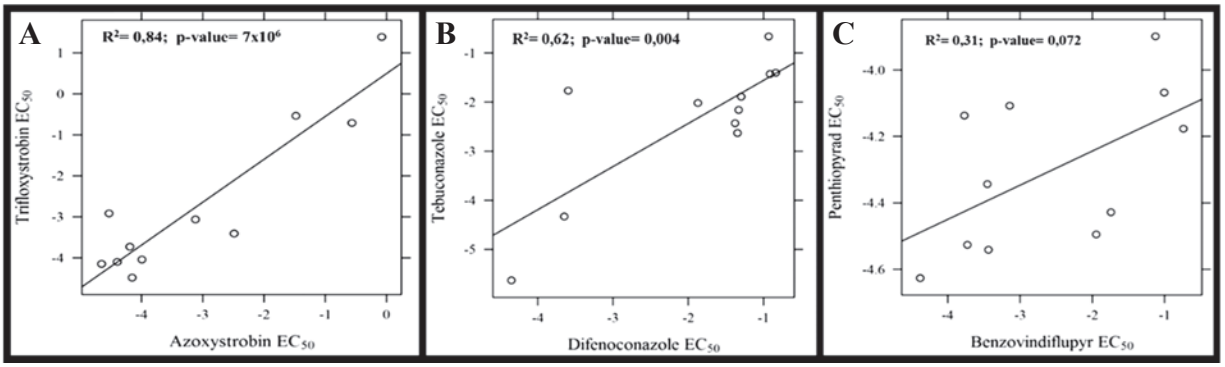


Figure 1. Cross-sensitivity of 16 isolates of *Colletotrichum* spp. to azoxystrobin and trifloxystrobin (A), tebuconazole and difenoconazole (B) and penthiopyrad and benzivindiflupyr (C).

CONCLUSION

Due to the moderate (DMI) and high (QoI) cross-sensitivity observed within chemical groups, it is expected that eventual resistance development will affect multiple active ingredients. Finally, the SDHI results represent the baseline data for monitoring the sensitivity for this group in eventual registration to control the anthracnose in persimmon.

REFERENCES

Blood RRY; Rozwalka LC; May De Mio LL (2015). Persimmon anthracnose caused by *Colletotrichum horii*: Incidence in branches, leaves, flowers and fruits in the field. *Revista Brasileira de Fruticultura* 37, 335-345.

Cox KD; Bryson PK; Schnabel G (2007). Instability of propiconazole resistance and fitness in *Monilinia fructicola*. *Phytopathology* 97, 448-453.

Chen SN; Luo CX; Hu MJ; Schanabel G (2016). Sensitivity of *Colletotrichum* species, including *C. fioriniae* and *C. nymphaeae*, from Peach to Demethylation Inhibitor Fungicides. *Plant Disease* 100, 2434-2441.

Lichtemberg PSF; Puckett R; Felts D; Luo Y; Doster L; Rodriguez D; Michailides TJ (2018). Survey of the pathogen of Alternaria late blight reveals different levels of carboxamide fungicide resistance in the main pistachio producing regions of California. In: *California Agriculture*, vol. 72, number 3.

Battistini G et al., Sensitivity of Italian *Zymoseptoria tritici* isolates to azoxystrobin and pyraclostrobin. In: Deising HB; Fraaije B; Mehl A; Oerke EC; Sierotzki H; Stammler G (Eds), "Modern Fungicides and Antifungal Compounds", Vol. IX, pp. 39-40. © 2020. Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-16-7

Sensitivity of Italian *Zymoseptoria tritici* isolates to azoxystrobin and pyraclostrobin

Battistini G[1], Gazzetti K[1], Menghini M[1], Frey R[2], Torriani SFF[2], Collina M[1]

[1]*DISTAL University of Bologna, Viale G. Fanin 40-42, 40127 Bologna (Italy)*

[2]*Syngenta Crop Protection Muenchwilen, Schaffhauserstrasse CH 4332 Stein (Switzerland)*

Email: marina.collina@unibo.it

INTRODUCTION

Septoria tritici blotch (STB), caused by *Zymoseptoria tritici*, is the most important and widespread disease on wheat in Northern and Central Europe. In Italy, during the last few years the incidence of the disease has increased. Resistant cultivars, cultural management and chemical control are the most common strategies to contain the disease (Fraaije et al., 2005). The main fungicide classes used are QoIs, DMIs, and SDHIs. In Europe, QoIs are often no longer recommended to control STB due to widespread resistance, but in Italy they are still commonly used in the management of *Z. tritici*. As no results from wide and specific sensitivity monitoring programmes in Italy are available, the aim of this study was to test the sensitivity of *Z. tritici* strains to QoIs in order to obtain first data on the Italian scenario.

MATERIAL AND METHODS

Infected leaves were collected during 2015 and 2016 from different kind of sites: commercial and experimental fields as well as one isolated field as reference. From each field, one population was obtained by selecting different picnidia from several leaves. Monoconidial isolates were obtained and tested *in vitro* by a microtiter assay. Sensitivity to azoxystrobin and pyraclostrobin was tested at the concentrations 0-0.001-0.003-0.01-0.03-0.1-0.3-1-3-10-30 mg/l of active ingredient for azoxystrobin and 0-0.01-0.1-1-2-10 mg/l of active ingredient for pyraclostrobin. Gathering the EC_{50} (mg/l) of azoxystrobin and pyraclostrobin for each type of fields, box-plots were obtained (by Excel 2013 software).

RESULTS AND CONCLUSION

The EC_{50} values of the isolates collected during 2015-2016 showed that the highest median value for azoxystrobin (Figure 1a) belongs to treated experimental fields, followed by treated commercial, untreated experimental, untreated commercial and the reference field. Statistical analysis revealed that the reference field strains differ to treated experimental and untreated fields. For pyraclostrobin (Figure 1b) the situation was different, with the highest median EC_{50}

value belonging to untreated experimental fields followed by treated experimental, treated commercial and untreated commercial fields.

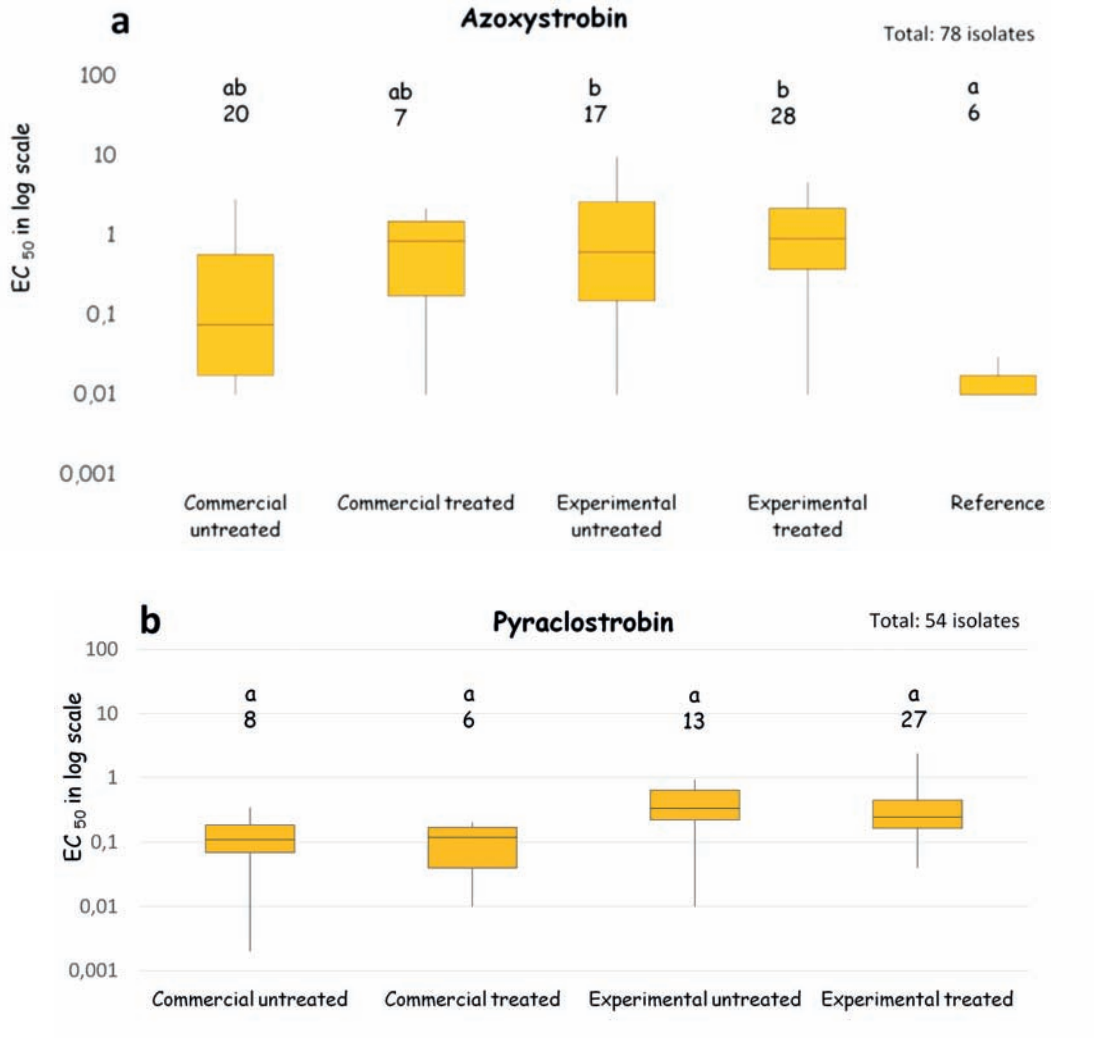


Figure 1 a-b Sensitivity of *Z. tritici* monoconidial isolates to azoxystrobin (a) and pyraclostrobin (b). The horizontal lines within the boxes indicate the medians; the edge of the box are the 25° and 75° percentiles; the whiskers indicate the lowest and highest EC_{50} value. Box plot characterized by the same letters are not statistically different for p= 0.05 (post-hoc Duncan test).

This study showed the selection imposed by fungicide treatments; in fact, the EC_{50} median value of treated fields was higher than the untreated ones, even if data do not show statistically significant differences. Selection imposed by the fungicide used could locally result in an increased frequency of resistance.

REFERENCES

Fraaije BA, Cools HJ, Fountaine J, Lovell DJ, Motterm J, West JS, Lucas JA (2005). Role of ascospores in further spread of QoI-resistant cytochrome b alleles (G143A) in field populations of *Mycosphaerella graminicola*. *Phytopathology* 95, 933-941.

Milosavljević Anja, Trkulja Nenad, Sensitivity monitoring of *Cercospora beticola* to fungicides in Serbia. In: Deising HB; Fraaije B; Mehl A; Oerke EC; Sierotzki H; Stammler G (Eds), "Modern Fungicides and Antifungal Compounds", Vol. IX, pp. 41-42. © 2020. Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-16-7

Sensitivity Monitoring of *Cercospora beticola* to Fungicides in Serbia

Milosavljević A[1], Trkulja N[1]

[1]*Institute for Plant Protection and Environment, Teodora Drajzera 9, 11000 Belgrade, Serbia*
Email: anjamilosavljevic@yahoo.com

INTRODUCTION

Cercospora leaf spot disease (CLS) caused by *Cercospora beticola* is the most destructive sugar beet (*Beta vulgaris* L.) disease worldwide (Holtschulte 2000). Decrease in fungicide efficacy during the last two years caused serious problems in disease management. Three groups of fungicides differing in mode of action, i.e. benzimidazoles (MBC), triazoles (DMI) and strobilurins (QoI), were intensively used. Three years after MBC introduction in Serbia, resistance developed (Marić et *al*. 1976). Triazoles were introduced at the begining of the 1980s and resistance was also recorded. QoIs have been used since 2006 and had excellent efficacies until resistance developed (Trkulja et *al*. 2017). Sensitivity assessment allowing to detect rapid development of populations resistant to all three fungicide groups requires constant monitoring (Trkulja et *al.* 2017).

MATERIAL AND METHODS

Samples of sugar beet leaves with sporulating lesions of *C. beticola* were collected after appearance of field symptoms and transferred to the laboratory. In 2016 isolates were collected from two different localities, Indjija and Kukujevci, and from locations Crvenka and Stari Tamis in 2017. Spores from leaves were placed onto potato dextrose agar (PDA) for 24h, single germinated conidia were excised and single spore isolates were established on PDA. Mycelial growth measurement at a discriminative dose (DC) of 1 mg/l was conducted for MBC and DMIs, i.e. thiophanate-methyl (Galofungin T 450SC, Galenika Phytopharmacy, Serbia) and tetraconazole (Eminent 125 EW, Isagro, Italy), respectively. Mycelial growth was measured after 7 days of incubation at 25 ºC, dark, and isolates with relative growth rates ≥50% of the control were considered as resistant. Tests of conidial germination (DC 5 mg/l) were conducted for measuring sensitivity to the QoIs, pyraclostrobin (Retengo, BASF, Germany). Isolates with more than 50% germinated conidia were considered as resistant.

RESULTS

Frequencies of *C. beticola* isolates resistant to MBC, QoI and DMI fungicides collected at Indjija in 2016 were 70%; 80%; and 70%, while at Kukujevci the frequencies of resistance to

these fungicide classes were 53%; 78%; and 95%, respectively. Isolates collected from Crvenka in 2017 showed frequencies of resistance to MBC, QoI and DMI fungicides corresponding to 76%; 100%; and 100%, while resistance rates of those sampled at Stari Tamis were 57%; 76%; and 92%, respectively. The fungicide thiophanate-methil showed the lowest frequency of resistance at all localities, ranging from 53%-76%, while tetraconazole had the highest on two localities, i.e. at Kukujevci in 2016 and at Stari Tamis in 2017. Pyraclostrobin had the high frequencys of resistance at all localities, ranging from 70%-100%. These results indicate a significant decrease in sensitivity of *C. beticola* population to fungicides with different modes of action (Trkulja et *al*. 2013; 2015; 2017). Also, emergence of *C. beticola* populations, multi-resistant to all three groups of fungicides (Trkulja et al. 2017) occurred, likely due to excessive use of these fungicides addressing the distinct modes of actions over the years. This led to decline of fungicides efficacy, which had direct impact on managing control of the pathogen in fields in Serbia.

REFERENCES

Holtschulte B (2000). *Cercospora beticola* – worldwide distribution and incidence. In: Advances in Sugar Beet Research. Vol. 2: *Cercospora beticola* Sacc. Biology, Agronomic Influence and Control Measures in Sugar Beet 2000, eds. MJC Asher, B Holtschulte, MR Molard, F Rosso, G Steinruecken, and R Beckers, pp. 5-16. International Institute for Beet Research: Brussels.

Marić A; Petrov M; Maširević S (1976). Pojava tolerantnosti kod *Cercospora beticola* Sacc. prema benomilu u Jugoslaviji i mogućnosti suzbijanja ovog parazita. Zaštita bilja 27, 227-236.

Trkulja N; Ivanović Ž; Pfaf-Dolovac E; Dolovac N; Mitrović M; Toševski I; Jović J (2013). Characterization of benzimidazole resistance of *Cercospora beticola* in Serbia using PCR-based detection of resistance-associated mutations of the ß-tubulin gene. European Journal of Plant Pathology 135, 889-902.

Trkulja N, Milosavljević A, Stanisavljević R, Mitrović M, Jović J, Toševski I, Bošković J (2015). Occurrence of *Cercospora beticola* populations resistant to benzimidazoles and demethylation-inhibiting fungicides in Serbia and their impact on disease management. Crop Protection 75, 80-87.

Trkulja N, Milosavljević A, Mitrović M, Jović J, Toševski I, Khan M, Secor G (2017). Molecular and experimental evidence of multi-resistance of *Cercospora beticola* field populations to MBC, DMI and QoI fungicides, European Journal of Plant Pathology, Springer, 149, 4, pp. 895 - 910, 0929-1873, 10.1007/s10658-017-1239-0, 2017.

Nanni IM et al., Dimethomorph acitivity on different oomycete species of economic and veterinary interest. In: Deising HB; Fraaije B; Mehl A; Oerke EC; Sierotzki H; Stammler G (Eds), "Modern Fungicides and Antifungal Compounds", Vol. IX, pp. 43-44. © 2020. Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-16-7

Dimethomorph Activity on Different Oomycete Species of Economic and Veterinary Interest

Nanni IM[1], Tedesco P[2], Galuppi R[2], Collina M[1]

[1]*Department of Agricultural and Food Sciences (DISTAL), University of Bologna - Viale G. Fanin 42, 40127 Bologna, Italy*

[2]*Department of Veterinary Medical Sciences (DIMEVET), University of Bologna – Via Tolara di Sopra 50, 40064 Bologna, Italy*

Corresponding Author Email: irenemaja.nanni2@unibo.it

INTRODUCTION

The class Oomycota comprises species that are pathogenic to plants and animals, including humans, and are able to cause severe economic losses in agriculture and aquaculture industry worldwide. *Phytophthora infestans* causes losses in potato and tomato crops for more than US-$ 6 billion per year, as well as damages to ecosystems (Fry 2008). The genus *Pythium* includes species that are pathogenic for both plants and animals, and particularly *Pythium insidiosum* was reported to cause diseases in humans and in other mammals (Gaastra et al. 2010). Oomycetes of the order Saprolegniales are widely distributed in freshwater environments, causing infections in different taxa of aquatic animals (Bruno et al. 2010). Among these, members of the genus *Saprolegnia* represent a severe problem in freshwater fish farms, where production losses from 10 to 50% are reported (van West 2006). Despite the wide distribution and the impact of oomycetes on economic activities and on animal health, there are no effective molecules available against these agents. Following the current classification of malachite green and formalin among carcinogens, there are limited possibilities to control oomycete infections in aquaculture (Sudova et al. 2007). The aim of this work was to test *in vitro* the activity of dimethomorph (DMM), a fungicide used in agriculture to control oomycete infections, on different species of *Saprolegnia* and *Pythium* isolated from fish and aquatic environment.

MATERIALS AND METHODS

In vitro tests were performed using two protocols according to Alderman (1982): one screening method in Agar (protocol I), aimed at assessing the minimum inhibitory concentration (MIC), and one hour bath in aqueous solution of mycelium growing on polycarbonate membrane (protocol II), to assess the minimum lethal concentration (MLC).

Tests were performed using two field strains of *Saprolegnia parasitica* and *Saprolegnia delica* isolated from brown trout (*Salmo trutta*) and rainbow trout (*Oncorhynchus mykiss*), respectively, one reference strain of *S. parasitica* (CBS 223.65 provided by CSIC-RJB), one strain of *Pythium pachycaule* isolated from *S. trutta*, one strain of *Pythium dissimile* isolated from European chub (*Squalius cephalus*), and two strains of *Pythium aquatile* and *Pythium*

rhizo-oryzae isolated from water. DMM was tested at concentrations of 0, 0.1, 1, 5, 10, 50, and 100 ppm. For each concentration, strains have been tested in triplicate.

RESULTS

Protocol I:

After 24 h, all tested strains showed reduced radial growth at 50 and 100 ppm DMM. At 100 ppm, radial growth of *S. parasitica* and *S. delica* slowed down for 72 h. After 6 d, growth of aerial mycelium of *S. parasitica* was inhibited at 50 ppm, while growth of aerial mycelium of *S. delica* was inhibited at 100 ppm. Out of the *Pythium* strains, *P. dissimile* and *P. rhizo-oryzae* showed highest sensitivity, and particularly with 100 ppm DMM, radial growth after 6 d hadn't reached the diameter of the control. No inhibition of aerial mycelium was observed.

Protocol II:

No lethal effect or reduced growth of *Saprolegnia* spp. and *Pythium* spp. was observed at all concentrations studied after one hour bath in aqueous DMM solution.

CONCLUSIONS

Although MIC and MLC values couldn´t be determined in this study, the results of protocol I showed always a reduction of mycelial growth at the highest test concentrations of 50 and 100 ppm DMM. Furthermore, our results suggest a different sensitivity to DMM of the tested oomycete species. Particularly the inhibitory effect of dimethomorph on the development of aerial mycelium, observed in all *Saprolegnia* strains tested but not in *Pythium* spp., may result from chemically induced morphological changes in the hyphae, and would require further investigation.

REFERENCES

Alderman DJ (1982). *In vitro* testing of fisheries chemotherapeutants. *Journal of Fish Diseases* 8, 289-298.

Bruno D; van West P; Beakes G (2010). *Saprolegnia* and other oomycetes. In: Fish diseases and disorders: Viral, bacterial and fungal infections. CABI International.

Fry, W. (2008). *Phytophthora infestans*: the plant (and R gene) destroyer. *Molecular plant pathology* 9 (3), 385-402.

Gaastra W; Lipman LJ; De Cock AW; Exel TK; Pegge RB; Scheurwater J; Vilela R; Mendoza L (2010). *Pythium insidiosum*: an overview. *Veterinary microbiology* 146 (1-2), 1-16.

Sudova E; Machova J; Svobodova Z; Vesely T (2007). Negative effects of malachite green and possibilities of its replacement in the treatment of fish eggs and fish: a review. *Veterinární Medicina* 52, 527-539.

Van West P (2006). *Saprolegnia parasitica*, an oomycete pathogen with a fishy appetite: new challenges for an old problem. *Mycologist* 20 (3), 99-104.

Jaklova P et al., Testing sensitivity of *Venturia inaequalis* populations to pyrimethanil from selected orchards in Czech Republic. In: Deising HB; Fraaije B; Mehl A; Oerke EC; Sierotzki H; Stammler G (Eds), "Modern Fungicides and Antifungal Compounds", Vol. IX, pp. 45-47. © 2020. Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-16-7

Testing the sensitivity of *Venturia inaequalis* to pyrimethanil from selected orchards in Czech Republic

Jaklova P, Hortova B, Kloutvorova J

Research and Breeding Institute Holovousy Ltd. Holovoousy 129, Hořice 508 01, Czech Rep.
Corresponding Author Email: pavlina.jaklova@vsuo.cz

INTRODUCTION

The fungus *Venturia inaequalis* (Cooke) G. Winter (1875) (anamorph - *Spilocea pomi* Fr., 1825) causes a disease in apple orchards worldwide – apple scab. It is the most serious and economically important disease of apple trees (*Malus* x *domestica* Borkh.) (MacHardy 1996). In order to ensure reliable protection against apple scab, it is necessary to apply 15 to 20 fungicide sprays during the season (Agrios 2005). Intensive use of chemical protection may lead to its failure, which may be due to lower sensitivity, or even resistance of the fungus (Brent &Hollomon 2007). One group of fungicides commonly used are known as anilino-pyrimidines (AP), with a moderate risk of developing resistence. Mutations in the regulation of methionine biosynthesis are a potential cause of AP fungicide resistance (Anonymous 2019a). Especially fungal pathogens such as *V. inaequalis* and *Botrytis cinerea* showed lower sensitivity or resistance to anilinopyrimidines in fruit crops (Köller et al. 2005; Weber 2011). Aim of this study was to find out the current state of sensitivity of *V. inaequalis* populations to the active substance pyrimethanil (compound Mythos 30 SC) from selected commercial orchards in the Czech Republic reporting nonsatisfactory management of apple scab.

METHODS

Greenhouse experiments were performed with Mythos 30 SC (300 g/kg pyrimethanil; BASF SE) at the concentrations 0 ppm, 100 ppm and 300 ppm of pyrimethanil. Apple seedlings, variety ´Golden Delicious´ were used (20 to 24 plants per treatment). These tests are based on the Fungicide Resistance Action Committee (FRAC) methodology: VENTIN in vivo-AP (BASF, 2006 V1). Seedlings were inoculated with a conidia suspension. Concentration of conidia was adjusted to 10 to 15 x 10^4 conidia per ml. The germination percentage of conidia was evaluated after 24 hours to control conidia viability. We tested a total of nine *V. inaequalis* populations from commercial orchards, where a reduced sensitivity to this active substance was suspected. Populations of *V. inaequalis* from Bříství, Dolany, Domašín, Choltice, Choustníkovo hradiště, Nosislav, Osík, Svinčany and Synkov (Figure 1) were tested. The results were compared with a sensitive population of *V. inaequalis* from Lázně Bělohrad (apple tree in road alley with no

history of fungicide treatment). In total, 50 leaves per treatment were evaluated and total percentage of leaf infestation was calculated.

RESULTS

According to the FRAC methodology, the sporulating scab lesions at 300 ppm indicate the presence of less sensitive spores in the population to pyrimethanil. Five *V. inaequalis* populations (Bříství, Dolany, Nosislav, Osík, Svinčany) were proved as resistant, the percentage of leaf infestation was in range 49 to 62 %, populations from Domašín, Choustníkovo hradiště and Synkov showed reduced sensitivity (6 to 16 % of leaf infestation). For comparison, percentage of leaf infestation of the sensitive population from Lázně Bělohrad was 1.3 %. Exact results of greenhouse tests for individual localities are presented in Figure 2. Currently, according to the results of monitoring carried out by FRAC, a trend of decreasing incidence of resistant *V. inaequalis* populations in apple orchards has been reported in European countries. Anilinopyrimidines still have relatively stable efficacy after several years of using (Anonymous 2019b).

CONCLUSION

Based on the results, we found that *V. inaequalis* populations with reduced sensitivity to pyrimethanil occurred in some commercial orchards in the Czech Republic. It would be appropriate to prevent the selection of mutant individuals and the principles of the anti-resistance strategies are highly recommend to growers.

The work was carried out within the project NPU LO1608.

REFERENCES

Agrios GN (2005). *Plant Pathology*. 4th ed. Elsevier Academic Press: London.

Anonymous (2018a). *AP fungicides – Introduction and General Information*. www.frac.info/working-group/ap-fungicides (5.3. 2019).

Anonymous (2018b). *AP fungicides – Introduction and General Information.* www.frac.info/docs/default-source/ap-wg/minutes-of-the-2018-ap-meeting-recommendations-for-2019.pdf (5.3. 2019).

Brent KJ, Hollomon DW (2007). *Fungicide Resistance: The Assessment of Risk*. 2nd ed. Aimprint, United Kingdom.

Köller W, Wilcox WF, Parker DM (2005). Sensitivity of *Venturia inaequalis* populations to anilinopyrimidine fungicides and their contribution to scab management in New York. *Plant Disease* 89, 357-365.

MacHardy WE (1996). *Apple Scab: Biology, Epidemiology, and Management*. APS Press, St. Paul, Minnesota.

Weber RWS (2011) Resistance of *Botrytis cinerea* to multiple fungicides in Northern Germany small-fruit production. *Plant Disease* 95, 1263-1269.

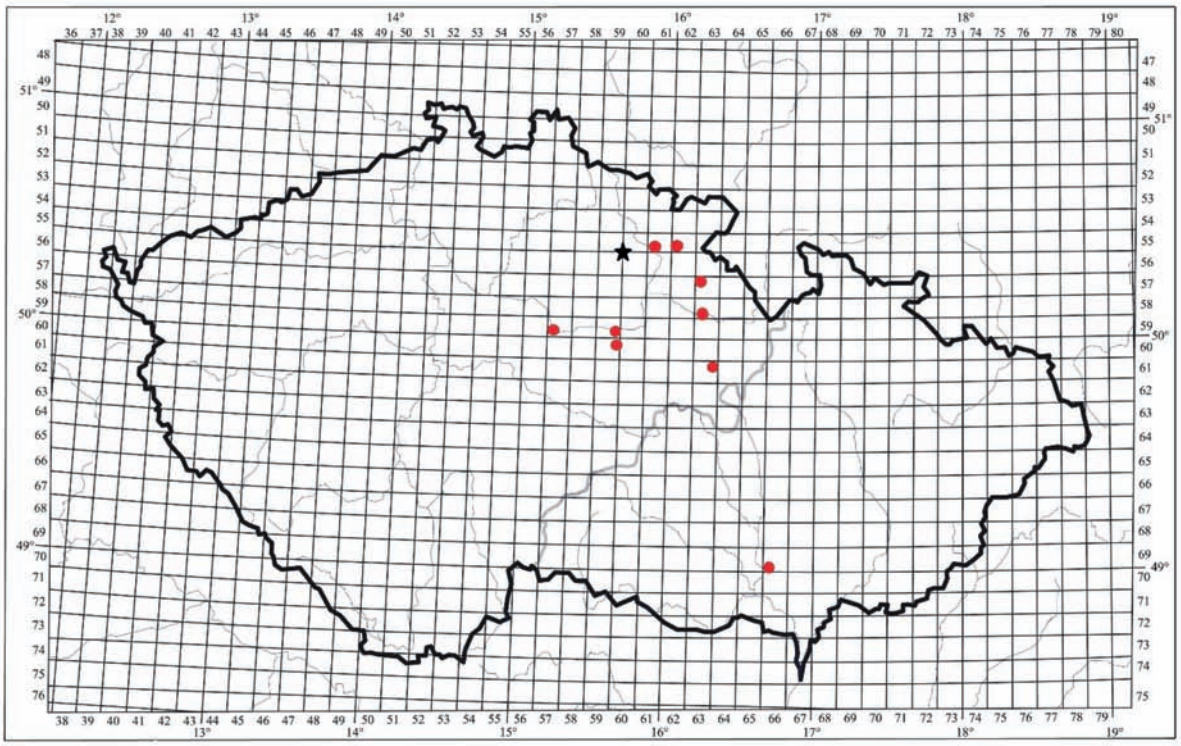


Figure 1. Map of localities, where populations of *V. inaequalis* were collected Populations from commercial orchards ● Sensitive population ★

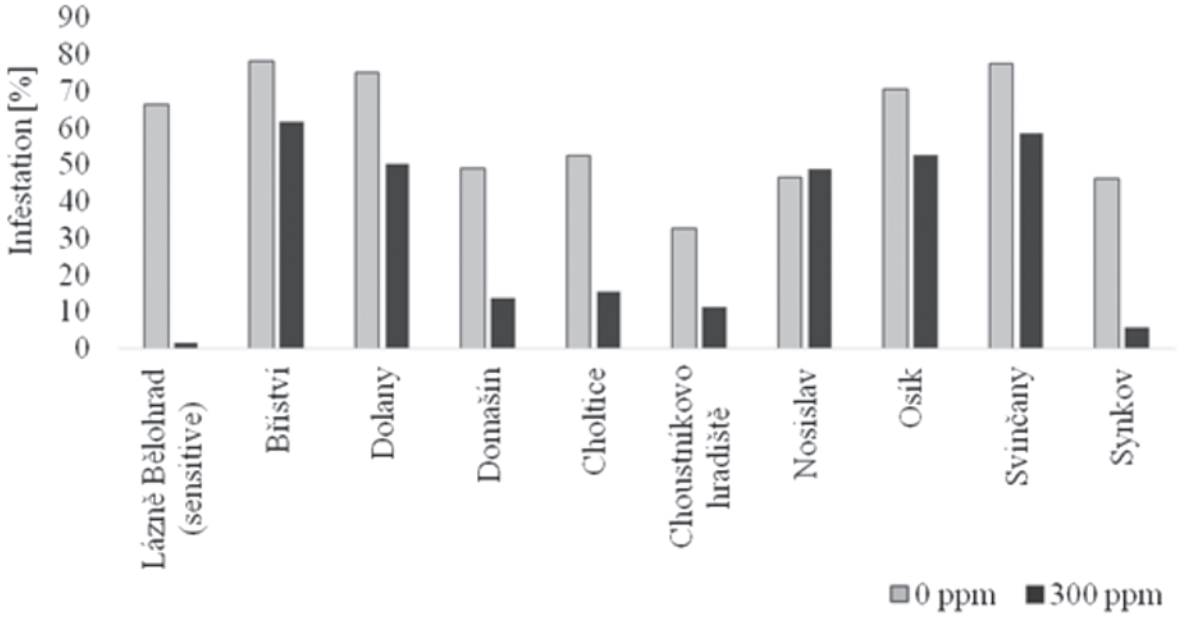


Figure 2. Results of glasshouse tests of *V. inaequalis* populations' sensitivity to pyrimethanil (Mythos 30 SC)

RESISTANCE RISK ASSESSMENT AND MANAGEMENT

Godoy CV; Meyer MC, Overcoming the threat of Asian soybean rust in Brazil. In: Deising HB; Fraaije B; Mehl A; Oerke EC; Sierotzki H; Stammler G (Eds), "Modern Fungicides and Antifungal Compounds", Vol. IX, pp. 51-56. © 2020. Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-16-7

Overcoming the threat of Asian soybean rust in Brazil

Godoy CV, Meyer MC

Embrapa, CEP 86001-970, Londrina, PR, Brazil
Email: claudia.godoy@embrapa.br

INTRODUCTION

Brazil harvested 236.7 million tons of grain in 2018/2019. The sown area was 62.8 million hectares. The estimated growth was 1.8% compared to the 2017/18 growing season. The largest increases in the area were soybean (652.8 thousand hectares), corn second summer crop season (741.4 thousand hectares), and cotton (415.6 thousand hectares) (Conab 2019).

The cultivated soybean area in 2018/2019 reached 35.8 million hectares (Conab 2019). One disease that threatens the sustainability of the crop and represents a breakthrough in the history of soybean in Brazil is Asian soybean rust, caused by the fungus *Phakopsora pachyrhizi*. The disease was first reported in Paraguay in 2001 and in the west of the state of Paraná, Brazil, spreading, within three years, throughout South America (Yorinori et al. 2005). In 2003, the disease was observed in more than 90% of the fields in Brazil, and the projected losses in Mato Grosso and Bahia alone were 2.2 MMT (US$ 487.3 million) (Yorinori et al. 2005). While yield losses decreased among the years, the use of fungicides was intensified. Costs with fungicide application in soybean were estimated in US$ 2.9 billion in 2018/2019, with an average of 2.75 fungicide applications per soybean crop season.

CHEMICAL CONTROL

The first fungicides labeled for soybean rust control were those that had already been used for other pathogens on soybean and which showed efficient control of soybean rust in previous trials. In the first years, the majority of the labeled fungicides belonged to the demethylation inhibitor (DMI) group and to a premix of DMIs and quinone outside inhibitors (QoIs). Since 2013, commercial mixtures of succinate dehydrogenase inhibitors (SDHIs) in double and triple mixtures were labeled for soybean rust (Godoy et al. 2016).

Since 2003/04, uniform field trials have been carried out in different soybean producing regions in order to compare the efficacy of fungicides registered and in the registration phase (Godoy et al. 2016). Besides the fungicides efficacy, the results of the uniform field trials allowed accompanying the change of sensitivity of the fungus to the different modes of action over the years, along with bioassays and molecular analyzes. Fungicides efficacy reduction in the uniform field trials was reported for the DMIs in 2007, QoIs in 2013, and for the SDHIs in 2016 (Godoy et al. 2016; 2018; 2019).

The decrease in efficacy of DMI fungicides became a general trend since 2007/08 (Figure 1). The only exception was prothioconazole, but in the recent crop season, a wide range of efficacy has been observed for this fungicide, that is used formulated in premix with QoI and QoI + SDHI (Godoy et al. 2018; 2019). At least six CYP51 mutations (Y131F/H; F120L; K142R; I145F; I475T) and overexpression is known to be involved in the sensitivity reduction towards DMIs (Schmitz et al. 2014).

For QoI, in 2013/2014, a reduced effectiveness was observed in the trials (Figure 1), although the EC50 in the monitoring bioassays has remained in the range reported in previous years. The F129L mutation was reported at high frequency (~90%) in 2013/14 *P. pachyrhizi* isolates (Klosowski et al. 2016a) and remained stable in the subsequent crop seasons. Studies with isolates showed that F129L mutation did not present fitness costs to *P. pachyrhizi* (Klosowski et al. 2016b).

In 2017/18 the fungicides registered for soybean rust were re-evaluated by the Ministry of Livestock Agriculture and Supply due to their low efficacy. All fungicides with a single active ingredients and products with low efficacy were suspended. Although not recommended as a disease management strategy, the efficacy of single active ingredient fungicides has been annually assessed in the uniformed fungicide trials to monitor the sensitivity of the fungus in different regions of Brazil (Figure 1).

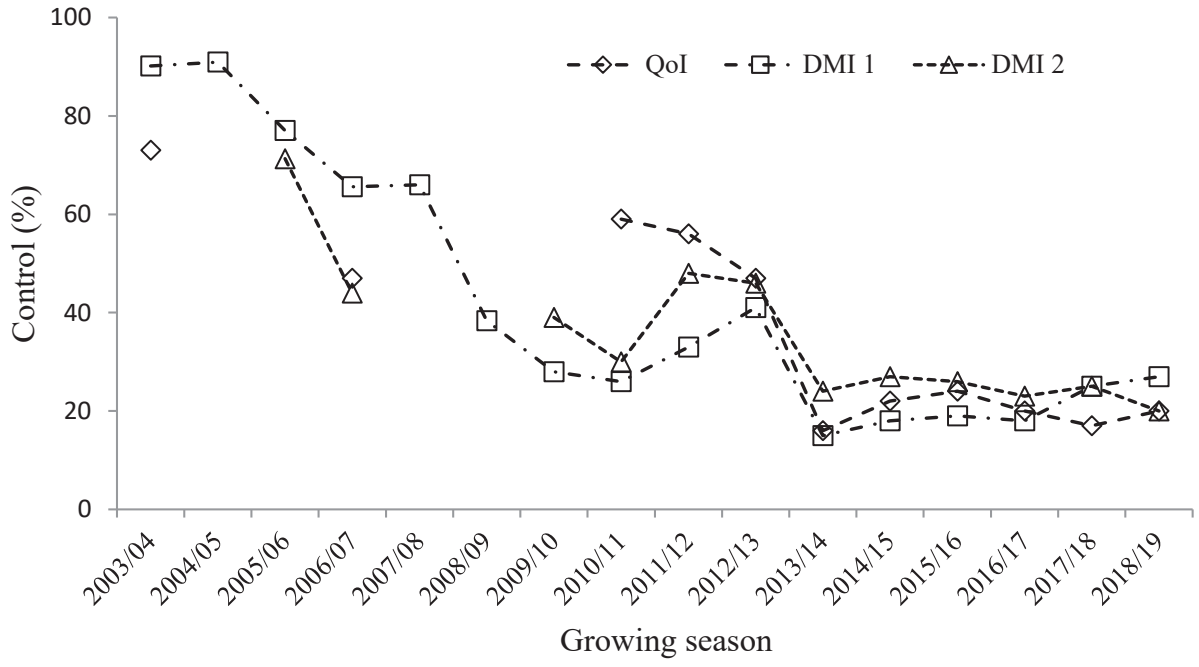


Figure 1 Average percentage control of soybean rust by fungicides in uniform field trials carried out in different growing seasons (number of trials): 2003/04 (11), 2004/05 (20), 2005/06 (15), 2006/07 (10), 2007/08 (7), 2008/09 (23), 2009/10 (15), 2010/11 (11), 2011/12 (11), 2012/13 (21), 2013/14 (16), 2014/15 (21), 2015/16 (23), 2016/17 (32), 2017/18 (26), and 2018/19 (25). DMI 1 - tebuconazole, DM 2 – cyproconazole, and QoI –azoxystrobin. Source: adapted from Godoy et al. (2016), and Godoy et al. (2018).

Athough cross-resistance in the same group occurs; the mutation F129L affected the active ingredients in different ways. To evaluate the efficacy of different QoI active ingredients (pyraclostrobin, azoxystrobin, trifloxystrobin, picoxystrobin, and metominostrobin) a field trial was carried out in Londrina, Paraná in 2018/19. Commercial fungicides were applied at 60 g a.i. ha^{-1} in a randomized completed block design with four repetitions. The first application was at 54 days after sowing and repeated at 15 and 17 days intervals with a CO^2 backpack sprayer, to deliver 150 liters ha^{-1}. Soybean rust symptoms started at R5 (beginning seed) and the average disease severity at R6 stage (full seed) reached 63% on the untreated check. Based on the disease severity at R6 stage, the control with picoxystrobin, trifloxystrobin and metominostrobin were higher than azoxystrobin and pyraclostrobin (Figure 2). This trend is observed in the trials with commercial premix with the same QoI fungicides (Godoy et al. 2018; 2019).

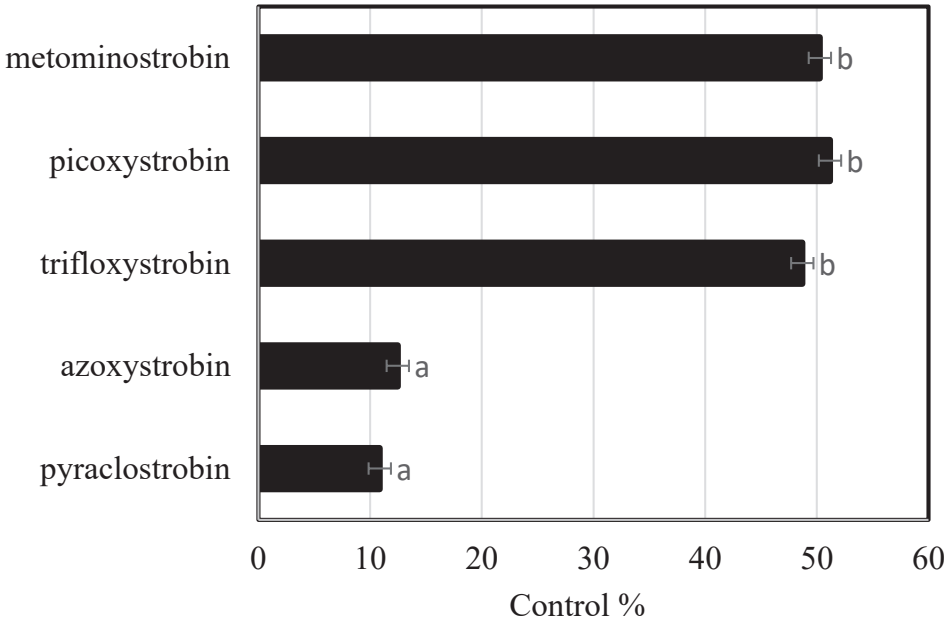


Figure 2 Percentage of soybean rust control at R6 stage (full seed) of different QoIs applied at 60g a.i. ha^{-1}, with applications starting at 54 days after sown and repeated at 15 and 17 days intervals. Means followed by the same letter are not significantly different by Tukey test at $P< 0.05$.

SDHI fungicides were used on soybean in Brazil for the first time in 2013/14 and strains of *P. pachyrhizi* with a lower sensitivity were found in monitoring studies in 2015/16, with a mutation in the C-I86F gene (Simões et al. 2018). In the subsequent growing season, the mutation I86F was detected in other regions and associated with lower efficacies of the SDHI fungicides in the uniform field trials (Godoy et al. 2018).

Since effective fungicides with a new mode of action are not readily available, multi-site fungicides, e.g. mancozeb, copper, and chlorothalonil, which have low resistance risk, have been recommended since 2014 for *P. pachyrhizi* control in premix or tank mixtures to increase control efficacy and reduce the risk of resistance to single-site fungicides (Godoy et al. 2016).

Even though all major single-site mode of action fungicides used for soybean rust control (DMI, QoI, and SDHI) have experienced adaptation by *P. pachyrhizi* in Brazil, they still contribute to disease control when associated with other management strategies. An important change that occurred with the efficacy reduction of the fungicides was a reduction in the application intervals of the fungicides from 21-28 days to 14 days.

CULTURAL AND GENETIC CONTROL

A cultural practice adopted in Brazil aiming to reduce the inoculum during the offseason is the soybean-free period. It has a minimum of 60 days and it is a public policy adopted by 13 states that started to be adopted in 2006. During this period, growers are forbidden to sow soybean under irrigation and it is mandatory to eliminate volunteer soybean plants. The soybean-free period provided a delay in the first onset of the disease during the regular crop season (Godoy et al. 2016).

Since 2005, the soybean cultivars cycle has been reduced in Brazil, and early sowing allowed a significant increase in the sowing of double summer crops (e.g. soybean-corn, soybean-cotton, soybean-cover crops). This strategy has contributed to the control of soybean rust by helping the plants to escape the disease. The soybean varieties in Brazil have been gradually replaced from determined to indeterminate, and the maturity groups, from south to north of Brazil changed from 6.8 - 9.4 to 4.5 - 8.2. These characteristics were the major contributions of soybean breeding programs in Brazil to the control of soybean rust although not targeting the disease as a goal.

The most important soybean rust control strategy in Brazil has been the early sowing of short-cycle varieties after the soybean-free period, escaping the higher inoculum pressure period. Most of the fungicide applications on soybean in Brazil is scheduled-based, starting at 40-45 days after sowing, but the crops sowed earlier escapes the soybean rust incidence.

Major resistance genes (*Rpp*) have been mapped and incorporated into the cultivars. Varieties with resistant genes, provided by Tropical Melhoramento & Genética (TMG) and Embrapa, have been available in the Brazilian market since 2009. Fungicide applications are recommended for these varieties due to the variability of the fungus, able to overcome the resistance genes in the same way than single-site fungicides. Some varieties present more than one gene stacked. In 2018/19, TMG 7062 IPRO, a variety with resistant gene to *P. pachyrhizi*, was one of the 10 most sowing varieties in Brazil. Varieties with *Rpp* genes are not available for all the Brazilian regions.

Studies of *P. pachyrhizi* diversity in Brazil, Argentina and Paraguay using a set of different varieties found out that PI 587880A (*Rpp1*), Shiranui (*Rpp5*), and 3 *Rpp*-unknown differentials (PI 587855, PI 587905, and PI 594767A) showed a resistant reaction to 78–96% of all populations. This study demonstrated that *P. pachyrhizi* populations from South America vary geographically and temporally in pathogenicity and that the known *Rpp* genes other than *Rpp1*

in PI 587880A and *Rpp*5 have been less effective against recent pathogen populations in the countries studied (Akamatsu et al. 2013).

Seven states in Brazil (Goiás, Mato Grosso, Paraná, Mato Grosso do Sul, Santa Catarina, Bahia, and Tocantins) adopted regional laws limiting the soybean-sowing period to curb late sowing of soybeans or avoid double soybean sowing, in order to reduce the number of spraying during the entire crop season, and delay the process of resistance to fungicides. Growers, who demand late sowing for producing seeds, frequently question these laws and claim for changes. The number of fungicides with acceptable efficacy to control Asian rust has been decreasing in the recent years due to resistance, although the yield losses have not been high because of the disease escape favored by the early sowing and the use of short-cycle cultivars. Not all regions in Brazil are able to sow early due to the temperate climate and dependence on rainfall. Consequently, yield losses due to soybean rust have been reported in the recent growing seasons.

REFERENCES

Akamatsu H; Yamanaka N; Yamaoka Y; Soares RM; Morel W; Ivancovich AJG; Bogado AN; Kato M; Yorinori JT; Suenaga K (2013) Pathogenic diversity of soybean rust in Argentina, Brazil, and Paraguay. *Journal of General Plant Pathology* 79, 28–40.

Conab (2019). Safras. www.conab.gov.br/info-agro/safras (21/09/2019).

Godoy CV; Seixas CDS; Soares RM; Marcelino Guimaraes FC; Meyer MC; Costamilan LM (2016) Asian soybean rust in Brazil: past, present, and future. *Pesquisa Agropecuária Brasileira* 51, 407-421.

Godoy CV; Utiamada CM; Meyer MC; Campos HD; Lopes ION; Dias AR; Deuner CC; Pimenta CB; Jaccoud Filho DS; Moreira EN; Borges EP; Andrade Junior ER; Siqueri FV; Juliatti FC; Juliatti FC; Favero F; Feksa HR; Araújo Júnior IP; Grigolli JPJ; Nunes Junior J; Belufi LMR; Carneiro LC; Carregal LH; Sato LN; Canteri MG; Volf MR; Goussain MM; Dias MD; Debortoli MP; Martins MC; Balardin RS; Furlan SH; Madalosso TM; Carlin V; Venancio WS (2018) Eficiência de fungicidas para o controle da ferrugem-asiática da soja, *Phakopsora pachyrhizi*, na safra 2017/2018: Resultados sumarizados dos ensaios cooperativos. Embrapa Soja. 8 p. (retr. 21/09/2019): https://ainfo.cnptia.embrapa.br/digital/bitstream/item/182754/1/CT138-ferrugem-OL-1.pdf

Godoy CV; Utiamada CM; Meyer MC; Campos HD; Lopes ION; Dias AR; Pimenta CB; Andrade Junior ER; Moresco E; Siqueri FV; Juliatti FC; Juliatti FC; Favero F; Araújo Júnior IP; Chaves ICPV; Roy JMT; Grigolli JFJ; Nunes Junior J; Navarini L; Senger M; Belufi LMR; Carregal LH; Sato LN; Goussain MM; Debortoli MP; Martins MC; Tormen NR; Balardin RS; Madalosso T; Carlin V; Venancio WS (2019). Eficiência de fungicidas para o controle da ferrugem-asiática da soja, *Phakopsora pachyrhizi*, na safra 2018/2019: Resultados sumarizados dos ensaios cooperativos. Embrapa Soja. 12 p. (retr. 21/09/2019): https://ainfo.cnptia.embrapa.br/digital/bitstream/item/199522/1/CT-148-OL.pdf.

Klosowski AC; May De Mio LL; Miessner S; Rodrigues R; Stammler G (2016a). Detection of the F129L mutation in the cytochrome *b* gene in *Phakopsora pachyrhizi*. *Pest Management Science* 72, 1211–1215.

Klosowski AC; Brahm L; Stammler G; May De Mio LL (2016b). Competitive fitness of *Phakopsora pachyrhizi* isolates with mutations in the CYP51 and CYTB genes. *Phytopathology* 106, 1278-1284.

Schmitz HK; Medeiros AC; Craig IR; Stammler G (2014). Sensitivity of *Phakopsora pachyrhizi* towards quinone-outside inhibitors and demethylation-inhibitors, and corresponding resistance mechanisms. *Pest Management Science* 7, 378-388.

Simões K; Hawlik A; Rehfus A; Gava F; Stammler G (2018). First detection of a SDH variant with reduced SDHI sensitivity in *Phakopsora pachyrhizi*. *Journal of Plant Diseases and Protection* 125, 21-26.

Yorinori JT; Paiva WM; Frederick RD; Costamilan LM; Bertagnolli PF; Hartman GL; Godoy CV; Nunes Junior J (2005). Epidemics of soybean rust (*Phakopsora pachyrhizi*) in Brazil and Paraguay. *Plant Disease* 89, 675-677.

Fouché G et al, Assessing the risk of resistance selection towards QiI fungicides in *Zymoseptoria tritici*. In: Deising HB; Fraaije B; Mehl A; Oerke EC; Sierotzki H; Stammler G (Eds), "Modern Fungicides and Antifungal Compounds", Vol. IX, pp. 57-62. © 2020. Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-16-7

Assessing the risk of resistance selection towards quinone inside inhibitor fungicides (QiIs) in *Zymoseptoria tritici*

Fouché G[1,3], Debieu D[1], Young D[3], Meunier B[2], Walker A S[1] and Fillinger S[1]

[1] *UMR Bioger, INRA, AgroarisTech, Université Paris-Saclay, F- 78850 Thiverval-Grignon*

[2] *CNRS Institute for Integrative Biology of the Cell (I2BC), Avenue de la Terrasse, F-91198 Gif-sur-Yvette, France*

[3] *Corteva Agriscience, 9330 Zionsville Road, Indianapolis, IN 46268, USA*

Email: guillaume.fouche@inra.fr

ABSTRACT

Zymoseptoria tritici is a phytopathogenic fungus responsible for septoria tritici blotch (STB) on wheat. With high disease pressure, yield losses can reach 50%, making STB the first threat for wheat production in Europe. Chemical control remains the most effective way to control STB in the field, but generalized resistance towards benzimidazoles, sterol demethylation inhibitors (DMIs) and quinone outside inhibitors (QoIs), as well as emerging resistance to succinate dehydrogenase inhibitors (SDHIs), stress the need for greater diversity in efficient modes of action and improved resistance management. In this challenging context, the launch of fenpicoxamid is highly anticipated since it provides a novel mode of action in the cereal fungicide market. Fenpicoxamid targets the Qi site of complex III in the mitochondrial respiratory chain, and is unaffected by target site-based resistance to other chemistries. Introduction of fenpicoxamid needs to be accompanied by implementation of anti-resistance strategies to preserve its long-term effectiveness. Our project aims to assess the risk of resistance towards fenpicoxamid before its introduction in the field. We have developed an experimental evolution protocol to select mutants resistant to complex III inhibitors in the laboratory, with the goal of mimicking potential field selection conditions. Our findings may provide insights into potential resistance mechanisms for optimizing anti-resistance strategies in the field.

INTRODUCTION

Wheat is the most economically important cereal crop in the European Union. Several fungal diseases can affect wheat yields but the major threat in Western Europe is septoria leaf blotch or septoria tritici blotch (STB), caused by the ascomycete *Zymoseptoria tritici*. Yield losses caused by STB usually range between 10 to 20%, but can reach 50% in years when conditions for disease are favorable (Torriani *et al.* 2015). Chemical control using fungicides remains the

best way to control STB in the field. The cereal fungicide market in Europe represents more than $2 bn and almost half aims at controlling *Z. tritici* (Torriani *et al.* 2015). Unfortunately, due to the extraordinary plasticity of its genome, its ability to perform sexual reproduction and to spread over long distances through ascospore production, *Z. tritici* has developed resistance towards all currently known modes of action (MoA) (Garnault *et al.* 2019). With the urgent need for MoA diversity, Corteva Agriscience has developed fenpicoxamid (Inatreq™ active), which provides a new MoA for STB control. Fenpicoxamid is a derivative of UK-2A; a natural antifungal compound produced by *Streptomyces* sp. 517.02, and is converted back to UK-2A in *Z. tritici* and wheat (Owen *et al*. 2017). UK-2A inhibits mitochondrial electron transport, binding to cytochrome *b* (complex III) at the ubiquinone inner binding site (Qi). Quinone inner binding site inhibition represents a new MoA on cereals, and is the first new MoA for STB control since the SDHI boscalid, in 2007 (Garnault *et al.* 2019). As new modes of action need to be protected by smart application strategies, our project aims at assessing the risk of resistance development under fenpicoxamid selection pressure in *Z. tritici* populations, especially in the western European context. In order to mimic field conditions as closely as possible, we designed an experimental evolution protocol to select strains resistant to QiIs, and decipher the underlying resistance mechanism(s).

MATERIALS AND METHODS

Zymoseptoria tritici strains and growth media

The strains used in this study (hereafter ancestor strains) were the reference strain IPO323 (Goodwin *et al*. 2011) and two progeny strains, C1 and C4, of a cross between IPO323 and a multidrug resistant (MDR) type I strain (Omrane *et al.* 2017) carrying the *cytb*^{G143A} allele conferring QoI resistance (StrR). Strains C1 and C4 were both StrR; C4 was also MDR. Both genotypes are commonly found in *Z. tritici* western European populations (Garnault *et al*. 2019; Kildea *et al.* 2019). All strains were grown in YPD liquid or solid medium (10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose and 20 g/L agar if needed).

Determination of strains sensitivity

10^7 spores were incubated in 25 mL of liquid YPD at 17°C and 150 rpm, in the dark, with increasing concentrations of fungicide, for 7 days. On the last day, optical density (OD) at 405 nm was determined. The minimal inhibitory concentration (MIC) and the 50% effective concentration (EC50) of fenpicoxamid were determined graphically.

Experimental evolution

10^7 spores were incubated in 25 mL of liquid YPD at 17°C, 150 rpm, in the dark, with fenpicoxamid at two different selection concentrations: MIC and 25 times MIC (25MIC), and 600 µg/mL propylgallate to inhibit the alternative oxidase (AOX). Four independent lines of each ancestral strain were grown simultaneously for 7-day cycles for each concentration. New

cycles were started with a 0.5% (125 µL) transfer rate, and complemented with spores from the corresponding control lines to reach 10^7. At the end of each cycle, 150 µL of each line were plated on selective medium to detect resistant isolates. The experiment was stopped after 8 selection cycles.

Characterization of resistant isolates

Resistant strains were incubated in 25 mL of liquid YPD at 17°C and 150 rpm, in the dark, for 3 days. After determining OD at 560 nm and standardizing spore concentration, strains were plated on YPD agar plates with selecting compounds. Compounds used were: 30 µg/mL fenpicoxamid, 2 µg/mL antimycin A, 2 µg/mL tolnaftate, 2.5 µg/mL azoxystrobin, 100 µg/mL propylgallate, 100 µg/mL salicylhydroxamic acid (SHAM), and mixtures of fenpicoxamid and propylgallate or fenpicoxamid and SHAM at the same concentrations.

Total DNA extraction and *Cytb* sequencing

Fungal material, grown on YPD agar at 17°C, in the dark, for 7 days, was harvested in 600 µL of sarkosyl buffer (50 mM Tris HCl at pH 8, 50 mM EDTA, 150 mM NaCl, 2% sarkosyl) before grinding. Total DNA was extracted from the aqueous supernatant by 3 successive phenol and/or chloroform extraction steps and then precipitated by the addition of 50 µL of 3M sodium acetate at pH 5.2. DNA pellets were resuspended in 200 µL of water. *Cytb* PCR was then performed using the primers 5'CCTGACTGGTATCATATTGTGT3' and 5'TATATTACTAGGTTATTTTTCGTG3', giving a 1562 kb amplicon. Sequencing was performed by Eurofins genomics, Ebersberg, Germany.

RESULTS

Resistance selection

Fenpicoxamid sensitivity of the ancestor strains was used to establish selecting fungicide concentrations for experimental evolution. In our conditions, the susceptibility of both IPO323 and C1 to fenpicoxamid was the same and the MIC value was estimated at 0.4 µg/mL. Sensitivity of C4 was lower and the MIC value was estimated at 1.2 µg/mL, giving a 3-fold resistance factor (Table 1).

Table 1 Inhibitor concentrations (µg/mL) used for experimental evolution.

	Fenpicoxamid		
Strain	**MIC**	**25MIC**	**Propyl gallate**
IPO323	0.4	10	600
C1	0.4	10	600
C4	1.2	30	600

Selection method of the fenpicoxamid resistant strains by experimental evolution is illustrated in Fig. 1. After 8 selection cycles, more than 100 strains were isolated, displaying normal growth on fenpicoxamid-containing YPD, but only 36 strains showed stable resistance after several subcultures on fenpicoxamid-amended agar plates. All of these 36 strains derived from the IPO323 ancestor strain.

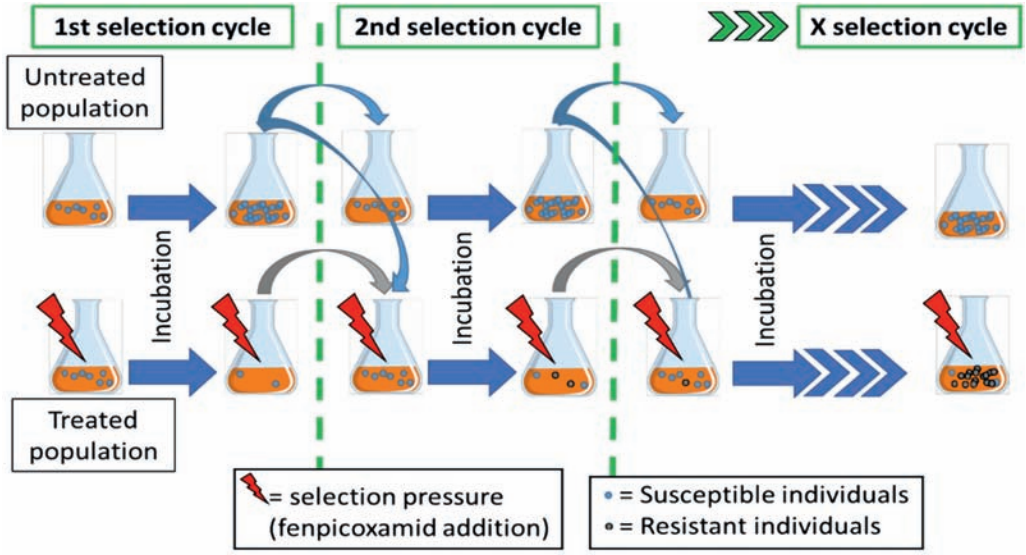


Figure 1 Selection of fenpicoxamid resistant *Z. tritici* strains by experimental evolution.

Resistance mechanism determination

In order to test the strains rapidly for their putative resistance mechanism(s), we performed a droplet growth test on YPD agar plates (Fig. 2). Resistant strains grew normally on fenpicoxamid-containing plates but were inhibited by azoxystrobin, meaning there was no cross-resistance between fenpicoxamid and the QoI. All strains were able to grow on medium containing fenpicoxamid with an AOX inhibitor, either SHAM or propylgallate, excluding AOX overexpression as a resistance mechanism. A similar test was performed on tolnaftate-amended plates, a fungicide used to discriminate MDR strains from others. The fenpicoxamid resistant strains did not grow on tolnaftate, showing that resistance was also not due to MDR.

Cytb gene sequences displayed no polymorphism except for one single point mutation that led to the substitution of glycine in position 37 by valine. This amino acid is located at the ubiquinone inner binding site (Qi site) and the G37V change was the only one found in all resistant strains selected by experimental evolution.

Sensitivity testings showed high levels of fenpicoxamid resistance in the G37V mutants. At 2 µg/mL fenpicoxamid, they showed growth similar to that on the control medium, indicating a resistance factor of at least 60. Resistance factors for antimycin A, another natural QiI fungicide, were calculated as approximately 10.

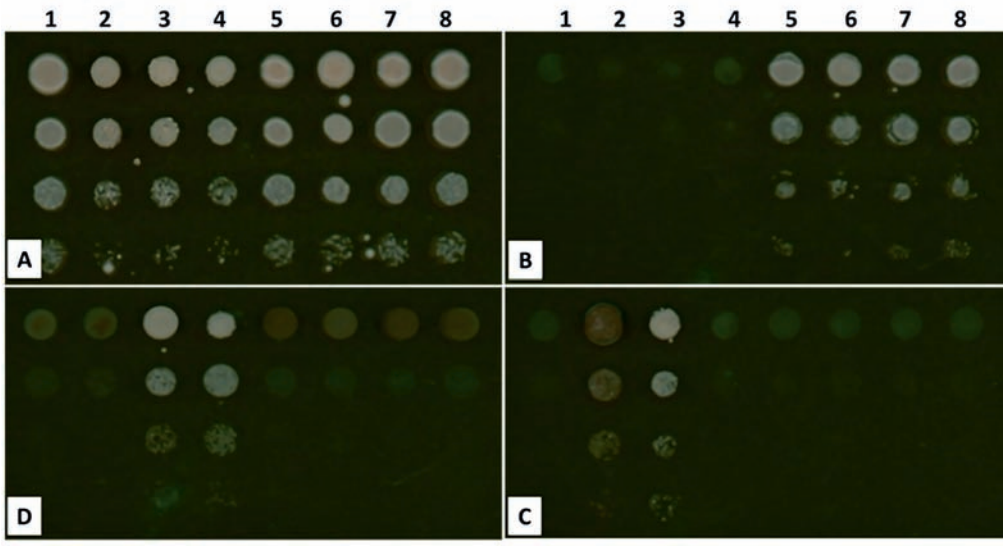


Figure 2 Strain growth on different selecting media reflecting their putative resistance mechanism. Strains obtained from experimental evolution displayed normal growth on fenpicoxamid but no growth on azoxystrobin or tolnaftate. 1: IPO323; 2: C1; 3: C4; 4: MDR strain; 5 to 8: strains from experimental evolution; A: YPD; B: 30 µg/mL fenpicoxamid; C: 2.5 µg/mL azoxystrobin; D: 2 µg/mL tolnaftate.

DISCUSSION

Experimental evolution provides a powerful new tool to assess the risk of resistance selection and the associated resistance mechanisms in *Z. tritici*. We have shown that the G37V change at the Qi site of cytochrome *b* is the most probable resistance mechanism to be developed by *Z. tritici* under fenpicoxamid selection pressure. However, conditions of experimental evolution do not allow us to predict if, and how fast, this mutation could actually be selected under field conditions. Initial data suggest that the G37V change does not significantly affect *in vitro* growth of *Z. tritici* strains (data not shown), but more precise measurements need to be performed. Pathogenicity and fitness of these strains, as well as efficiency of respiration and bc_1 complex activity should also be explored to assess carefully whether strains containing the $Cytb^{G37V}$ allele are likely to compete effectively under field conditions. Since most of the European *Z. tritici* population is resistant to strobilurin fungicides containing the G143A change in cytochrome *b*, another important question requiring study concerns the fitness of strains containing both G37V and G143A changes.

At the Qi binding site, glycine 37 is very near UK-2A in its proposed binding pose, being located in close proximity to the exocyclic methyl group (3.79 Å) and ester tail (4.06 Å) of the bislactone ring of UK-2A. The substitution of glycine by valine introduces a much bigger isopropyl group, which may interfere with binding of UK-2A by steric hindrance. Cross-resistance to antimycin A in the G37V mutants is consistent with the overall similar binding pose for antimycin A and UK-2A at the Qi site, and proximity of G37 to antimycin A (Young *et al*. 2018).

Glycine 37 is known as a key residue associated with mutations conferring resistance to Qi site ligands in lab mutants. In the yeast *Saccharomyces cerevisiae*, G37V confers resistance to

antimycin A, G37D or G37S confer resistance to ilicicolin H, while G37C confers resistance to both antimycin A and fenpicoxamid (Mounkoro *et al.* 2018). The G37V exchange has also been reported in a strain of *Z. tritici* selected for resistance to antimycin A (Fehr *et al*. 2015), although its reported resistance factor of >100 differs from the ~10-fold loss in sensitivity found in our study.

CONCLUSION

Fenpicoxamid is a QiI fungicide acting on complex III of the mitochondrial electron transport chain. It provides a new MoA for STB control and is not cross-resistant to current products. Using experimental evolution, we identified the G37V change in cytochrome *b* as the predominant resistance mechanism in laboratory mutants. The likelihood of selection under field conditions remains unknown. Future work will explore the impact of G37V on strain pathogenicity and fitness at both the organism and enzyme level. These insights into resistance mechanisms should help to guide anti-resistance strategies in the field, keeping in mind that evolution of resistance, and especially target site resistance, is always case specific and influenced by application strategies.

REFERENCES

Fehr M; Wolf A; Stammler G (2016). Binding of the respiratory chain inhibitor ametoctradin to the mitochondrial bc_1 complex. *Pest Manag Sci*. 72, 591-602.

Garnault M; Duplaix C; Leroux P; Couleaud G; Carpentier F; David O; Walker AS (2015). Spatiotemporal dynamics of fungicide resistance in the wheat pathogen *Zymoseptoria tritici* in France. *Pest Manag Sci.* 75(7), 1794-1807.

Goodwin SB; M'Barek SB; Dhillon B; Wittenberg AH; Crane CF; *et al.* (2011). Finished genome of the fungal wheat pathogen *Mycosphaerella graminicola* reveals dispensome structure, chromosome plasticity, and stealth pathogenesis. *PLoS Genet.* 7(6).

Kildea S; Bucar DE; Hutton F; de la Rosa S; Welch TE; Phelan S (2019). Prevalence of QoI resistance and mtDNA diversity in the Irish *Zymoseptoria tritici* population. *Irish J Agr Food Res.* 58, 27-33.

Mounkoro P; Michel T; Benhachemi R; Surpateanu G; Iorga I B; Fisher N; Meunier B (2018). Mitochondrial complex III Qi site inhibitor resistance mutations found in laboratory selected mutants and field isolates. *Pest Manag Sci.* 75, 2107–2114.

Omrane S; Audéon C; Ignace A; Duplaix C; Aouini L; Kema G; Walker AS; Fillinger S (2017). Plasticity of the *MFS1* Promoter Leads to Multidrug Resistance in the Wheat Pathogen *Zymoseptoria tritici*. *mSphere* 2(5): e00393-17.

Torriani SFF; Melichar JPE; Mills C; Pain N; Sierotzki H; Courbot M (2015). *Zymoseptoria tritici*: A major threat to wheat production, integrated approaches to control. *Fungal Genet. Biol.* 79, 8-12.

Young D; Wang N; Meyer S; Avila-Adame C (2018). Characterization of the mechanism of action of the fungicide fenpicoxamid and its metabolite UK-2A. *Pest Manag Sci.* 74, 489–498.

Koehler et al., Characterization of the *cytb* gene structure in *Macrophomina phaseolina* to assess fungicide resistance risk. In: Deising HB; Fraaije B; Mehl A; Oerke EC; Sierotzki H; Stammler G (Eds), "Modern Fungicides and Antifungal Compounds", Vol. IX, pp. 63-68. © 2020. Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-16-7

Characterization of the *cytb* gene structure in *Macrophomina phaseolina* to assess fungicide resistance risk

Koehler AM[1], Rogers LW[2], Shew HD[2]

[1]*University of Delaware, 16483 County Seat Highway, Georgetown, DE 19947, USA*
[2]*North Carolina State University, 100 Derieux Place, Raleigh, NC 27695, USA*
Corresponding Author Email: akoehler@udel.edu

ABSTRACT

Macrophomina phaseolina, the causal agent of charcoal rot, is a soilborne fungal pathogen widespread throughout the southern United States. The phase-out of preplant fumigants such as methyl bromide has led to increased incidence of *M. phaseolina* in numerous commercial crops and there is a need for new management options. Quinone outside inhibitor (QoI) fungicides are frequently used to control many pathogens of field crops and may be useful for management of charcoal rot. Due to the site-specific mode of action of QoI fungicides, multiple fungi have developed varying levels of resistance to QoIs based on mutations that occur at amino acid positions 129, 137, and 143 in the cytochrome b (*cytb*) gene. Primers were developed to determine the structure of the *cytb* gene in *M. phaseolina* and assess the likelihood of mutation development associated with QoI fungicide resistance. *M. phaseolina* isolates used in this study were collected from *Stevia rebaudiana*, an herbaceous perennial new to the US. None of the isolates screened in this study contained base pair mutations at position 129, 137, or 143. In the *cytb* gene structure, an intron was identified following the 143-position indicating a reduced risk for mutation and subsequent fungicide resistance at this position.

INTRODUCTION

Macrophomina phaseolina is a destructive, soilborne fungal pathogen with a host range of over 500 plant species. *M. phaseolina* is an Ascomycete in the family Botryosphaeriaceae and is the causal agent of many canker diseases including charcoal rot. Infection begins as light gray, spindle-shaped lesions that form on stems and eventually coalesce to lead to wilting and necrosis of the plant (Kaur 2012). In the early stages of infection, small black microsclerotia form on the base of the plant. Following plant death and decay, microsclerotia are deposited back into the soil to accumulate and are able to persist in the soil for up to 15 years (Short 1980). Significant damage by this pathogen has been reported in major crops such as maize, sorghum, strawberry, cotton, and soybean (Islam et al. 2012). Rapid accumulation of inoculum each year in the soil can be an issue for perennial crops (Ammon et al. 1974). *Stevia rebaudiana*, an herbaceous perennial native to Paraguay with leaves containing glycosides around 300 times sweeter than sucrose, is rapidly emerging as a new crop in the United States (US). As products containing

stevia glycosides expand globally, there has been interest in establishing commercial production of stevia in the Southeastern US. Stevia was first planted in North Carolina (NC) in 2011, and *M. phaseolina* was first isolated from stevia roots in 2016 (Koehler & Shew 2018).

Currently, there are no fungicides registered for management of *M. phaseolina* on stevia in the US, including quinone outside inhibitor (QoI) fungicides, which inhibit fungal growth by binding the quinol oxidation (Qo) site of the mitochondrial cytochrome bc_1 complex of the electron transport chain to halt ATP production. Resistance mutations to QoI fungicides were first identified in *Blumeria graminis* isolates (Bartlett 2002). Since their initial discovery, QoI fungicide resistance mutations have been reported in over 20 genera of fungal pathogens including *Alternaria alternata*, *Botrytis cinerea, Mycosphaerella graminicola*, *Rhizoctonia solani*, and *Venturia inaequalis* (FRAC 2012). Three point mutations in the *cytb* gene have been identified that confer varying levels of resistance to QoI fungicides. A substitution from glycine to alanine at codon 143 (G143A) confers complete resistance to QoIs. Substitutions from phenylalanine to leucine at 129 (F129L), and glycine to arginine at 137 (G137R) confer partial resistance to QoIs. Repeated use of QoI fungicide applications to control other fungal pathogens may inadvertently place selection pressure on *M. phaseolina* populations. In order to evaluate the resistance potential of *M. phaseolina* to QoI fungicides, this study examined a partial fragment of the *cytb* gene of *M. phaseolina* isolated from stevia. The objectives of this project were to develop primers that amplified the region of the *cytb* gene that allowed for screening of SNPs and other gene elements associated with QoI fungicide sensitivity.

MATERIALS AND METHODS

Isolate collection

Stevia plants exhibiting symptoms of *M. phaseolina* infection were collected from fields in Kinston and Rocky Mount, NC that received QoI fungicide applications to manage other fungal pathogens in 2015 and 2016. Symptomatic roots were washed under tap water, pulled from the root crown, and cut into 2 cm pieces. Root pieces were surface disinfected in 0.825% NaOCl for 30 s, rinsed with sterile deionized H_2O, and plated onto potato dextrose agar (PDA, Difco) amended with streptomycin (100 $\mu g\ mL^{-1}$) and penicillin G (100 $\mu g\ mL^{-1}$). Isolates were grown for seven days at room temperature and actively growing hyphae with characteristics of *M. phaseolina* were transferred to new PDA Petri dishes.

DNA extraction and isolate confirmation

Three 6.75 mm plugs of 40 putative *M. phaseolina* isolates were transferred into 20 mL of potato dextrose broth (PDB, Difco). The isolates were grown for five days at ~21°C and the mycelial mat was aspirated, transferred into 1.5 mL microcentrifuge tubes, and lyophilized. Genomic DNA was extracted by grinding tissue with Qiagen cell lysis solution and 3 µL of Proteinase K and incubated at 55°C for 1 h. Samples were then treated with RNase (3 µL) and incubated for 30 min at 37°C before adding Qiagen protein precipitation solution (Qiagen, Hilden, Germany).

The sample was added to isopropanol to precipitate DNA and washed with 70% ethanol. DNA was eluted with 100 µL of sterile dH_2O and analyzed by spectrophotometry.

rDNA internal transcribed spacer (ITS) regions ITS1, 5.8S rRNA, and ITS2 were amplified by polymerase chain reaction (PCR) using primers *ITS4/5* (White et al. 1990). PCR reactions were 50 µL in volume and consisted of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 µM each dNTPs, 4 µM of each primer, 1.25 U of Taq polymerase (Applied Biosystems, Branchburg, NJ, USA), and 80 ng of genomic DNA. Thermal cycling parameters included an initial denaturation at 95°C for 3 min, followed by 34 cycles of DNA denaturation at 95°C for 30 s, primer annealing at 55°C for 30 s, and primer extension at 72°C for 1 min. A final extension at 72°C for 10 min completed the cycling parameters. Amplified products were cleaned using ExoSap-IT (Affymetrix® Thermo Fisher Scientific, Waltham, MA, USA). Samples were sent for sequencing to Eton Bioscience Inc. (Research Triangle Park, NC, USA). Sequences were identified through a BLAST search in the GenBank database (National Center for Biotechnology Information). Analysis of sequence data was completed using CLC Main Workbench 7.7 (CLC Bio: a Qiagen Company, Germany).

RNA isolation and cDNA synthesis

Three 6.75 mm plugs of ten *M. phaseolina* isolates were grown on PDB as described above. Total RNA was extracted from each sample using the Qiagen RNeasy Mini Kit according to manufacturer's instructions (Qiagen, Hilden, Germany). First-strand complementary DNA (cDNA) was synthesized from 1 µg of total RNA from each sample using the Invitrogen ThermoScript reverse-transcription polymerase chain reaction system following manufacturer's instructions (Invitrogen, Thermo Fisher Scientific, Carlsbad, CA).

Primer development, amplification, and characterization of *cytb* gene fragments

A *M. phaseolina* genome scaffold (accession number: AHHD00000000.1) was imported into CLC Main Workbench. Conserved *cytb* sequences from *Botrytis cinerea* (accession number: KP795071.1) and *Venturia inaequalis* (accession number: LC279540.1) were used to design primers to amplify the conserved regions of the *cytb* gene in *M. phaseolina* that would allow for detection of amino acid positions 129, 137 and 143. The primer pairs were designed to determine different aspects of the *cytb* gene structure in *M. phaseolina* (Table 1).

Macrophomina phaseolina cDNA was PCR amplified using primer set *Mpcytb_F1/R1* according to manufacturer's instructions in the SuperScript™ IV First-Strand Synthesis System PCR amplification protocol (Invitrogen, Thermo Fisher Scientific, Carlsbad, CA, USA). PCR reactions using primer sets *MPCB_143F/Mpcytb_R1* and *MPCB_FV2/RV2* were 20 µL in volume with final concentrations of 1X of 2X Phusion Master Mix, 0.5 µM each of 10 µM primers, 0.6 µL DMSO, and 100 ng of template DNA. Thermal cycling parameters included an initial denaturation at 98°C for 30 s, followed by 30 cycles of denaturation at 98°C for 5 s, primer annealing (Table 1) for 30 s, and primer extension at 72°C for 1.5 min. A final extension at 72°C for 8 min completed the cycling parameters. PCR products were visualized on a 1%

agarose gel and cleaned using ExoSap-IT (Affymetrix® Thermo Fisher Scientific, Waltham, MA, USA). Samples were sent for sequencing to Eton Bioscience Inc. (Research Triangle Park, NC, USA). Using the *M. phaseolina* genome scaffold, cDNA PCR products of the *M. phaseolina cytb* gene were aligned to determine exon, intron, and amino acid positions using CLC Main Workbench 7.7 (CLC Bio: a Qiagen Company, Germany).

Table 1. Primers developed to characterize *cytb* gene fragments.

Primer Pairs	Primer	Primer sequence 5′ to 3′	Annealing temperature (°C)	Orientation
cDNA	Mpcytb_F1	GTGATTGACTCACCTCAACC	53	Forward
	Mpcytb_R1	CTTGTCCAACTCAAGGTATAGC	53	Reverse
Intron	MPCB_143F	ACTAATGGGTGGCTGAA	62	Forward
	Mpcytb_R1	CTTGTCCAACTCAAGGTATAGC	62	Reverse
gDNA screening	MPCB_FV2	CTTACACATAGGGAGAGGTC	55	Forward
	MPCB_RV2	CCGTAGGCAGTCTACTGACT	55	Reverse

RESULTS

Molecular characterization of *M. phaseolina* isolates

In total, 40 isolates of *M. phaseolina* were collected from sites in both Kinston and Rocky Mount, NC spanning four years (2016-2019). Genomic DNA was successfully extracted from all isolates and nucleotide sequences of the ITS region shared 100% sequence identity with *M. phaseolina* (accession number: KY680345.1).

Primers *Mpcytb_F1/R1* successfully amplified a single 400 bp cDNA amplicon spanning amino acid positions 27 through 159 of the *cytb* gene in each of the isolates. The cDNA sequences of each isolate shared 100% homology with the coding regions annotated in the *M. phaseolina* reference genome (accession number: AHHD00000000.1). Intron and exon positions were determined by aligning the cDNA amplicon sequences to the reference genome. An intron was identified (1099 bp) immediately downstream of codon 143 (Figure 1).

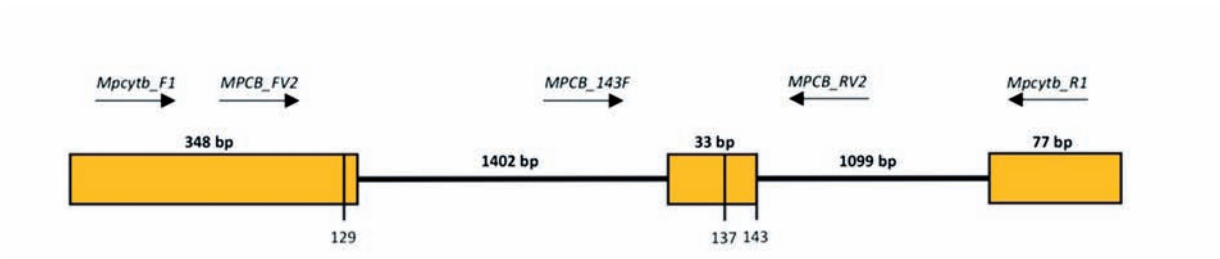


Figure 1 *M. phaseolina cytb* gene structure and primer binding sites

Primers *MPCB_143F/Mpcytb_R1* amplified a ~1200 bp fragment of gDNA covering amino acid positions 137 and 143 and spanning the third exon of the gene for detection of group I introns. Group I introns were found in all isolates studied, and a BLASTp search of the intronic sequences revealed a conserved protein motif belonging to the LAGLIDADG endonuclease superfamily for all isolates sequenced.

Primers *MPCB_FV2/RV2* amplifed a ~1100 bp fragment of gDNA spanning positions 129, 137 and 143. No base pair mutations were identified in any of the isolates screened.

DISCUSSION

QoI fungicides are considered to have a high risk of losing antifungal efficiency (FRAC 2019), with an increasing number of phytopathogenic fungi developing mutations conferring resistance. Due to the wide host range of *M. phaseolina* and widespread use of QoIs to control other fungal pathogens, *M. phaseolina* populations in stevia fields have likely been exposed to QoIs. To date, no studies have examined the *cytb* gene of *M. phaseolina* for resistance development to QoI fungicides.

This study investigated a fragment of the *cytb* gene that corresponds to amino acid positions 27 through 159 of the *cytb* protein in *M. phaseolina*. None of the mutations associated with QoI resistance were identified in the *M. phaseolina* isolates used in this study. An important discovery of this project was a 1099 bp intron identified immediately downstream of the 143 position. Several studies have correlated the presence of an intron immediately next to the 143 position with a decreased risk of G143A mutation occurrence (Standish 2016; Hily 2011). Mutation development seems to be decreased due to the splice-site sequences found at the intron/exon junctions of genes. Within the *cytb* gene, the 143 codon contains the splice site of the adjacent group I intron. If a mutation was to occur here, this would lead to incorrect mRNA processing and be lethal to the organism. Additionally, the translated group I intron sequences obtained from this study contained the LAGLIDADG endonuclease protein motif. This type of endonuclease, although uncommon, is usually found within group I introns near conserved nucleotides that correspond to functionally critical amino acids of cellular genes, like the 143 codon of the *cytb* (Celis et al. 2017). While the presence of an intron following position 143 reduces the chance of G143A mutation, fungal *cytb* introns encoding an endonuclease are frequently lost and gained during evolutionary events due to their ability to cleave DNA, and move laterally across genomes (Yin et al. 2012). This may suggest that while the position of this intron decreases the chance of G143A mutation development, it is possible for pathogens to lose these introns. Therefore, it is still critical for fungicide resistance management strategies to be followed. Future research should determine the prevalence of this intron in other *M. phaseolina* populations, and monitor its movement throughout the genome if products containing QoI components are ever labelled for control of *M. phaseolina.*

REFERENCES

Ammon V; Wyllie TD; Brown Jr MF (1974). An ultrastructural investigation of pathological alterations induced by *Macrophomina phaseolina* (Tassi) Goid in seedlings of soybean, Glycine max (L.) Merill. *Physiological Plant Pathology* 4(1), 1-4.

Celis JS; Edgell DR; Stelbrink B; Wibberg D; Hauffe T; Blom J; Kalinowski J; Wilke T (2017). Evolutionary and biogeographical implications of degraded LAGLIDADG endonuclease functionality and group I intron occurrence in stony corals (Scleractinia) and mushroom corals (Corallimorpharia). *PLoS One* 12(3), e0173734.

FRAC (2012). List of pathogens with field resistance towards QoI fungicides. *Fungicide Resistance Action Committee*, online publication. https://www.frac.info/docs/default-source/working-groups/sdhi-fungicides/group/list-of-resistant-plant-pathogens_2012-edition.pdf.

FRAC (2019). FRAC Code List 2019: Fungicides sorted by mode of action. *Fungicide Resistance Action Committee*, online publication. https://www.frac.info/docs/default-source/publications/frac-code-list/frac-code-list-2019.pdf.

Hily JM; Singer SD; Villani SM; Cox KD (2011). Characterization of the cytochrome b (cyt b) gene from Monilinia species causing brown rot of stone and pome fruit and its significance in the development of QoI resistance. *Pest Management Science* 67(4), 385-396.

Islam MS; Haque MS; Islam MM; Emdad EM; Halim A; Hossen QMM; Hossain MZ; Ahmed B; Rahim S; Rahman MS; Alam MM (2012). Tools to kill: genome of one of the most destructive plant pathogenic fungi *Macrophomina phaseolina*. *BMC Genomics* 13(1), 493.

Kaur S; Dhillon GS; Brar SK; Vallad GE; Chand R; Chauhan VB (2012). Emerging phytophathogen *Macrophomina phaseolina*: biology, economic importance and current diagnostic trends. *Critical Reviews in Microbiology* 38(2), 136-151.

Koehler AM; Shew HD (2018). First report of charcoal rot of stevia caused by *Macrophomina phaseolina* in North Carolina. *Plant Disease* 102(1), 241-241.

Short GE; Wyllie TD; Bristow PR (1980). Survival of Macrophomina phaseolina in soil and in residue of soybean. *Survival* 7(13), 17.

Standish JR; Avenot HF; Brenneman TB; Stevenson KL (2016). Location of an intron in the cytochrome b gene indicates reduced risk of QoI fungicide resistance in Fusicladium effusum. *Plant Disease* 100(11), 2294-2298.

White TJ; Bruns T; Lee SJWT; Taylor JL (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *PCR protocols: a guide to methods and applications* 18(1), 315-322.

Yin LF; Hu MJ; Wang F; Kuang H; Zhang Y; Schnabel G; Li GQ; Luo CX (2012). Frequent gain and loss of introns in fungal cytochrome b genes. *PLoS One* 7(11), e49096.

Dowling M et al., Eight years of fungicide resistance monitoring of *Botrytis cinerea* from United States strawberry fields. In: Deising HB; Fraaije B; Mehl A; Oerke EC; Sierotzki H; Stammler G (Eds), "Modern Fungicides and Antifungal Compounds", Vol. IX, pp. 69-74. © 2020. Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-16-7

Eight years of fungicide resistance monitoring of *Botrytis cinerea* from United States strawberry fields

Dowling M[1]; Fernández-Ortuño[2] D; Hu M[3]; Schnabel G[1]

[1]*Clemson University, 105 Collings Street/217 BRC, 29634, Clemson, USA*

[2]*Universidad de Málaga, Departamento de Microbiología, Facultad de Ciencias, 29071, Malaga, Spain*

[3]*University of Maryland, Department of Plant Science and Landscape Architecture, 20742, College Park, USA*

Corresponding Author Email: schnabe@clemson.edu

ABSTRACT

Resistance management practices preserve chemical efficacy over time, but growers often lack the information or training required to implement them appropriately. In response to grower-reported lack of fungicide efficacy in the field, a yearly resistance monitoring program began in 2012 for *Botrytis cinerea* on strawberry. The primary purpose was to identify potential resistance issues in existing spray programs, provide grower education, and modify resistance management strategies if needed. Over 50 strawberry farms across the United States sent blossom samples for testing and hyphal growth was analyzed on artificial media amended with discriminatory doses of fungicides representing Fungicide Resistance Action Committee (FRAC) codes most commonly used for gray mold management in the field. Results and management recommendations were then provided to growers. Monitoring led to (1) identification of FRAC codes becoming ineffective due to widespread, qualitative resistance, (2) grower awareness of the need for implementing resistance management strategies, and (3) adjustments in resistance management recommendations. Through the monitoring service, *Botrytis fragariae*, a species with higher frequencies and levels of resistance to fludioxonil, was first detected in the USA.

INTRODUCTION

Gray mold of strawberry drives the spray program for strawberry producers in the southern United States. The pathogen, *Botrytis cinerea,* enters the field on transplants used in plasticulture production every fall (Oliveira et al. 2017; Peres 2015; Schnabel et al. 2015) and mixes with local populations spreading from decaying plant tissue. The fungus can quickly adapt to fungicide pressure, and the presence of fungicide resistant phenotypes has been frequently detected in nurseries (Schnabel et al. 2015) and local populations (Fernández-Ortuño et al. 2014). Applications of fungicides start at the nursery level, to generate healthy planting stock,

are resumed in production fields in fall, prior to putting on row covers, and in spring, to protect flowers and fruit from infection. Unless growers spray multi-site fungicides such as captan or thiram, there is no assurance that control will occur when fungicides at risk for resistance development are applied. Resistance to multiple FRAC codes is often already present in fields (Fernández-Ortuño et al. 2014) and lack of control by these FRAC codes can occur even if they have not yet been applied that season.

To provide growers with an idea of the fungicide resistance profile at their location, we assessed sensitivity to 7 different classes of fungicides in early spring using isolates from frost-damaged flowers. The testing required a minimum of six but most often 10 samples (isolates) per field. While this sample size was insufficient for a scientific population analysis, it made sample collection feasible for growers and county agents and also provided them with useful information. If resistance to a specific FRAC code was detected in multiple isolates from a single field within the ten samples, this resistance was present and likely widespread in the field. The grower was then advised to use different FRAC codes in the spray program for that year. Furthermore, growers were exposed to the concepts of FRAC codes and resistance management, providing an educational component to the project.

MATERIALS AND METHODS

Resistance profiling was conducted as described previously (Li et al. 2014). Briefly, flowers with a black torus were submitted to the lab, petals were removed, the tori were surface sterilized, and incubated for 2 to 3 days in a moist chamber (Fig. 1).

Figure 1 Processing profile samples: **(A)** flowers from the field were **(B)** received **(C)** sepals were removed and flowers were sterilized then **(D)** placed in a moist chamber **(E)** spores were collected from the surface of blossoms and **(F)** transferred to a discriminatory dose of fungicide amended media **(G)** growth was rated and results sent to the grower and/or county agent.

Spores emerging from conidiophores were carefully transferred with a toothpick to fungicide-amended growth medium and mycelial growth was assessed in relation to an untreated control (Fig. 2). Results were shared with the grower and regional county agent and disease and

fungicide resistance management recommendations were provided based on the results. Also, the recommendations explained that resistance profiles were likely to change with each following application of a site-specific product.

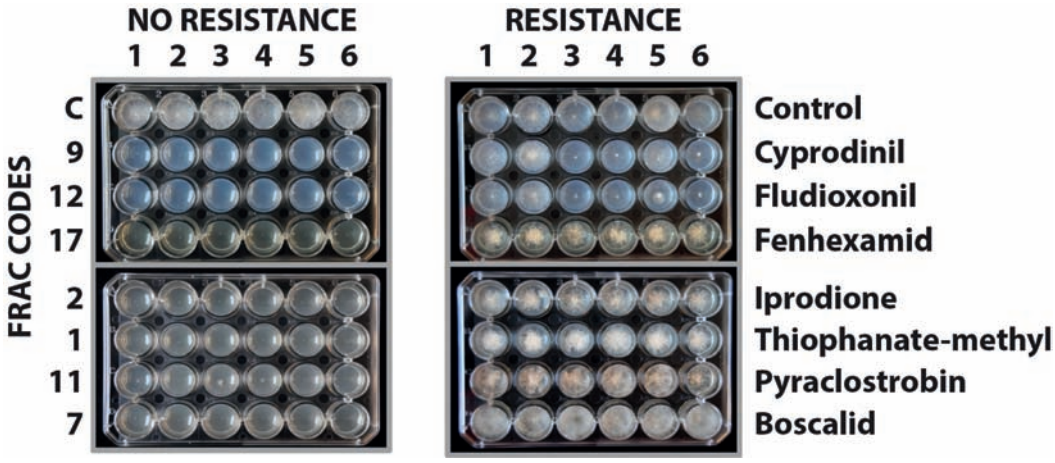


Figure 2 An example of profile plates containing six fully sensitive isolates (left) and six fully resistant isolates (right). FRAC codes and fungicides used for testing are also provided.

RESULTS AND DISCUSSION

During the monitoring program (2012 to 2019), over 50 farms were profiled and 5,359 individual *Botrytis cinerea* isolates tested. A total of 16 states were represented, including: Florida, Georgia, South Carolina, North Carolina, Virginia, Alabama, Louisiana, Missouri, and Arkansas from the Southern United States; Maryland, Pennsylvania, Delaware, and Connecticut from the Northeast; Ohio and Michigan from the Midwest; and California from the West Coast. Growers and specialists provided overwhelmingly positive feedback with statements such as "This service is not good…. It is excellent," "Thank you for the screen results of the fungicides on *Botrytis*. It is very informative and helps in making a decision on spray materials...we have a better chance at efficacy!" and "It (the resistance profile service) has saved me a lot of money personally from not buying a chemical that I would have bought otherwise. It is a great service you provide." Clearly, growers benefited from this program by learning to use the FRAC code system for resistance management. While *B. cinerea* was by far the most common species isolated from flowers, some cultures did not match the description of this species. Nucleotide sequence analysis of *G3PDH*, *NEP2*, *NEP1*, *HSP60*, and *RPB2* gene regions revealed some isolates belonging to *B. fragariae, B. caroliniana,* and *B. mali* species (Dowling et al. 2017a; Dowling & Schnabel 2017b; Fernández-Ortuño et al. 2012).

One of the most striking initial results was the high frequency of resistance to thiophanate-methyl (Fig. 3). Almost 70% of isolates were resistant to thiophanate-methyl in 2012 and this level was maintained across experimental years despite growers discontinuing use of FRAC 1 fungicides in 2013. This illustrates the selective power of the fungicide and stability of this phenotype even in the absence of selection pressure. Later, we confirmed that thiophanate-methyl was present in virtually every multi-fungicide resistant phenotype. Therefore, resistance

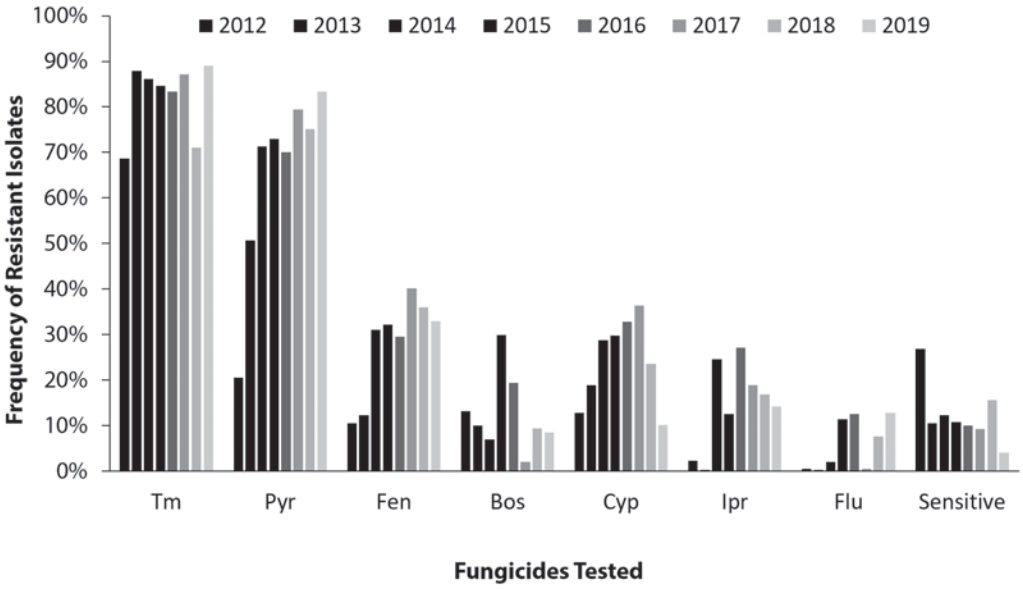


Figure 3 Frequency of resistance to the fungicides Thiophanate-methyl (Tm), Pyraclostrobin (Pyr), Fenhexamid (Fen), Boscalid (Bos), Cyprodinil (Cyp), Iprodione (Ipr), and Fludioxonil (Flu) in the 8 years that strawberry flower samples were profiled.

continued to be selected in the following seasons not directly by any application of thiophanate-methyl, but indirectly by 'selection by association' (Hu et al. 2016). Another striking result was the rapid increase of resistance to QoI fungicides (FRAC 11) for the first three years, and the maintenance of high resistance frequencies for the remainder of the monitoring program. Although FRAC 11 fungicides are not technically 'botryticides,' they do have suppressive action on sensitive isolates and are therefore used in tank mixtures with FRAC 7 products to help slow down selection for this fungicide class. Our results clarified that this resistance management strategy was no longer working and that the SDHIs were completely 'unprotected' with regard to resistance selection in *B. cinerea*. Furthermore, spraying the 7/11 mixture for gray mold control had become ineffective and potentially detrimental in the absence of other pathogens controlled by QoI fungicides (such as powdery mildew, leaf spots, or anthracnose).

Resistance frequencies to fenhexamid, cyprodinil, iprodione, and boscalid, were lower than those of thiophanate-methyl or QoI fungicides and remained relatively stable over the 8 years of monitoring. Frequencies ranged from 10% (or less) to peaks of up to 40%. For some products, such as fenhexamid, the resistance frequencies quickly rose, but then plateaued at approximately 30 to 40%. For others (i.e. cyprodinil, boscalid, and iprodione) the frequencies rose but then dropped back down to acceptable levels (10 to 20%) where a single use of the product might still do more good than harm in managing the disease. For fenhexamid and iprodione, the management of resistance frequencies may have been due to a tighter restriction on the number of applications per season (one) compared to label recommendations. The stable and low frequency of resistance to boscalid can perhaps be explained by the unique and not always consistent action of FRAC 7 fungicides. FRAC 7 products continue to be applied multiple times per season. However, new FRAC 7 products have been released that do not necessarily select for the same resistance genotypes. For example, boscalid and fluxapyroxad select for isolates with *H272Y*, *H272R*, and *H272L* mutations in the *SDHB* gene, but fluopyram and isofetamid do

not. It is, therefore, possible that the introduction of next generation SDHIs, such as fluopyram and isofetamid, helped de-select isolates with resistance to boscalid due to incomplete cross resistance (Ishii and Hollomon 2015).

Fludioxonil continues as a grower favorite with its high efficacy against gray mold and anthracnose. This action against both diseases is particularly important since anthracnose outbreaks can wipe out an entire crop under conducive conditions. Fludioxonil use has increased significantly in southern strawberry fields with up to 5 applications per season. However, resistance frequencies remained low (at about 10%) over the course of this study. It is still unclear whether isolates resistant to fludioxonil experience a fitness penalty. If they do, it could explain the observed results. The fungicide is sold in combination with cyprodinil (FRAC 9), which may also contribute to resistance management. Resistance to cyprodinil has remained around 10 to 20%. We do believe, however, that it is of utmost importance to tightly regulate how often this combination product is applied each season. The current, regional recommendation (by specialists) is to use it no more than twice per season rather than the maximum allowed by the label of 4 to 5 applications (depending on dose) and to rotate this product with multi-sites or with other FRAC codes.

In contrast to our concerns at the beginning of this study, the frequency of isolates with multi-fungicide chemical class resistance (CCR) has stabilized over time (Fig. 4). At first, we saw a dramatic increase in the frequency of isolates with resistance to 3 and 4 FRAC codes (3CCR; 4CCR) and emergence of 5CCR, 6CCR, and 7CCR isolates. However, the number of 5CCR isolates has returned to low levels in recent years and frequencies of 6 and 7CCR did not increase for our sampled isolates. One possible explanation is that the monitoring program recommendations were successful and resulted in stricter control of the number of applications

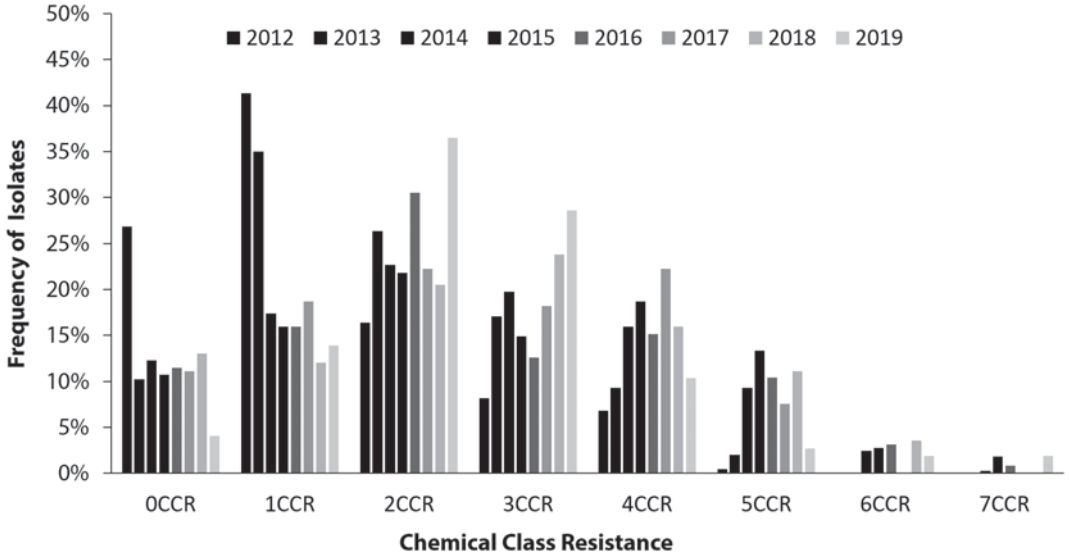


Figure 4 Frequency of isolates with differing chemical class resistance (CCR), where 0CCR refers to isolates sensitive to all 7 chemical classes tested and 7CCR refers to isolates with resistance to all 7 chemical classes tested in the 8 years that strawberry flower samples were profiled.

per season for each FRAC code, a larger focus on using multi-site fungicides such as thiram and captan, and avoidance of FRAC codes that were projected to be of little use due to very high resistance frequencies. Another is that the more chemical classes an isolate becomes resistant to, the more likely it is for fitness penalties to occur. A previous study, conducted under controlled conditions, indicated substantial competitive disadvantages of 5CCR and 7CCR isolates compared to 0CCR isolates (Chen et al. 2016).

REFERENCES

Chen SN; Luo CX; Hu MJ; Schnabel G (2016). Fitness and competitive ability of Botrytis cinerea isolates with resistance to multiple chemical classes of fungicides. Phytopathology 106, 997–1005.

Dowling ME; Hu MJ; Schnabel G (2017a). Identification and characterization of Botrytis fragariae isolates on strawberry in the United States. Plant Disease 101, 1769–1773.

Dowling ME; Schnabel G (2017b). First report of Botrytis mali causing gray mold on strawberry in the United States. Plant Disease 101, 1034.

Fernández-Ortuño D; Grabke A; Bryson PK; Amiri A; Peres NA; Schnabel G (2014). Fungicide resistance profiles in Botrytis cinerea from strawberry fields of seven Southern U.S. States. Plant Disease 98, 825–833.

Fernández-Ortuño D; Li X; Wang F; Schnabel G (2012). First report of gray mold of strawberry caused by Botrytis caroliniana in North Carolina. Plant Disease 96, 914.

Hu MJ; Cox KD; Schnabel G (2016). Resistance to increasing chemical classes of fungicides by virtue of "selection by association" in Botrytis cinerea. Phytopathology 106, 1513-1520.

Stammler G; Wolf A; Glaettli A; Klappach K (2015). Respiration inhibitors: complex II. In Fungicide Resistance in Plant Pathogens: Principles and a Guide to Practical Management; eds H Ishii and D W Hollomon; pp. 105-117. Springer: Tokyo.

Li X; Fernández-Ortuño D; Chen S; Grabke A; Luo CX; Bridges WC; Schnabel G (2014). Location-specific fungicide resistance profiles and evidence for stepwise accumulation of resistance in Botrytis cinerea. Plant Disease 98, 1066–1074.

Oliveira MS; Amiri A; Zuniga AI; Peres NA (2017). Sources of primary inoculum of Botrytis cinerea and their impact on fungicide resistance development in commercial strawberry fields. Plant Disease 101, 1761–1768.

Peres NA (2015). 2015 Florida Plant Disease Management Guide: Strawberry. http://edis.ifas.ufl.edu/pg056 (date of access 08.08.2016).

Schnabel G; Hu M; Fernández-Ortuño D (2015). Monitoring resistance by bioassay: Relating results to field use using culturing methods. In Fungicide Resistance in Plant Pathogens: Principles and a Guide to Practical Management; eds. H Ishii and D W Hollomon, pp. 281–293. Springer: Tokyo.

Khan M F R, Managing *Cercospora beticola* on Sugar Beet with Fungicides. In: Deising HB; Fraaije B; Mehl A; Oerke EC; Sierotzki H; Stammler G (Eds), "Modern Fungicides and Antifungal Compounds", Vol. IX, pp. 75-78. © 2020. Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-16-7

Managing *Cercospora beticola* on Sugar Beet with Fungicides

Khan M F R

North Dakota State University and University of Minnesota. Fargo, North Dakota, 58104-6050, USA

Email: Mohamed.khan@ndsu.edu

INTRODUCTION

Cercospora beticola causes Cercospora leaf spot (CLS) which is one of the most damaging foliar disease of sugar beet worldwide. The disease reduces tonnage, increases impurities leading to lower sugar concentration, and reduces recoverable sucrose while slowing the sugar extraction process resulting in higher processing costs (Shane and Teng 1992; Khan and Smith 2005). Roots of diseased plants do not store well in storage piles that are processed in a 7 to 9 month period in North Dakota and Minnesota (Smith and Ruppel 1973). CLS is managed by planting improved *C. beticola* resistant varieties, using crop rotation, planting away from the previous sugar beet crop, and fungicide applications (Miller et al. 1994; Khan et al 2007). It is difficult to combine high levels of Cercospora leaf spot resistance with resistance to other major sugar beet diseases and produce high yielding varieties (Smith and Campbell, 1996). Consequently, commercial varieties generally have moderate levels of resistance to *C. beticola* and require fungicide applications to obtain adequate levels of protection against CLS and economic returns (Miller et al. 1994).

During the period 1999 to 2014, growers in the US typically used single site mode of action fungicides such as tetraconazole, pyraclostrobin and triphenyl tin hydroxide in a rotation program to effectively control CLS. In 2015, a few fields in the Minn-Dak Factory District had fungicide failures, especially after use of a quinone outside inhibitor (QoI) fungicide. Secor et al. (2015) reported that the *C. beticola* population collected from North Dakota and Minnesota had reduced sensitivity to triphenyltin hydroxide, thiophanate methyl and QoI fungicides.

C. beticola infected leaves were collected from fields with fungicide failure in 2015, dried and used as inoculum in 2016. The objective of this research was to evaluate the efficacy of fungicides to control a mixed population of *C. beticola* with reduced sensitivity and/or resistance to fungicides.

MATERIALS AND METHOD

Field trial was conducted near Foxhome, MN in 2016. The experimental design was a randomized complete block with four replicates. Field plots comprised of six 9 m long rows spaced 56 cm apart. Plots were planted on 24 April with SesVanderHave 633RR sugar beet

seeds. Seeds were treated with Tachigaren (45 g/100,000 seeds), Poncho beta, and Kabina to protect against *Aphanomyces cochlioides*, insect pests, and *Rhizoctonia solani*, respectively. Seed spacing within rows was 12 cm. Weeds were controlled with two applications (27 May and 7 June) of glyphosate. Azoxystrobin fungicide was applied to all plots on 22 May to control *Rhizoctonia solani*. Plots were inoculated on 29 June with grounded *C. beticola* inoculum (Khan et al. 2009) collected from growers' fields in the Foxhome area where the pathogen was not effectively controlled during the previous growing season.

Fungicide spray treatments were applied with a CO_2 pressurized 4-nozzle boom sprayer with 11002 TT TwinJet nozzles calibrated to deliver 159 liter ha^{-1} of solution at 414 kPa pressure to the middle four rows of plots. Fungicide treatments were initiated on 1 July with three additional fungicide applications on 20 July, 3 and 17 August. Treatments were applied at rates indicated in Table 1.

Cercospora leaf spot severity was rated on the leaf spot assessment scale of 1 to 10 (Jones and Windels, 1991). A rating of 1 indicated the presence of 1- 5 spots/leaf or 0.1% disease severity and a rating of 10 indicated 50% or higher disease severity. Cercospora leaf spot severity was assessed during the season and the ratings provided were taken on 1 September, 2016.

Plots were defoliated mechanically and harvested with a mechanical harvester on 28 September. The middle two rows of each plot were harvested, weighed for root yield, and 12 to 15 representative roots from each plot, not including roots on the ends of the plot, were analyzed for quality at the American Crystal Sugar Company Quality Tare Laboratory, East Grand Forks, MN. Statistical analysis was conducted using the SAS general linear models (Proc GLM) procedure (Version 9.4, SAS Institute Inc.; Cary, NC, USA) and treatment means for root yield, sucrose concentration, sugar loss to molasses and recoverable sucrose were separated by calculating Fisher's Least Significant Difference (LSD) at 95% confidence level. CLS ratings were analyzed by non-parametric analysis where mean rank was calculated by running the SAS procedures of Proc Rank and Proc Mixed.

RESULTS AND DISCUSSIONS

Environmental conditions were favorable for good plant growth which led to row closure early as well as rapid development of *C. beticola* in early July. Cercospora leaf spot progressed very rapidly in the non-treated check and reached economic injury level by early-August. At the beginning of September, the non-treated check had severe disease and a Cercospora leaf spot rating of 10 where all the mature leaves were killed and with regrowth of new leaves (Table 1). All fungicide treatments provided significantly greater disease control compared to the non-treated check in July (data not shown). However, by 2 August Topsin (thiophanate methyl) and Priaxor (pyraclostrobin + fluxapyroxad) became ineffective at controlling CLS, followed by Badge (copper oxychloride + copper hydroxide), Kocide (copper hydroxide) and Manzate (mancozeb) around 1 September. It should be noted that the Manzate and Priaxor treatments did result in significantly higher recoverable sucrose that the non-treated check suggesting that they did provide some level of protection especially early in the season. The fungicides that were

Table 1. Efficacy of four applications of fungicides used alone or in mixtures at controlling CLS on sugar beet yield, quality and recoverable sucrose at Foxhome, MN, 2016

Treatments[a]	**Rate (L/ha)**	**CLS Rating (1-10)**[b]	**Yield (t/ha)**	**Sucrose concen-tration (%)**	**Sugar Loss to Molasses (%)**	**Recoverable Sucrose (kg/ha)**
Non-inoculated Check		10.0	58.5	11.1	1.9	5363.4
Agri Tin	0.6	8.5	70.8	12.9	1.7	7894.4
Topsin	1.5	10.0	61.7	11.5	1.8	5892.9
Topsin	0.7	10.0	56.7	11.6	1.7	5572.3
Manzate	3.7	9.8	66.8	12.3	1.6	7182.2
Eminent	0.9	9.3	70.2	12.5	1.7	7466.8
Inspire XT	0.5	8.8	71.1	11.9	1.9	7169.7
Proline	0.4	8.0	73.5	12.9	1.8	8192.8
Priaxor	0.5	10.0	62.1	12.1	1.7	6470.7
Badge SC	0.5	10.0	59.4	11.5	1.7	5823.4
Kocide 3000	2.2	9.5	63.4	11.3	1.9	5943.0
Agri Tin + Topsin	0.4 + 0.6	9.3	69.5	12.1	1.5	7353.5
Agri Tin + Eminent	0.4 + 0.7	7.0	69.5	12.3	1.8	7319.6
Agri Tin Proline	0.4 + 0.3	6.0	76.2	13.3	1.6	9218.5
Agri Tin + Inspire XT	0.4 + 0.4	6.5	72.6	13.6	1.6	8692.6
Agri Tin + Priaxor	0.4 + 0.4	8.3	67.7	12.3	1.7	7158.1
Topsin + Proline	0.6 + 0.3	8.3	72.6	13.0	1.6	8318.4
Minerva Duo	1.2	7.0	77.8	13.7	1.6	9341.1
Manzate + Inspire XT	3.0 + 0.4	7.8	73.8	12.6	1.7	7972.3
Priaxor + Badge SC	0.4 + 0.4	9.3	59.0	12.1	1.6	6158.0
Badge SC + Inspire XT	3.7 + 0.4	9.0	61.0	11.7	1.8	6038.6
Manzate + Priaxor	3.0 + 0.4	8.8	66.8	11.9	1.6	7069.6
LSD (0.05)		0.7	7.8	0.9	0.3	1116.0

[a] Treatments were applied on 1, 20 July, 3 and 17 August.

[b] CLS rating was done on a 1 to 10 scale where 1 indicated no leaf infection and 10 indicated that all mature leaves were killed with regrowth of new leaves.

most effective at significantly reducing disease severity and increasing recoverable sucrose when used alone compared to the check were Agri Tin (triphenyltin hydroxide), Proline (prothioconazole), Eminent (tetraconazole), Inspire XT (difenoconazole + propiconazole) and Manzate. Fungicide mixtures (Agri Tin and Proline, Agri Tin and Inspire XT, Minerva Duo which is a pre-mix of triphenyltin hydroxide and tetraconazole, and Inspire XT and Manzate) provided better disease control and higher recoverable sucrose.

This research indicated that triphenyltin hydroxide, triazoles (prothioconazole, difenoconazole + propiconazole) and the ethylene-bis-dithiocarbamate (mancozeb) fungicides provided effective control when used alone and in mixtures. Since the field population of *C. beticola* has reduced sensitivity and or resistance to the major fungicides used for its control (Secor et al. 2015), the use of mixtures in a rotation program at about 14 days intervals should provide effective control of CLS. The second fungicide application in this trial was delayed by five days because of wet field conditions. It is believed that if the second applications were done in a timely manner, disease control would have been better especially for fungicide mixtures. As such, in field conditions where ground rigs could not be operated because of unfavorable field conditions, it is advisable to use aerial applicators (where legal, as in the USA) so as as to provide protection when necessary.

REFERENCES

Jones RK, Windels CE (1991). A management model for Cercospora leaf spot of sugarbeets. Minnesota Extension Service. University of Minnesota. AG-FO-5643-E.

Khan J; del Rio LE; Nelson R; Khan MFR (2007). Improving the Cercospora leaf spot management model for sugar beet in Minnesota and North Dakota. *Plant Dis*. 91, 1105-1108.

Khan J; Qi A; Khan MFR (2009). Fluctuations in the number of *Cercospora beticola* conidia in relationship to environment and disease severity in sugar beet. *Phytopathology* 99, 796-801.

Khan MFR; Smith LJ (2005). Evaluating fungicides for controlling Cercospora leaf spot on sugarbeet. *J. Crop Prot*. 24, 79-86.

Miller SS; Rekoske M; Quinn A (1994). Genetic resistance, fungicide protection and variety approval policies for controlling yield losses from Cercospora leaf spot infection. *J. Sugar Beet Res*. 31, 7-12.

Secor G; Rivera V; Bolton M; Khan MFR (2015). Sensitivity of *Cercospora beticola* to foliar fungicides in 2015. 2015 Sugarbeet Res. Ext. Rep. 46, 128-136.

Shane WW; Teng PS (1992). Impact of Cercospora leaf spot on root weight, sugar yield and purity. *Plant Dis*. 76, 812-820.

Smith GA; Campbell LG (1996). Association between resistance to Cercospora and yield in commercial sugarbeet. *Plant Breed*. 115, 28-32.

Smith GA; Ruppel EG (1973). Association of Cercospora leaf spot, gross sugar, percentage sucrose and root weight in sugarbeet. *Can. J. Plant Sci*. 53, 695-696.

Massi F et al., Temporal analysis of mandipropamid resistance in *Plasmopara viticola* populations isolated from grapevine fields treated according to anti-resistance strategies. In: Deising HB; Fraaije B; Mehl A; Oerke EC; Sierotzki H; Stammler G (Eds), "Modern Fungicides and Antifungal Compounds", Vol. IX, pp. 79-84. © 2020. Deutsche Phytomedizinische Gesellschaft, Braunschweig,
ISBN: 978-3-941261-16-7

Temporal analysis of mandipropamid resistance in *Plasmopara viticola* populations isolated from grapevine fields treated according to anti-resistance strategies

Massi F[1], Maddalena G[1], Bianco PA[1], Borsa P[2], Coatti M[2], Sierotzki H[3], Torriani SFF[3], Toffolatti SL[1]

[1]*Università degli Studi di Milano, Dipartimento di Scienze Agrarie e Ambientali; via Celoria 2; 20133 Milano; Italy*

[2]*Syngenta Italia Spa, Milan, Italy*

[3]*Syngenta Crop Protection Münchwilen AG, Stein, Switzerland*

Email: federico.massi@unimi.it

ABSTRACT

Downy mildew, caused by the oomycete *Plasmopara viticola*, is one of the major diseases affecting grapevine cultivation worldwide. Chemical control is unavoidable in regions characterized by a high disease pressure, such as those of Northern Italy. In this study, the effectiveness of two anti-resistance strategies in real field conditions was analyzed. Two spray programs, including or not the CAA fungicide mandipropamid, were applied in two commercial vineyards with different initial *P. viticola* populations (sensitive and resistant) over six seasons (2013–2018). The programs were compared in terms of disease control and fungicide sensitivity of the pathogen by a combination of biological and molecular (G1105S/V mutations) assays on different structures (oospores and sporangia). Both programs showed an equally good field performance, even if the one including mandipropamid in mixture did not completely prevent an increase in the frequency of insensitive strains. On the other side, wide fluctuations in the G1105S/V mutations occurred through time due to recombination. Therefore, the characterization of the pathogen population in field is crucial for an appropriate planning of the mixtures and to keep anti-resistance strategies effective.

INTRODUCTION

Grapevine downy mildew is one of the most devastating diseases of *Vitis vinifera* and represents a threat to viticulture in regions characterized by high precipitation rate and mild temperatures. *Plasmopara viticola*, the causal agent of grapevine downy mildew, is an obligate parasite and a polycyclic pathogen, able to undergo numerous infection cycles during a single growing season if weather conditions are favorable. The primary infections are a consequence of the germination of the oospores, the overwintering structures differentiated by sexual reproduction, whereas the

secondary cycles depend on the differentiation of asexual structures (sporangia). Disease management requires the application of up to 20 fungicide treatments per season, therefore single site modes of action are under strong risk of selecting resistant individuals. The carboxylic acid amide (CAA) fungicide class, which includes dimethomorph, flumorph, pyrimorph, iprovalicarb, benthiavalicarb, valifenalate and mandipropamid, is commonly employed in spray programs to control *P. viticola* (Gisi et al. 2012). Resistance to CAAs is associated with two SNPs (Single Nucleotide Polymorphism) at codon 1105 of gene *PvCesA3*, which determine the substitution of glycine with serine (mutation G1105S) or valine (mutation G1105V) in the aminoacidic sequence (Sierotzki et al. 2011; Blum et al. 2012). The resistance character is recessive, and only homozygous individuals for a single mutation or heterozygous individuals for the two mutations are resistant (Blum et al. 2010; Toffolatti et al. 2018). For a correct evaluation of the proposed anti-resistance strategies, it is necessary to gather information on the dynamics of pathogen populations in real field conditions. In this study, two anti-resistance strategies, differing only for including or not the CAA mandipropamid, were compared over six seasons (2013-2018) to evaluate the efficacy in controlling the disease and the diffusion of resistant strains in the pathogen population.

MATERIALS AND METHODS

Field assay

The commercial vineyards, named 'R' and 'S', are located in two northern Italian regions. *P. viticola* strains resistant to mandipropamid were already present in the R vineyard, while only sensitive strains were initially present in the S vineyard. Starting in Spring 2013, each vineyard was divided into three plots. The first plot (untreated plot) was not treated with fungicides against downy mildew. The other two plots (1 ha each) were treated against downy mildew by using strategies differing only for including (strategy A) or not (strategy B) commercial products containing mandipropamid in mixture with an anti-resistance partner (Table 1).

Disease assessment was performed each year at berry touch in the three plots by estimating the disease severity (I%I) (Toffolatti et al. 2018) to calculate the protection index, I%P (Abbott 1925). The effectiveness of the two strategies was compared by ANOVA on I%Ps.

Sensitivity and molecular assays

P. viticola populations were tested for sensitivity to mandipropamid by sampling infected leaves in different periods of the growing season: 1) autumn; 2) spring; 3) early summer; and 4) late summer. Oospores were collected at sampling period 1, sporangia at sampling periods 2-4. Sensitivity was assessed as described by Toffolatti and coworkers (2018) through oospore germination assays and experimental inoculations of sporangia on leaf discs. The EC_{50} value, *i.e.* the concentration of fungicide able to inhibit the oospore germination or the pathogen sporulation by 50% compared to the control, were calculated for oospores and sporangia

Table 1 List of the treatments performed in vineyard at each phenological stage

Treatment	Phenological stage	Strategy A	Strategy B
1	Shoot length 10 cm	metiram	metiram
2	5-6 leaves unfolded	mandipropamid+mancozeb	cymoxanil+mancozeb
3	Inflorescence development	metalaxyl M+mancozeb	metalaxyl M+mancozeb
4	Pre-bloom/bloom	metalaxyl M+mancozeb	metalaxyl M+mancozeb
5	Fruit set	mandipropamid+zoxamide*	ametoctradin+metiram
6	Fruits swelling	mandipropamid+zoxamide	ametoctradin+metiram
7	Before berry touch	mandipropamid+zoxamide	ametoctradin+metiram
8	Berry touch	copper oxychloride	copper oxychloride
9	Berry touch	copper oxychloride	copper oxychloride

*Treatment not performed in 2015 and moved at stage 3 from 2016 to 2018

samples, respectively. Molecular assays were carried out on oospores and sporangia, following the method described by Toffolatti and coworkers (2018), to calculate the percentages of the three allelic variants associated to sensitivity (G1105) or resistance (S1105 or V1105).

RESULTS

Field trials

Generally, a very high disease severity (48<I%I<100%) occurred on leaves of the untreated plots of both vineyards as a consequence of the high precipitation rates (rainfall rate=244-521 mm) occurring from April to July (Table 2). In S vineyard, disease severity was lower than 18 % on both leaves and bunches only during 2016 and 2017, when low precipitation rates and frost damages occurred, respectively. On bunches, disease severity showed very high values in R vineyard (I%I>66 %) and fluctuated between 10 and 90% in the S vineyard.

Table 2 Rainfall amount (mm) and disease severity (I%I) of the untreated plots of R and S vineyards.

Year	R vineyard			S vineyard		
	Rainfall (mm)	I%I leaves	I%I bunches	Rainfall (mm)	I%I leaves	I%I bunches
2013	406	66	97	278	48	70
2014	521	92	95	255	50	10
2015	376	92	96	159	85	90
2016	411	100	100	134	18	16
2017	450	90	100	204	0.6	0
2018	302	91	95	244	71	76

Despite the high disease pressure, the protection indices of the two strategies were always greater than 80% on both leaves and bunches (Figure 1), and no significant differences were found among the efficacy of strategy A and strategy B (P>0.05).

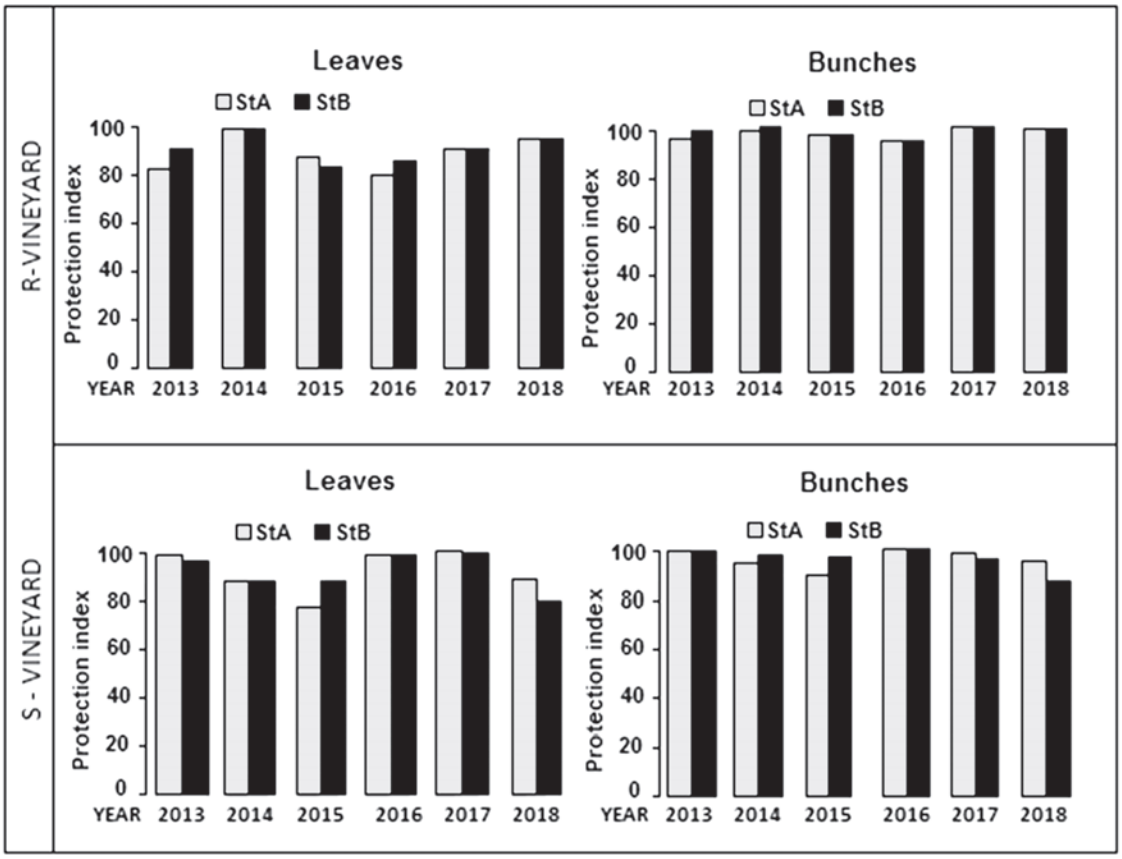


Figure 1 Protection index of the strategy A (StA) and B (StB)

Sensitivity and molecular assays

In R vineyard, all the samples collected during the period of investigation have always shown EC_{50} values above the threshold for resistance (10 mg/L) (Table 3). Only two samples collected from the untreated and strategy B plots in 2015 and 2016 showed EC_{50} values typical of sensitivity. Molecular assays showed the presence of the V1105 allele at the highest frequency (Table 3), indicating the potential presence of resistant homozygous strains. A wide range in the percentages of the three allelic variants could be observed, even if the percentage of the wild type allele (G1105) was generally very low.

The EC_{50} values recorded in S-vineyard showed that *P. viticola* population remained fully sensitive to mandipropamid in the untreated and strategy B plots during the whole period (Table 3). In these plots, the wild type allele G1105 predominated over the others (Table 3). In contrast, in the strategy A plot, the population started to show EC_{50} values higher than 10 mg/L from 2014 onwards. Molecular assays in this plot showed that resistance is mainly due to G1105S mutation.

Table 3 Sensitivity profiles, expressed in terms of EC_{50} values and percentage of allelic variants encoding for glycine (G), serine (S) and valine (V), of *P. viticola* populations in the three plots of R and S vineyards

Plot	Year	SP	R vineyard				S vineyard			
			EC_{50}	%G	%S	%V	EC_{50}	%G	%S	%V
Untreated	2013	2	>100	8	13	78	0.29	88	10	1
	2013	3	>100	8	12	81	0.11	100	0	0
	2013	1	>100	11	28	61	0.001	85	5	10
	2014	2	>100	10	74	16	0.02	99	1	0
	2014	3	>100	6	2	92	1.7	89	11	0
	2014	1	>100	22	76	1	0.1	79	21	0
	2015	2	>100	12	76	12	0.3	85	8	7
	2015	3	53.6	12	8	81	0.7	58	28	14
	2015	4	>100	20	1	79	0.8	72	11	17
	2015	1	0.01	44	46	10	7.8	85	5	10
	2016	2	>100	21	17	61	0.1	98	2	0
	2016	3	0.2	31	57	12	0.05	99	1	0
	2016	1	64.1	2	4	94	–	–	–	–
	2017	2	>100	–	–	–	–	–	–	–
	2017	3	>100	–	–	–	0.02	–	–	–
	2018	2	>100	0	1	99	28.7	42	40	18
	2018	3	>100	0	4	96	15.6	44	34	22
Strategy A	2013	3	>100	2	5	93	0.03	96	4	0
	2013	1	>100	10	72	19	0.2	79	21	1
	2014	4	>100	6	5	89	>100	26	74	0
	2014	1	>100	39	47	14	>100	14	74	12
	2015	3	>100	16	5	79	>100	24	60	15
	2015	4	>100	4	8	88	0.14	52	31	18
	2015	1	>100	8	16	76	>100	24	64	12
	2016	3	90.9	5	20	75	>100	37	57	6
	2016	1	–	2	6	92	–	–	–	–
	2017	3	200	–	–	–	>100	–	–	–
	2018	3	>100	2	4	94	>100	1	72	27
Strategy B	2013	3	>100	10	20	70	0.0005	100	0	0
	2013	1	>100	14	52	34	0.2	56	33	11
	2014	4	>100	6	2	92	4.5	99	0	1
	2014	1	>100	14	81	5	0.04	86	6	8
	2015	3	27.1	34	14	53	0.1	86	9	6
	2015	4	>100	12	1	87	1	98	1	1
	2015	1	0.01	32	16	52	0.08	68	27	5
	2016	3	8.8	45	41	14	0.005	86	6	8
	2016	1	NC	0.73	3.96	95.32	–	–	–	–
	2017	3	>100	–	–	–	0.001	–	–	–
	2018	3	>100	9.7	3.8	86.5	0.2	75.8	14.6	9.6

CONCLUSIONS

At the beginning of the experimental activity, *P. viticola* population was different in the two vineyards considered: in S-vineyard it was composed by sensitive strains, whereas in R vineyard resistant strains were already present. Despite this, anti-resistance strategies proved to be effective, as demonstrated by the statistically analogous protection observed in the treated plots. The temporal analysis of *P. viticola* populations showed that resistance in R vineyard did not change over time and was mainly related to the V1105 allele. On the contrary, in the mandipropamid-treated plot of S vineyard, the pathogen populations shifted from sensitivity to resistance, that was associated with S1105 allele, during the second year of the fungicide application. Overall, these results indicate that anti-resistance strategies are highly effective in controlling the disease but do not prevent the selection of resistant strains in *P. viticola* population, that can be characterized by different mutations in different locations. Characterizing the pathogen population in field is crucial for an appropriate planning of the mixtures and to keep anti-resistance strategies effective.

REFERENCES

Abbott WS (1925). A method for computing the effectiveness of an insecticide. *Journal of Economic Entomology* 18, 265-267.

Blum M; Waldner M; Gisi U (2010). A single point mutation in the novel *PvCesA3* gene confers resistance to the carboxylic acid amide fungicide mandipropamid in *Plasmopara viticola*. *Fungal Genetics and Biology* 47, 499–510.

Blum M; Gamper HA; Waldner M; Sierotzki H; Gisi U (2012). The cellulose synthase 3 (*CesA3*) gene of oomycetes: structure, phylogeny and influence on sensitivity to carboxylic acid amide (CAA) fungicides. *Fungal Biology* 116, 529–542.

Gisi U (2012). Resistance to Carboxylic Acid Amide (CAA) Fungicides and Anti-Resistance strategies. In: *Fungicide resistance in crop protection: risk and management,* ed TS Thind, pp-191-207. CABI: Wallingford.

Sierotzki H; Blum M; Olaya G; Waldner-Zulauf M; Buitrago C; Wullschleger J; Cohen Y; Gisi U (2011). Sensitivity to CAA fungicides and frequency of mutations in cellulose synthase (*CesA3*) gene of oomycete pathogen populations. In: *Modern Fungicides and Antifungal Compounds VI*, eds HW Dehne, U Gisi, KH Kuck, PE Russell, & H Lyr, pp. 103-110. DPG-Verlag, Braunschweig

Toffolatti SL; Russo G; Campia P; Bianco PA; Borsa P; Coatti M; Torriani SFF; Sierotzki H (2018). A time-course investigation of resistance to the carboxylic acid amide mandipropamid in field populations of *Plasmopara viticola* treated with anti-resistance strategies. *Pest Management Science* 74, 2822–2834.

Lichtemberg PSFet al., With stable sensitivity and not clear disadvantages, the management of SDHI resistant mutant of Alternaria alternata will rely on an effective pistachio spray program. In: Deising HB; Fraaije B; Mehl A; Oerke EC; Sierotzki H; Stammler G (Eds), "Modern Fungicides and Antifungal Compounds", Vol. IX, pp. 85-90. © 2020. Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-16-7

With stable sensitivity and unclear disadvantages, the management of SDHI resistant mutant of *Alternaria alternata* will rely on an effective pistachio spray program

Lichtemberg PSF, Carraro TA, Doussoulin H, Velasco J, Puckett R, Michailides TJ

University of California Davis at Kearney Agricultural Research and Extension Center, 9240 South Riverbend Avenue, Parlier, California, 93648, United States

Corresponding Author Email: plichtemberg@ucdavis.edu

INTRODUCTION

The Alternaria late blight (ALB) disease of pistachio is mainly caused by *Alternaria alternata* and affects not only fruit quality but also orchard productivity over time. Management of ALB is performed with two or three fungicide applications, made between June and July. Among the different fungicide groups used, succinate dehydrogenase inhibitors (SDHI) are prevalent and represented by four active ingredients (a.i.): boscalid, fluxapyroxad, penthiopyrad and fluopyram. Resistance by *A. alternata* to SDHI was observed only a few years after their introduction and has been associated with multiple mutations of three Sdh subunits: Sdh-B, -C and -D (Avenot et al. 2014; Lichtemberg et al. 2018a). Currently, 54% of the population of pistachio *A. alternata* possess some mutation associated with SDHI resistance (Lichtemberg et al. 2018b). Considering this, efforts to manage resistance are necessary to extend the useful life of registered products and to maintain disease levels below the economic threshold. Fitness stability and the ability of different sensitivity genotypes to compete are key elements for determining whether resistant mutants will decrease in frequency within the population and if fungicide applications will remain effective. Ishii (2015) described several similar studies where different Sdh mutations and pathogens have different effect on fitness. Our objectives are, to determine the fitness cost and *in vitro* competitiveness of the $SdhB^{H277Y}$, $SdhC^{H134R}$ and wild-type (WT) genotypes in the absence of SDHI, evaluate the population dynamic after discontinuing SDHI sprays in the field, and test the multi drug resistance (MDR) of *A. alternata* genotypes.

MATERIAL AND METHODS

Isolate Collection

The isolate set 1 was used to evaluate the fitness stability and competition capacity of three *A. alternata* genotypes (H134R, H277Y and wild-type-WT). For each genotype group, three isolates were used. The isolate set 2 included 12 isolates for each genotype group and were used for the multi-drug resistance studies. The isolates were collected from commercial pistachio

orchards in California and were previously identified and single-spore purified prior to using in the experiments below.

Fitness

The fitness components included the area under mycelial growth curve (AUMGC), sporulation, germination, and pathogenicity on detached pistachio leaves. Evaluations occurred after the first and twelfth successive transfer *in-vitro* on acidified potato dextrose agar (APDA) plates. Each isolate was tested in triplicates and experiments were performed twice. AUMGC was determined by measuring the colony size over the course of 10 days and calculating the area using the trapezoidal rule (Leader 2004). Sporulation was determined with a hematocytometer from two 10 µl droplets per combination of isolate and repetition. Germination frequency was tested after 6 hours incubation of spore suspensions spread onto the surface of a 2% water agar plate (WA) at 23ºC. Pathogenicity in pistachio leaves was evaluated after 10 days incubation by measuring the lesion size. For that, spore suspensions were adjusted to 10^3 spores/ml and 20 µl was used to inoculate 30 wounded leaves placed inside humidified plastic containers at 23ºC.

Competition *in-vitro*

The H277Y, H134R and WT competitiveness was compared during 5 sucessive transfers *in-vitro*. For that, spores were harvested and adjusted to 10^3 spores/ml. Suspensions were grouped by genotype at a 1:1 proportion. Subsequently, 1:1 mixtures of H277Y:WT and H134R:WT were provided. Pure genotype suspensions were used as controls. Mixtures and controls were plated onto APDA media without fungicide and incubated over 7-days at 23ºC. Spores were harvested and used to start a new cycle. At the end of each cycle, the frequency of spores exhibiting a resistant phenotype was estimated by a germination assay on WA plates amended with boscalid at 10 µg/ml. At that dose, resistant spores were those that formed a germ tube.

Discontinuing SDHI from field spray program

One commercial orchard of Kerman pistachio planted in 1989 was used to evaluate the effect of discontinuing SDHI for three years starting in 2016. Twice a year (early-May and early-September), we randomly collected leaf samples from a ten-acre plot where SDHI was discontinued. Up Subsequently, up to 24 colonies of *A. alternata* were identified, single-spored, and genotyped for the presence of H277Y and H134R using the AS-PCR method (Avenot et al. 2008; Lichtemberg et al. 2018a).

Multi-Drug Resistance *in-vitro*

The sensitivity of three genotypes (H277Y, H134R and WT) were estimated with the mycelium growth assay using the spiral gradient dilution method proposed by Förster et al (2004) and modified by Torres-Londoño et al. (2016). The commercial formulations tested were: Gem (Bayer Crop Science LP), PhD (Arysta LifeScience North America LLC), Quash (Valent USA Coorporation), Scala (Bayer Crop Science LP) and Bravo Weather Stik (Syngeta). All fungicides were diluted in water to prepare stock solution of 10,000 mg of a.i. per ml.

Statistics

Fitness was analysed by the correspondent linear model fitted for AUMGC, sporulation, germination and lesion size with effects tested by ANOVA to evaluate the significance of genotype and transfer. The false discovery rate method (FDR) was used to separate the means of AUMGC (powered 2 transformed), sporulation (logarithmic transformed), germination (powered 2 transformed) and lesion size (not transformed). Competition studies analysed the main transfer effect on genotype mixtures and controls with ANOVA and the Fisher's LSD test was used to separate the means. Only descriptive statistics were made for isolates recovered from the test plot where SDHI was discontinued. MDR had their effects tested by ANOVA and means separated by the Fisher's LSD test (logarithmic transformed) for genotypes. For all statistical inferences, P value <0.05 was considered as the nominal significance level. The statistical software R (version 3.2.1335) was used for all analysis.

RESULTS

Fitness

The H134R resistant genotypes were significantly affected for AUMGC (P=0.049), sporulation (P =0.049), germination (P=7×10^{-4}) and lesion size (P=8×10^{-11}). After the 12th transfer, increased AUMGC and lesion size by 12% and 41% were respectively observed. Moreover, its sporulation and germination were reduced by 63% and 8.5%, respectively (Fig. 1). The H277Y genotype showed no transfer effect on AUMGC (P=0.147), lesion size (P=0.194) or germination (P=0.509). Only the sporulation was reduced (P=0.001) to 79% capacity within 12 transfers (Fig. 1B). No WT fitness parameters were affected by the successive transfer *in-vitro.*

Competition *in-vitro*

After one transfer cycle in the absence of SDHIs, the frequency of H134R (P=2.2×10^{-16}) and H277Y (P=3.3×10^{-14}) on mixtures with WT were increased by 17.3% and 16.5% respectively. After 5 transfers, these genotypes reached 76.5% and 74.8%, respectively (Fig. 2). Controls made with resistant genotypes were not changed throughout the experiment. Significant (P=0.017) WT control change was observed after the fifth transfer cycle.

Discontinuing SDHI from field spray program

In the absence of SDHI pressure the frequency of H134R genotype within the population seems to decrease. The H134R genotype was not observed during the late evaluation of years 1 and 2 and showed a 49% reduction in year 3. In contrary, the genotype H277Y increased its frequency by 9.2- and 4.5-fold in years 1 and 3, while in year 2 it was reduced from 43.8% to 0%. The only frequency reduction of WT isolates was observed in the first year. In the second and third years the WT frequency increased by 3.2- and 2.3-fold (Table 1).

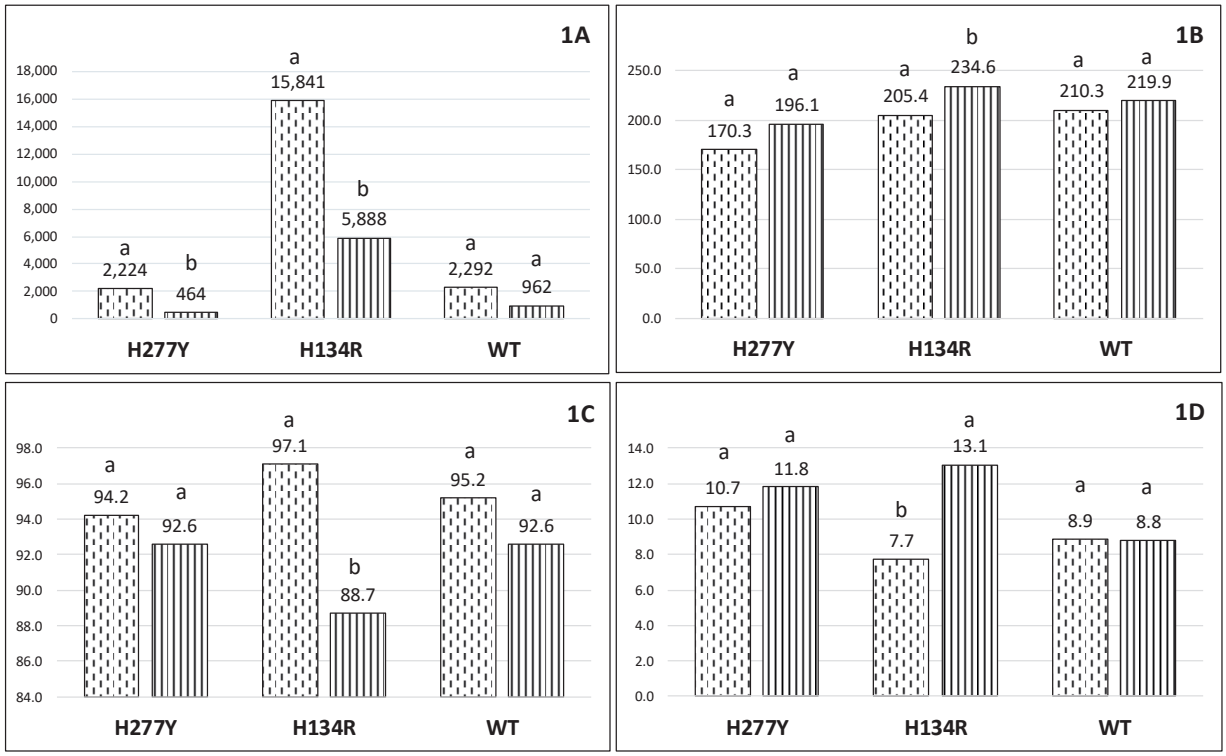


Figure 1 Effect of sucessive transfers on *Alternaria alternata* fitness components. (A) sporulation (spore/ml), (B) AUMGC, (C) germination (%) and (D) lesion size (mm). Dash-lines = evaluation made after 1st transfer. Solid-line = evaluation made after 12th transfer. Genotypes H277Y (n=3), H134R (n=3) and WT (n=3). Means with same letter are not significantly different (α=0.05) when compared with False Discovery Rate (FDR) method.

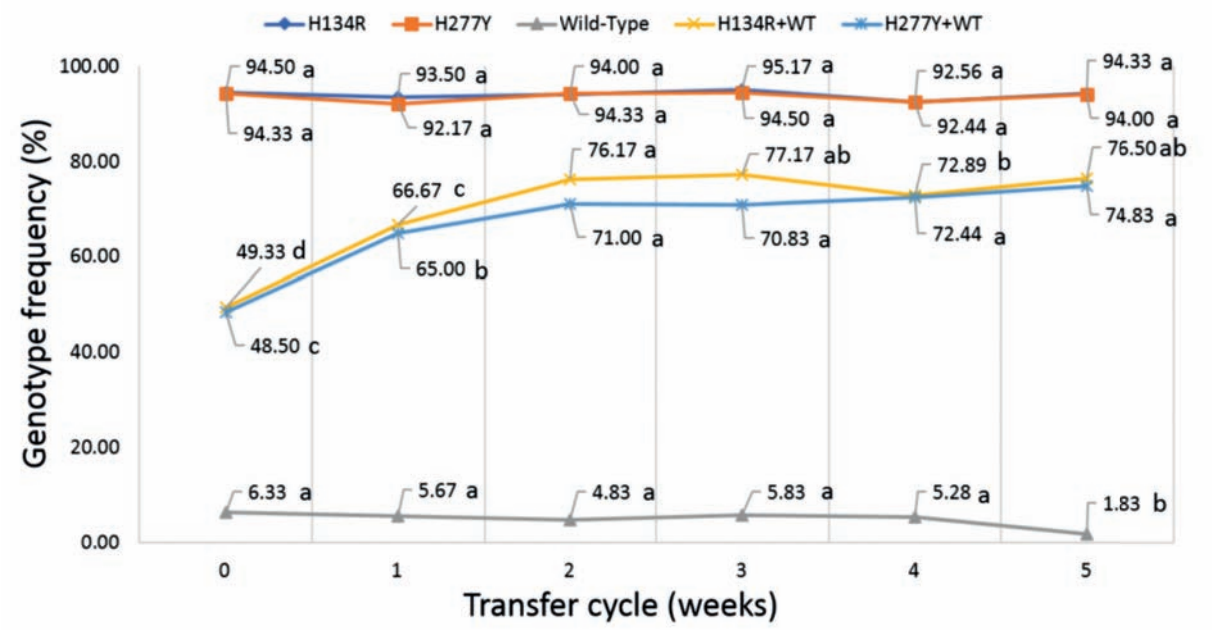


Figure 2 Germination frequency of resistant spores of *Alternaria alternata* mixed with wild-type isolates. Diamond = H134R control, square = H277Y control, triangle = WY control, x = 1:1 mixture of H134R:WT and * = 1:1 mixture H277Y:WT. Means with same letter are not significantly different (α=0.05) when compared with Fisher's Least significant Difference (LSD) method.

Table 1. Frequency of *Alternaria alternata* genotypes after discontinuing SDHI applications in a commercial pistachio orchard for three sucessive years.

Genotype	Frequency (%) of genotypes for each sampling event					
	May' 16	Sep' 16	May' 17	Sep' 17	May' 18	Sep' 18
H134R	37.5	0.0	25.0	0.0	80.0	31.8
H277Y	8.3	76.9	43.8	0.0	10.0	45.5
WT	54.2	23.1	31.3	100.0	10.0	22.7

Multi-Drug Resistance *in-vitro*

Significant genotype effects on sensitivities were occurred for cyprodinil (Scala, P=0.003) and trifloxystrobin (Gem, P=2.2×10^{-16}). For all other fungicides tested, there were no significant differences among H134R, H277Y and WT genotypes (Table 2). For cyprodinil, the H134R EC_{50} value differed from the H277Y and the WT results were similar to both resistant genotypes. For trifloxystrobin, the WT EC_{50} value differed from both resistant genotypes (Table 2).

Table 2. *Alternaria alternata* sensitivity value for five active ingredients by SDHI resistant genotype.

Genotype	Mean EC50 value (µg/ml)				
	Chlorothalonil	Cyprodinil	Metconazole	Polyoxin	Trifloxystrobin
H134R	4.86 a	2.09 a	0.16 a	26 a	101.46 a
H277Y	4.55 a	0.90 b	0.18 a	17.68 a	99.72 a
WT	4.50 a	1.36 ab	0.14 a	23.68 a	25.73 b

DISCUSSION

In the current study, we observed that *Alternaria alternata* carrying the H134R mutation had its fitness penalized in the absence of fungicide pressure (Fig. 1). Also, the H134R genotype revealed higher than or similar sporulation, germination and growth rate *in vitro* as compared to other SDHI-genotypes, and to the wild-type (data not shown). Except for sporulation, the H277Y showed no fitness costs, which may explain the stability for resistant SDHI-phenotypes in similar populations reported by Avenot and Michailides (2007). At that time, the H277Y was the most frequent SDHI mutation found in *A. Alternata* populations in California pistachio, but the increased use of fluopyram over boscalid has changed this population dynamic (Avenot et al. 2014; Lichtemberg et al. 2018b). Our competition studies revealed that both H134R and H277Y can increase their frequency by approximately 25% when coinoculated with wild-type isolates (Fig. 2). However, only the H277Y genotype was able to numerically increase its frequency in the field after discontinuing SDHI applications in a commercial pistachio orchard. The H134R fitness cost observed *in-vitro* seems to also occur in the field, revealing a great oportunity for managing this genotype that is associated with high resistance levels. Furthermore, the MDR results should be taken into consideration, since half of the SDHI products registered for controlling ALB on pistachio are mixed with QoI. We are currently testing different spray programs on three commercial orchards to evaluate their effect on ALB

control and SDHI/QoI mutations. Additionally, the SDHI genotypes used in this study are currently being characterized with respect to spore respiration.

ACKNOWLEDGEMENT

We thank the California Pistachio Reseach Board for financial support of this research project.

REFERENCES

Avenot H., Sellam, A., Karaoglanidis, G., and Michailides, T. (2008). Characterization of mutations in the iron-sulphur subunit of succinate dehydrogenase correlating with boscalid resistance in *Alternaria alternata* from California pistachio. Phytopathol 98:736-742.

Avenot, H., Van den Biggelaar, H., Morgan, D., Moral, J., Joosten, M., and Michailides, T. (2014). Sensitivities of baseline isolates and boscalid-resistant mutants of *Alternaria alternata* from pistachio to fluopyram, penthiopyrad, and fluxapyroxad. Plant Dis 98:197-205.

Avenot, H. F., and Michailides, T. J. (2007). Resistance to boscalid fungicide in *Alternaria alternata* isolates from pistachio in California. Plant Dis 91:1345-1350.

Förster, H., Kanetis, L., and Adaskaveg, J. E. (2004). Spiral gradient dilution, a rapid method for determining growth responses and 50% effective concentration values in fungus-fungicide interactions. Phytopathology 94:163-170.

Ishii, H. (2015). Stability of Resistance. Page 490 in: Fungicide Resistance in Plant Pathology. H. Ishii and D. W. Hollomon, eds. Springer, Tokyo, Japan.

Leader, J. (2004). Numerical Analysis and Scientific Computation. Pearson Addison Wesley, Boston.

Lichtemberg, P., Luo, Y., Doussoulin, H., and Michailides, T. (2018a). Using Allele-specific PCR for detecting multiple amino acid substitutions associated with SDHI resistance in *Alternaria alternata* causing Alternaria late blight in pistachio. Letters in Applied Microbiology 67: 506-512.

Lichtemberg, P., Puckett, R., Felts, D., Luo, Y., Doster, L., Rodriguez, D., and Michailides, T. (2018b). Survey of the pathogen of Alternaria late blight reveals different levels of carboxamide fungicide resistance on the main producing regions of California. California Agriculture 72:170-178.

Torres-Londoño, G. A., Hausbeck, M., and Hao, J. (2016). ECX: An R package for studying sensitivity of antimicrobial substances using spiral plating technology. Plant Health Progress 17:188-194.

MOLECULAR MECHANISMS OF FUNGICIDE RESISTANCE

Huf A et al., Distribution and changes of genotypes associated to DMI sensitivity of *Zymoseptoria tritici* in Europe. In: Deising HB; Fraaije B; Mehl A; Oerke EC; Sierotzki H; Stammler G (Eds), "Modern Fungicides and Antifungal Compounds", Vol. IX, pp. 93-98.
© 2020. Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-16-7

Distribution and changes of genotypes associated to DMI sensitivity in *Zymoseptoria tritici* in Europe

Huf A[1,2], Pfleger S[1], Strobel D[1], Bryson R[1], Voegele R T[2], Stammler G[1]

[1]BASF SE, Agricultural Research Station, 67117 Limburgerhof, Germany, [2]University of Hohenheim, Faculty of Agricultural Sciences, Institute of Phytomedicine, Department of Phytopathology, 70599 Stuttgart, Germany

Corresponding Author Email: gerd.stammler@basf.com

INTRODUCTION

Septoria tritici blotch (STB), caused by *Zymoseptoria tritici* (previously *Mycosphaerella graminicola*) is one of the most yield limiting diseases on wheat worldwide. Management of STB mainly relies on the application of fungicides with different modes of action, including demethylation inhibitors (DMIs). DMIs inhibit the enzyme lanosterol 14α-demethylase (Yoshida & Aoyama 1987) encoded by the *CYP51* gene and have been a key component in STB control in Europe for many years. However, a continuous shift of the *Z. tritici* population towards a reduced sensitivity to DMIs has been observed (Clark 2006; Cools & Fraaije 2013). This reduction in sensitivity is mainly driven by the accumulation of mutations in the *Z. tritici CYP51* gene (*MgCYP51*) resulting in the development of various *CYP51* haplotypes (Stammler et al. 2008; Cools & Fraaije 2013; Huf et al. 2018). Additionally, *MgCYP51* overexpression and an increased efflux activity has been shown to affect the sensitivity to DMIs (Cools et al. 2012; Omrane et al. 2015). In isolates overexpressing *MgCYP51* a 120 bp insert in the promotor region was identified (Cools et al. 2012). In addition, 862 and 866 bp inserts were also identified in the *MgCYP51* promotor. However, it is thought that these two inserts do not lead to an overexpression of the gene (Omrane et al. 2015; Kildea et al. 2019). An increased efflux activity in *Z. tritici* was shown to rely on a constitutive overexpression of the *MgMFS1* gene coding for an MFS1 transporter. Inserts of different sizes in the promotor of *MgMFS1* are considered to be responsible for this overexpression (Omrane et al. 2017).

In this study, the occurrence and frequency of different mechanisms conferring a reduced sensitivity of *Z. tritici* towards DMIs in Europe was examined. *CYP51* haplotypes and inserts in the *MgCYP51* and *MgMFS1* promotor region were identified in a collection of isolates from 2016 and 2017. Frequency and distribution of *CYP51* haplotypes in Europe in 2016 was previously published in association with a proposal for a new nomenclature to describe *CYP51* haplotypes (Huf et al. 2018). In this paper results of *CYP51* haplotype studies are shown for 2017, the distribution of *CYP51* overexpression and distribution of increased efflux mechanisms in Europe are also described for 2016 and 2017.

MATERIAL AND METHODS

Isolate collection

STB-infected leaves were collected at commercially treated fields across Europe in 2016 and 2017 as part of the BASF SE routine sensitivity monitoring and sent to the company EpiLogic (Freising, Germany). EpiLogic generated single pycnidia isolates. In 2016, out of the total amount of isolates of the routine monitoring, a representative collection was selected and transferred to BASF SE (see Huf et al. 2018 for more details). In 2017 all isolates of the routine monitoring were transferred to BASF SE for further investigations.

Molecular biological methods

DNA of all isolates was extracted using NucleoSpin DNA Plant II (Macherey-Nagel, Düren, Germany) or NucleoSpin 8/96 Plant II kits (48-/96-well scale, Macherey-Nagel) according to the manufacturer's protocols. For isolates from 2016 *CYP51* haplotypes were determined as described in Huf et al. (2018). For isolates from 2017 this method was slightly modified. PCR clean-up was performed directly after PCR with the NucleoSpin 96 PCR Clean-up kit (Macherey-Nagel) using the protocol for manual vacuum processing and sequencing was subsequently done with primer KES 2283 (5'-ATCCTCTGTGCCAATTTCTC-3') instead of KES 2192. In order to identify *CYP51* overexpression, isolates were checked for inserts in the *CYP51* promotor region. For this purpose, the PCR method described by Cools et al. (2012) was used with slight modifications. Instead of primer Mg51-seqR (Cools et al. 2012), primer KES 2188 (5'- GTCTGGCCGAATTGCGCGTC-3') was used with an annealing temperature of 64°C. For identification of inserts in the *MgMFS1* promotor region leading to an *MgMFS1* overexpression, PCR methods described by Omrane et al. (2015) with an annealing temperature of 58°C and Omrane et al. (2017) with an annealing temperature of 63°C were used. PCR product size was checked on 1% agarose gels using ethidium bromide.

RESULTS

In this paper the key focus is on results from countries in which samples were taken in both years and more than ten isolates were tested, namely: Germany (DE), France (FR), Ireland (IE), Netherlands (NL), Poland (PL) and the United Kingdom (UK).

CYP51 haplotypes

In total, 42 different *CYP51* haploytpes were identified from across DE, FR, IE, NL, PL and the UK in both years (Table 1). In 2016 it was observed that various *CYP51* haplotypes could be found in Europe. However only nine haplotypes were found to represent ~85% of all tested isolates. These nine most frequent haplotypes were C8, E3, E4, E5, F2, F8, G1, H4, and H6. Further information, including DMI sensitivity are described in Huf et al. 2018.

Table 1 All *CYP51* haplotypes found in Germany, France, Ireland, Netherlands and the United Kingdom in 2016 and 2017. Nine most frequent *CYP51* haplotypes are underlined.

Haplo type	Amino acid position															
	L 50	K 95	D 134	V 136	S 188	N 284	A 318	I 377	A 379	I 381	A 410	Y 459	G 460	Y 461	N 513	S 524
C2	S									V		S				
C4	S									V		D				
C6	S			A										S		
C7	S			A										H		
C8	S									V				H		
D7	S			A										S		
D9	S		G	A										H		
D10	S			A						V						
D13				C										H		T
D26	S						G			V				H		
D27	S							V		V				H		
E3	S			A						V				S		T
E4	S		G	A						V				H		
E5	S			A						V				H		T
E7	S			C						V				H		T
E9	S		G	A						V		S				
E25				A					G	V				S		T
E26	S		G	A						V		Del				
E28	S	M	G	G										S		
E29	S			C						V				S		T
F1	S				N				G	V		D				
F2	S				N					V		Del	Del		K	
F3	S			C	N							Del	Del		K	
F4	S			C	N					V				H		T
F5	S			A	N							Del	Del		K	
F6	S			A	N							Del	Del			T
F7	S			A					G	V				S		T
F8	S		G	A						V				H		T
F9				C					G	V		Del	Del			T
F10	S		G	A						V				S		T
F19	S			A								Del	Del		K	T
G1	S				N				G	V		Del	Del		K	
G2	S				N				G	V		Del	Del			T
G6	S		G	A						V		Del	Del		K	
G7	S			A	N				G	V				S		T
H3	S				N	H			G	V		Del	Del		K	
H4	S			A	N				G	V		Del	Del			T
H5	S				N				G	V	T	Del	Del		K	
H6	S			C	N				G	V		Del	Del			T
H9	S				N				G	V		Del	Del		K	T
I1	S			A	N				G	V		Del	Del		K	T
I2	S		G	A					G	V		Del	Del		K	T

In Figure 1 changes in the occurrence and frequency of *CYP51* haplotypes from 2016 to 2017 are shown. Haplotypes were grouped according to relevance: the nine most frequent (w/o arrow) and other haplotypes (below arrow). The frequency of other haplotypes, shown below the arrow, increased substantially in 2017 in IE and NL (2016: IE: 17.2%, NL: 18.2%, 2017: IE: 40.6%,

NL: 41.7%). There was only a slight increase in DE (2016: 9.1%, 2017: 12.5%) and in FR (2016: 12.3%, 2017: 14.8%) and even a decrease in Poland (2016: 18.4%, 2017: 5.81%). Therefore, the composition of *CYP51* haplotypes of *Z. tritici* populations changed strongly in IE and NL from 2016 to 2017. In the other countries the populations were mainly composed of the nine most frequent *CYP51* haplotypes found in 2016. However, frequency shifts were observed regarding these haplotypes with mainly E4 and F2 showing higher frequencies in 2017. In FR the composition and frequency of *CYP51* haplotypes stayed relatively stable.

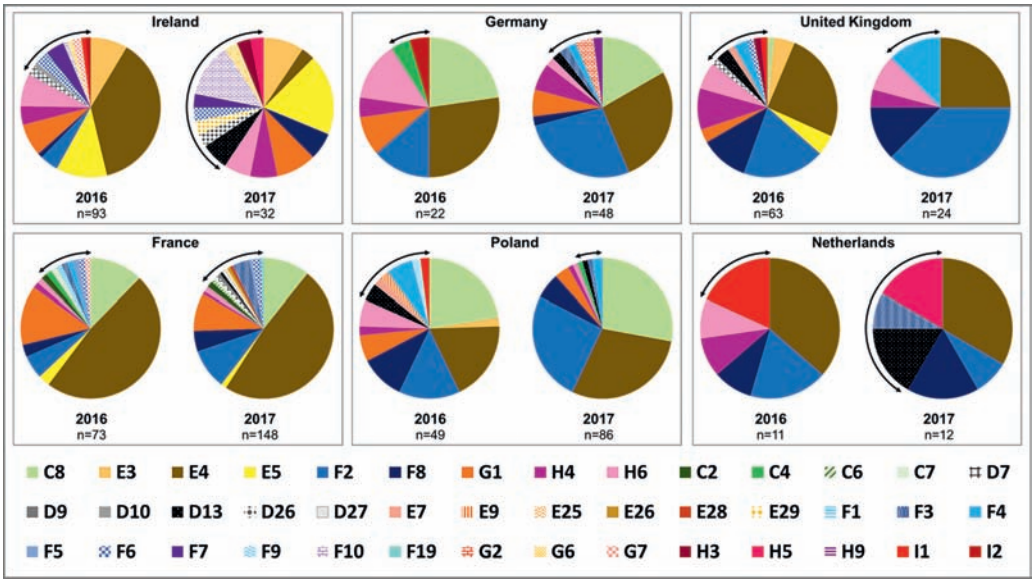


Figure 1 Ocurrence and frequency of *CYP51* haplotypes of *Z. tritici* in 2016 and 2017 in different countries. Haplotypes were grouped according to relevance: the nine most frequent (w/o arrow) and other haplotypes (below arrow). The number of tested isolates is given below each pie chart ('n').

Identification of *CYP51* overexpression and an increased efflux activity

Isolates were checked for inserts in the *MgCYP51* promotor region to identify *CYP51* overexpression and for inserts in the *MgMFS1* promtor region to identify an increased efflux activity. In Figure 2 the distribution and frequency of inserts in the *MgCYP51* and *MgMFS1* promotor regions are shown. The 120 bp insert in *CYP51* promotor region which correlates with *CYP51* overexpression was found in 2016 in frequencies ranging from 4 to 22% across the different countries (Figure 2). In 2017, frequencies between 3 and 38% were detected. The frequency of the 120 bp insert increased (about 9-15%) in UK, DE, PL and slightly (about 3%) in FR and decreased in NL (about 10%) as well as slightly (about 1%) in IE. The 120 bp insert was mainly found in the F2 haplotype with the exception of a few single isolates (data not shown). In addition, a new insert of 300 bp was found in both years but at a very low level (2016: 3.2%, 2017: 2.1%). High frequencies (2016: 73-86%, 2017: 63-88%) of inserts around 900 bp, were also detected in 2016 and 2017 but are believed not to be associated with *CYP51* overexpression.

In 2016 inserts of 150, 267, 338, 369, 377, and 519 bp were identified in the *MgMFS1* promotor region of *Z. tritici* (Figure 2). Occurrence of 150, 338, 369, and 519 bp inserts in field isolates of *Z. tritici* were recently reported, the 519 bp insert was found to have the highest impact on *MgMFS1* expression. The effects of smaller inserts have been shown to be weaker (Omrane et al. 2017). In 2016 the 519 bp insert was detected in IE, NL, DE, FR and PL in low frequencies ranging from 5 to 9%. Inserts smaller than 519 bp were only detected in IE, UK and in low frequency in Poland. In 2017 an increase of the 150 bp insert (2016: 10%, 2017: 34%) was detected in IE and the 267 bp (16.7%), 338 bp (16.7%) and 369 bp (16.7%) inserts were found in higher frequencies in NL compared to 2016. Single isolates with either the 150, 267 or 338 bp insert were detected in FR, DE and PL. In 2017 the 519 bp insert was again found in IE, DE, NL, FR and additionally in UK, however not in PL anymore. Frequencies of the 519 bp insert were observed to be relatively stable at a low level or even slightly decreased ranging from 4 to 8% in 2017.

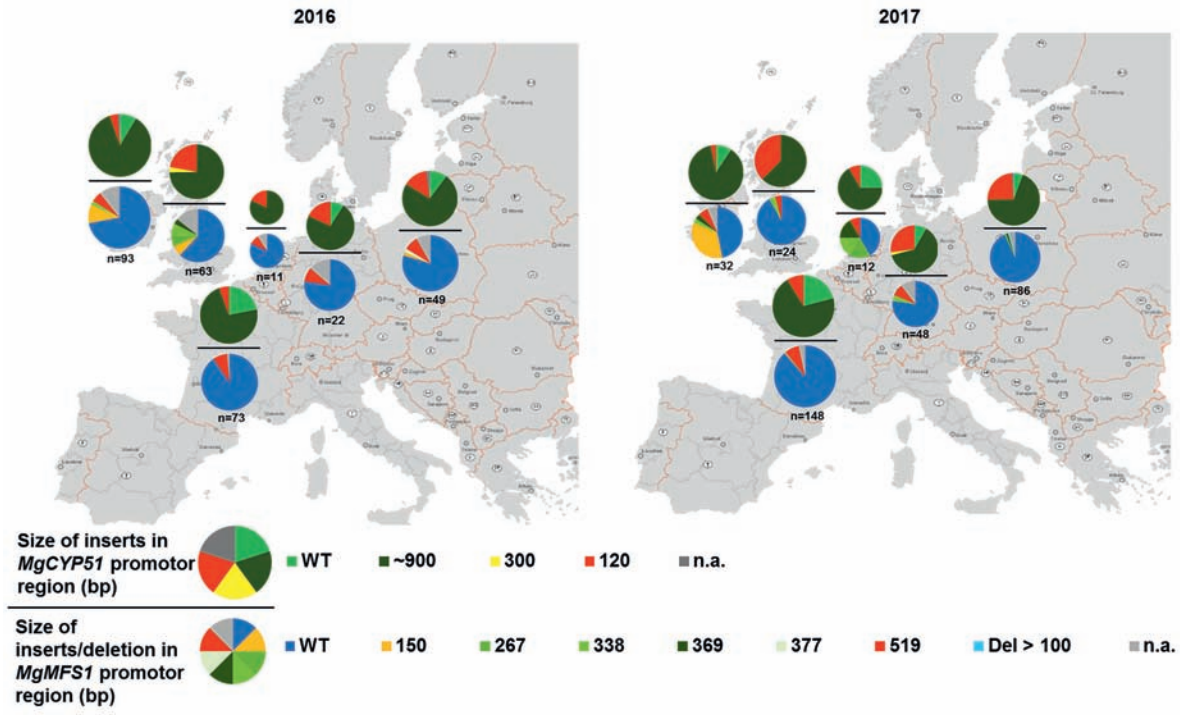


Figure 2 Frequency and distribution of inserts in the *MgCYP51* promotor region (pie chart above black line) and in the *MgMFS1* promotor region (pie chart underneath black line) of *Z. tritici* in different European countries in 2016 compared to 2017. The number of tested isolates for each country is given below the pie chart ('n').

CONCLUSION

In this paper the occurrence and frequency of different mechanisms leading to a reduced DMI sensitivity of *Z. tritici* were examined in isolates from 2016 and 2017 monitoring. It was observed that evolution of *CYP51* is still ongoing and changes in composition and frequency of *CYP51* haplotypes are still happening in European populations. Varying frequencies of *CYP51*

overexpression and an increased efflux activity, based on promotor insertion studies, were identified across different European countries. It was observed that occurrence of *CYP51* haplotypes is still the most frequent mechanism leading to a reduced DMI sensitivity. Frequency of *CYP51* overexpression, however, increased in some countries in 2017, whereas frequency of an increased efflux activity conferred by the 519 bp insert with the strongest effects, stayed relatively stable but at a low level across both years. An increase of inserts with weaker effects on an increased efflux activity was identified in some countries, however, with the exception of UK and NL, still at a very low level. Sensitivity monitoring should be continued to observe further evolution of *CYP51* haplotypes and spreading of additional mechanisms and to optimise resistance management strategies.

REFERENCES

Clark WS (2006). Fungicide resistance: are we winning the battle but losing the war? *Aspects of Applied Biology* 78, 119-126.

Cools HJ; Bayon C; Atkins S; Lucas JA; Fraaije BA (2012). Overexpression of the sterol 14α-demethylase gene (*MgCYP51*) in *Mycosphaerella graminicola* isolates confers a novel azole fungicide sensitivity phenotype. *Pest Management Science* 68, 1034-1040.

Cools HJ; Fraaije BA (2013). Update on mechanisms of azole resistance in *Mycosphaerella graminicola* and implications for future control. *Pest Management Science* 69, 150-155.

Huf A; Rehfus A; Lorenz KH; Bryson R; Voegele RT; Stammler G (2018). Proposal for a new nomenclature for *CYP51* haplotypes in *Zymoseptoria tritici* and analysis of their distribution in Europe. *Plant Pathology* 67, 1706-1712.

Kildea S; Marten-Heick T; Grant J; Mehenni-Ciz J; Dooley H (2019). A combination of target-site alterations, overexpression and enhanced efflux activity contribute to reduced azole sensitivity present in the Irish *Zymoseptoria tritici* population. *European Journal of Plant Pathology* 154, 529-540.

Omrane S; Sghyer H; Audéon C; Lanen C; Duplaix C; Walker AS; Fillinger S (2015). Fungicide efflux and the MgMFS 1 transporter contribute to the multidrug resistance phenotype in *Zymoseptoria tritici* field isolates. *Environmental Microbiology* 17, 2805-2823.

Omrane S; Audéon C; Ignace A; Duplaix C; Aouini L; Kema G; Walker AS; Fillinger S (2017). Plasticity of the *MFS1* promoter leads to multidrug resistance in the wheat pathogen *Zymoseptoria tritici*. *mSphere* 2, e00393-17.

Stammler G; Carstensen M; Koch A; Semar M; Strobel D; Schlehuber S (2008). Frequency of different CYP51-haplotypes of *Mycosphaerella graminicola* and their impact on epoxiconazole-sensitivity and-field efficacy. *Crop Protection* 27, 1448-1456.

Yoshida Y; Aoyama Y (1987). Interaction of azole antifungal agents with cytochrome P-450_{14DM} purified from *Saccharomyces cerevisiae* microsomes. *Biochemical Pharmacology* 36, 229-235.

Rehfus A, Katamadze A, Strobel D, Bryson R, Stammler G, Evolution of SDHI adaptation in cereal pathogens in Europe. In: Deising HB; Fraaije B; Mehl A; Oerke EC; Sierotzki H; Stammler G (Eds), "Modern Fungicides and Antifungal Compounds", Vol. IX, pp. 99-104. © 2020. Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-16-7

Evolution of SDHI adaptation in cereal pathogens in Europe

Rehfus A, Katamadze A, Strobel D, Bryson R, Stammler G

BASF SE, Agricultural Research Station, 67117 Limburgerhof, Germany
Corresponding Author Email: gerd.stammler@basf.com

INTRODUCTION

Presently, three single-site fungicide classes dominate the control of a broad range of phytopathogenic fungi in European cereal crops: the DMIs (C14-demethylation inhibitors), the QoIs (quinone outside inhibitors) and the SDHIs (succinate dehydrogenase inhibitors). To date, SDHI adapted isolates have been found in three cereal pathogens, namely *Zymoseptoria tritici*, causal agent of Septoria blotch of wheat (Dooley et al. 2016; Rehfus et al. 2018), *Pyrenophora teres*, causal agent of net blotch of barley (Rehfus et al. 2016) and *Ramularia collo-cygni*, causal agent of Ramularia leaf spot of barley (Rehfus et al. 2019). Several mutations in the target genes of SDHIs (Sdh genes), which lead to amino acid exchanges in the SDH complex, have been found to cause SDHI adaptation. Different mutations might have different effects to various SDHIs. In this study, we present recent SDHI monitoring data for the three cereal pathogens mentioned above, which, due to their occurance and yield impact, are of major economic importance in Europe.

MATERIAL AND METHODS

Wheat leaves infected with *Z. tritici* were collected at commercially treated fields across Europe in 2017 as part of the BASF routine sensitivity monitoring and sent to the company EpiLogic (Freising, Germany). EpiLogic generated single isolates and determined the sensitivity to SDHIs in the form of EC_{50} values by using microtiter tests. All isolates with EC_{50} values >0.3 ppm were considered resistant and forwarded to BASF for further analysis on mutations in the SDH genes, as previously described (Rehfus *et* al. 2018). Isolates were also evaluated for insert(s) in the promotor of the *MSF1* efflux transporter gene as described by Omrane et al. (2017) and Huf et al. (2019). In spring 2018, wheat leaves from fields with *Z. tritici* infections were sampled across Europe and SDH-mutations were quantitatively detected from the plant tissue using pyrosequencing assays, as previously described (Rehfus 2017).

Barley leaves infected with *P. teres* were collected and analyzed in BASF for the frequency of various mutations in the SDH genes conferring SDHI resistance, as previously described (Rehfus et al. 2016).

Barley leaves with clear symptoms of Ramularia leaf spot caused by *R. collo-cygni* were collected in 2014-2017 and sent to the company Agrotest Fyto (Kromeriz, Czech Republic) for

isolation of strains and subsequent SDHI sensitivity evaluation by using Petridish assays, as described by Rehfus et al. (2019). Afterwards, all *R. collo-cygni* isolates were then forwarded to BASF, where they were analyzed for mutations in the SDH genes, as previously described (Rehfus et al. 2019).

RESULTS AND DISCUSSION

Zymoseptoria tritici

Frequency and ratios of mutations causing SDHI resistance

475 isolates from the 2017 European random monitoring were analyzed for mutations in the SDH genes. All isolates with fluxapyroxad resistance (EC_{50} >0.3 ppm) carried mutations, whereas-all sensitive isolates with EC_{50} <0.3 ppm were wildtype for SDH B, C and D. Most frequent were the mutations C-T79N and C-N86S, which cause low to moderate resistance levels and to a lower frequency the C-H152R mutation, which is responsible for higher resistance levels (Rehfus et al. 2018). A genetic monitoring of samples collected in spring 2018 showed that the mutations C-T79N and C-N86S were even more frequent than the C-H152R (Figure 1), which suggests a reduced fitness of the C-H152R compared to C-T79N and C-N86S.

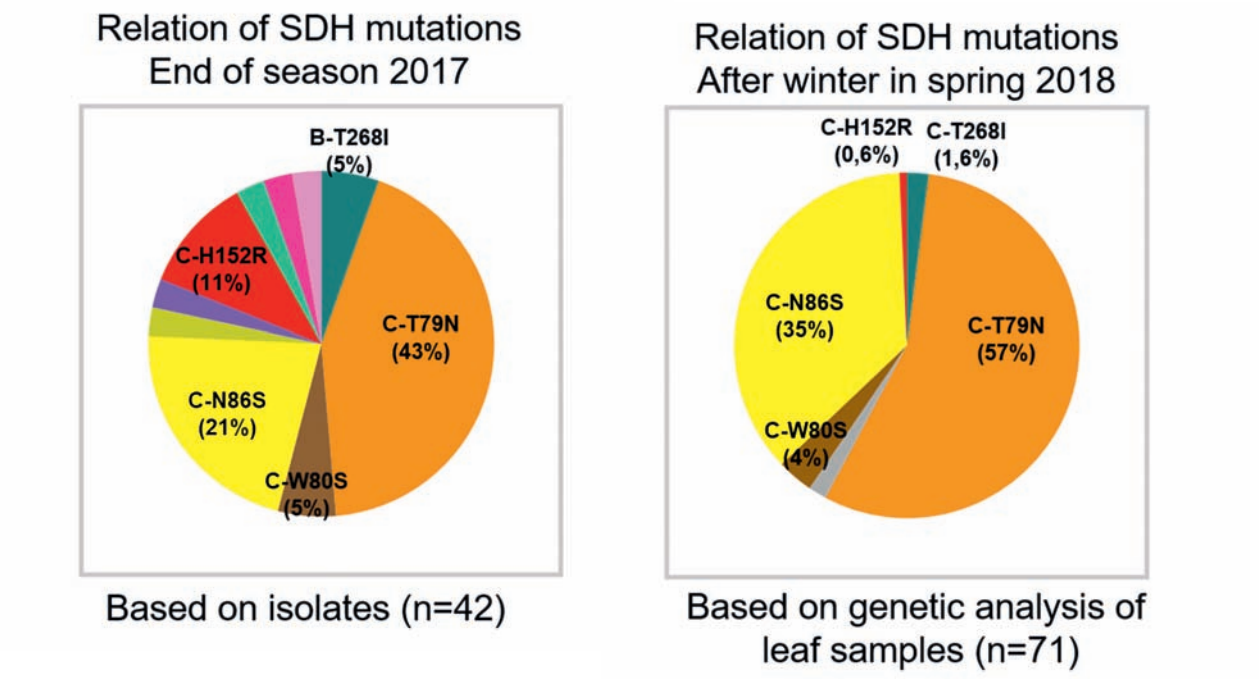


Figure 1 Ratio of mutations in SDHI adapted isolates end of 2017 season (42 out of 475 isolates were adapted). In the following spring, mutations were quantitatively detected in infected plant tissue by pyrosequencing. Most frequent mutations are mentioned, others are indicated by different colours. Results showed a decrease of C-H152R from late 2017 season to spring monitoring 2018 from 11% to 0.6% indicating a fitness penalty caused by this mutation.

Role of enhanced efflux on SDHI sensitivity

Sixteen wildtype-, 11 C-T79N-, 8 C-N86S- and 8 C-H152R-isolates were analyzed for the presence of introns in the *MFS1* promotor region and classified into Wildtype, type I, II, III and "no band" (i.e. no PCR product obtained) according to Omrane *et* al. (2017). The EC_{50} values were evaluated for 6 different SDHIs. The data show that in the background of SDH wildtype, isolates with inserts in the *MFS1* promotor region had a tendency to higher EC_{50} values for all 6 SDHIs. This trend was lower for isolates which had the C-T79N or C-N86S amino acid exchange in the SDH-C gene, but those with type 1 insert were in the upper range of EC_{50} values for these two SDH-groups. In isolates with the C-H152R mutation, the inserts in *MFS1* transporter did not lead to higher EC_{50} values. However, no type 1 insert was in this group, which has the strongest effects on sensitivity reduction. The sensitivity reduction effect of the C-H152R mutation alone is obviously so high for all tested SDHIs that the effect of the enhanced efflux is only marginal.

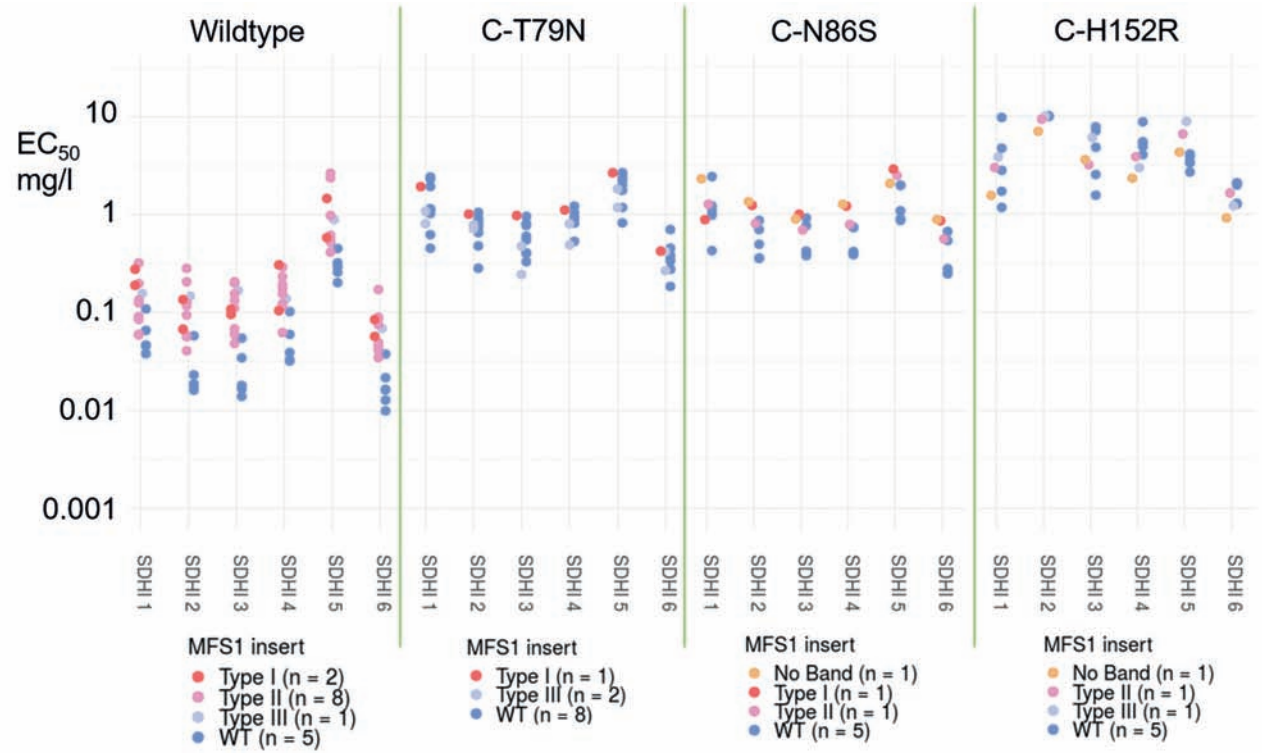


Figure 2 EC_{50} values of isolates with no or different intron sequences in the *MFS1* promotor regions (labelled with different colours), separated by their SDH background (wild-type, C-T79N, C-N86S and C-H152R). EC_{50} was determined for six different SDHIs. Efflux effects are strongest in SDH gene wildtype, followed by C-T79N, C-N86S and no additional sensitivity reduction was recognized for the C-H152R isolates.

Pyrenophora teres

The 2018 monitoring showed a high frequency of SDH mutations in Germany, France and UK (Figure 3). The most frequent mutation in France is C-G90R, the mutation pattern in Germany

is more heterogenous. The evolution of mutations over the years has shown an increase in the frequency of mutations since 2012 in France and Germany, with some differences in the pattern of these mutations (Figure 4).

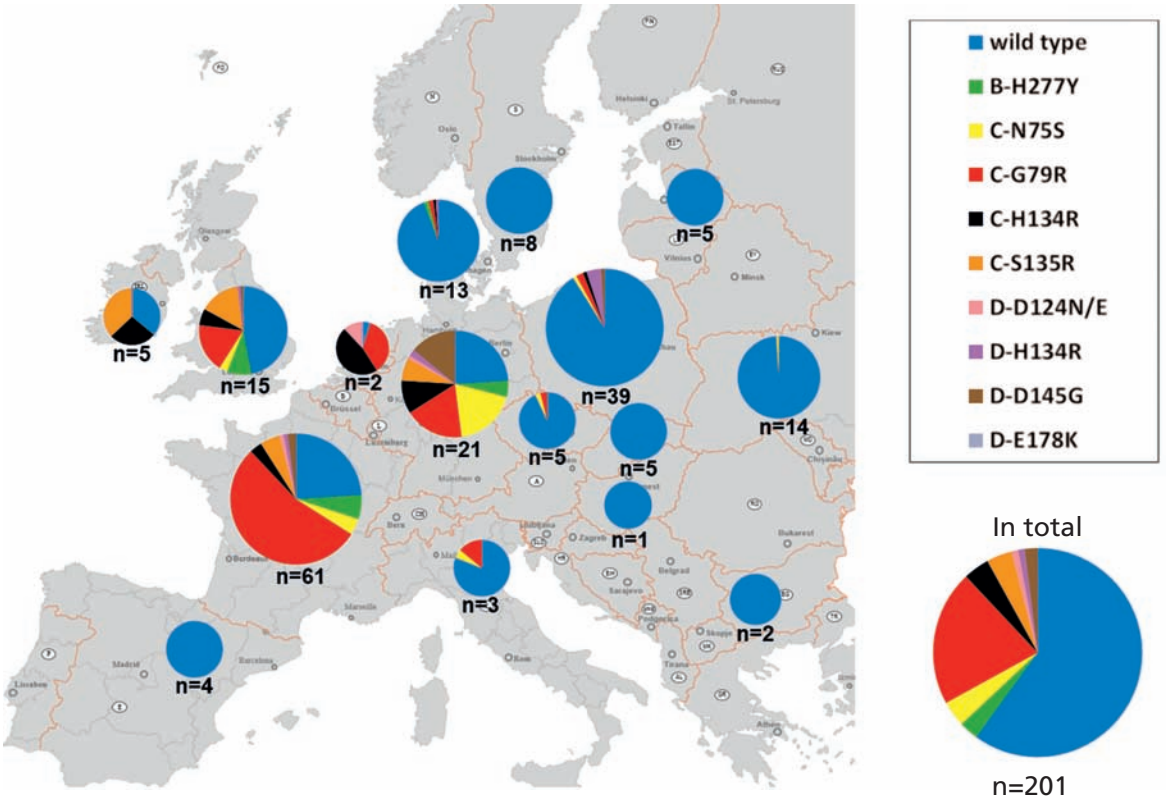


Figure 3 Frequency of SDH mutations in field samples collected in different European countries in the season 2018, determined by pyrosequencing.

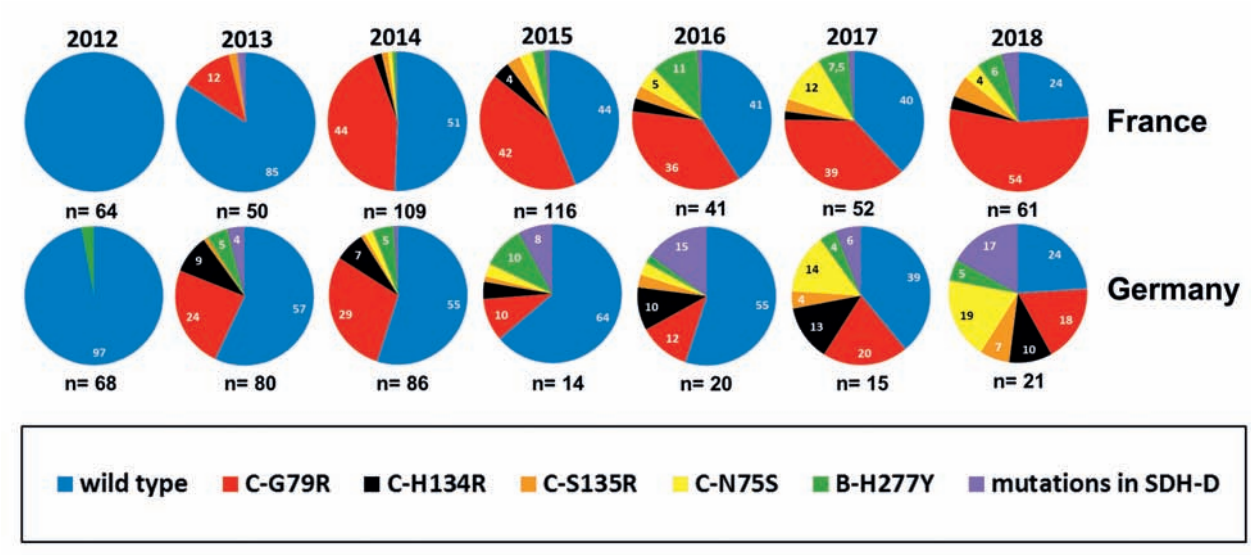


Figure 4 Frequency of SDH mutations in field samples from France and Germany from 2012 to 2018, determined by pyrosequencing.

Ramularia collo-cygni

Mutation analysis of isolates from 2014-2017 indicated the presence of SDHI resistance in various European countries, which had been caused by different mutations in the SDH-B or SDH-C genes (please refer to the European map shown in Rehfus et al. 2019). This was confirmed by the 2017 and 2018 reports of the SDHI Working Group, in which SDHI resistance has been detected in several European countries. SDHI mutations cause different levels of SDHI adaptation also in case of *R. collo-cygni*, which is well documented for other pathogens. In 2017, the most frequent mutations were the C-N87S, C-H146R and C-H153R. Especially the latter two result in higher resistance levels towards various SDHIs (Figure 5).

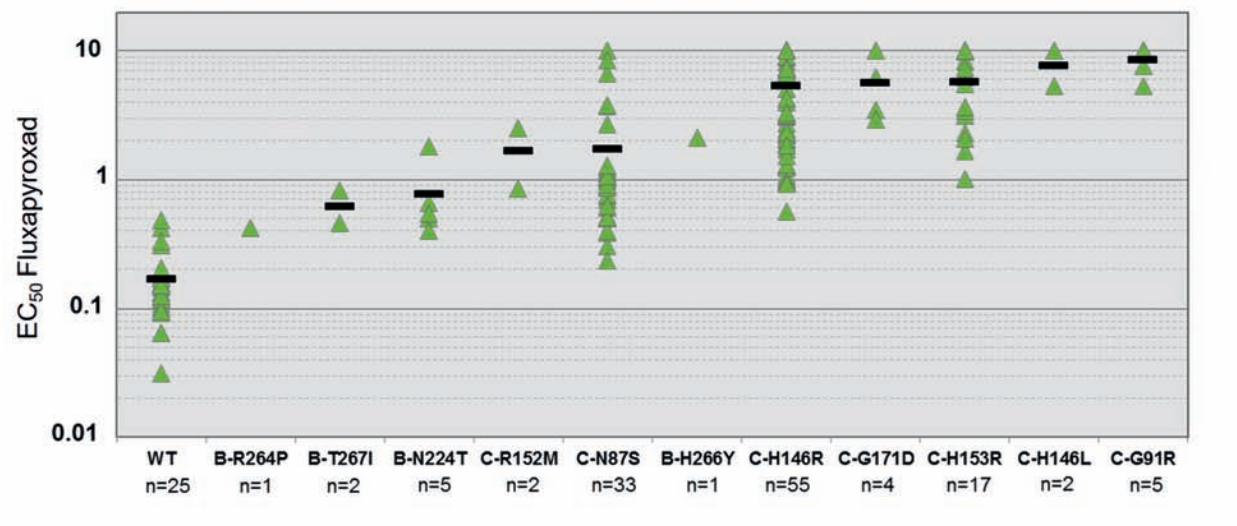


Figure 5 EC_{50} values for fluxapyroxad of isolates of *R. collo-cygni* from the 2017 monitoring, categorized by SDH-haplotypes.

Some of the mutations described for the different pathogens are homologous (e.g. C-N86S in *Z. tritici*, C-N75S in *P. teres* and C-N87S in *R. collo-cygni*). More details on the homologous mutations for these three pathogens are provided in the paper of Rehfus et al. (2019).

The results over the past years of the monitoring programmes have shown a rapid evolution of SDHI resistance in *R. collo-cygni* in many European countries, an evolution of SDHI resistance also in *P. teres* mainly in France, Germany, UK and some other regions, while in many remaining European countries the majority of *P. teres* populations are still fully sensitive. *Z. tritici* has shown the slowest increase in SDHI resistance over Europe in our monitoring studies. These findings are confirmed by a wealth of data published by the FRAC SDHI Working Group (FRAC 2019).

REFERENCES

Dooley H, Shaw MW, Mehenni-Ciz J, Spink J; Kildea S (2016). Detection of *Zymoseptoria tritici* SDHI insensitive field isolates carrying the SdhC-H152R and SdhD-R47W substitutions. *Pest Management Science* 72, 2203-2207.

FRAC (2019). www.frac.info.com

Huf A; Pfleger S; Strobel D; Bryson R; Voegele R T; Stammler G (2019). DMI sensitivity of *Zymoseptoria tritici* and studies on contributing mechanisms in Europe. *Modern Fungicides and Antifungal Compounds* Vol. IX.

Omrane S; Audéon C; Ignace A; Duplaix C; Aouini L; Kema G; Walker AS; Fillinger S (2017). Plasticity of the *MFS1* promoter leads to multidrug resistance in the wheat pathogen *Zymoseptoria tritici*. *mSphere* 2, e00393-17.

Rehfus A; Miessner S; Achenbach J; Strobel D; Bryson R; Stammler G (2016). Emergence of succinate dehydrogenase inhibitor resistance of *Pyrenophora teres* in Europe. *Pest Management Science* 72, 1977-1988.

Rehfus A (2017). Analysis of the emerging situation of resistance to succinate dehydrogenase inhibitors in *Pyrenophora teres* and *Zymoseptoria tritici* in Europe. Dissertation Hohenheim 2017.

Rehfus A; Strobel D; Bryson B; Stammler G (2018). Mutations in the sdh genes in field isolates of *Zymoseptoria tritici* and impact on the sensitivity to various succinate dehydrogenase inhibitors. *Plant Pathology* 67, 175-180.

Rehfus A; Matusinsky P; Strobel D; Bryson R; Stammler G (2019). Mutations in target genes of succinate dehydrogenase inhibitors and demethylation inhibitors in *Ramularia collo-cygni* in Europe. *Journal of Plant Disease and Protection* doi 10.1007/s41348-019-00246-4.

Eisermann et al, A single amino acid exchange in the transcription factor Azr1 governs azole tolerance in *Fusarium graminearum*. In: Deising HB; Fraaije B; Mehl A; Oerke EC; Sierotzki H; Stammler G (Eds), "Modern Fungicides and Antifungal Compounds", Vol. IX, pp. 105-110. © 2020. Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-16-7

A single amino acid exchange in the transcription factor Azr1 governs azole tolerance of *Fusarium graminearum*

Eisermann I, Gottschling D, Kemen E, Karlovsky P, Tryono R, Abou-Ammar G, Deising H.B, Wirsel S.G.R

Naturwissenschaftliche Fakultät III, Institut für Agrar- und Ernährungswissenschaften, Martin Luther Universität Halle-Wittenberg, Betty-Heimann-Str. 3, D-06120 Halle, Germany

Email: stefan.wirsel@landw.uni-halle.de

ABSTRACT

Epidemics caused by *Fusarium graminearum* can threaten the production of wheat and other cereals. Possible outbreaks of Fusarium Head Blight (FHB) at conducive weather conditions are typically controlled by spray application of triazoles. Quantitative resistance against these fungicides has been reported from several countries. Here, we investigate the molecular events that led to quantitative resistance in previously selected azole-adapted strains. By genome-sequencing of adapted strains, we discovered a candidate SNP causing an amino acid exchange in a gene encoding a transcription factor named *FgAZR1* and introduced this SNP into a wild type (WT) reference strain. Transgenic lines harbouring such an engineered *FgAZR1* allele showed no difference to the WT in several assays assessing vegetative growth, virulence, asexual and sexual reproduction. However, these mutants acquired azole tolerance at levels earlier seen in adapted strains. Furthermore, compared to the WT these mutants produced lower levels of zearalenone *in vitro*.

INTRODUCTION

F. graminearum (Schwabe) is the most common species causing Fusarium head blight (FHB) on cereals in central Europe. FHB infections do not only result in diminished harvests but also in contamination of grains with mycotoxins threatening human and animal health. In addition to the compliance with appropriate agronomic practices, the treatment of wheat with azole fungicides during flowering is used to protect crops against FHB (Becher et al. 2013). During many years of triazole usage, emergence of qualitative resistance has not been reported in *F. graminearum*. However, quantitative resistance has been detected in field isolates originating in several countries, which may facilitate FHB outbreaks in the future (Klix et al. 2007; Spolti et al. 2014; Talas & McDonald 2015; Yin et al. 2009). Previously, *F. graminearum* strains had been selected with increased tolerance to tebuconazole in an *in vitro* adaptation experiment that lasted for one month. Resistant strains exhibited two distinct phenotypes, P1 and P2, differing in their degree of tolerance to triazoles (Resistance factors: ≤ 10.9 for P1 isolates, ≤ 2.6 for P2

isolates), cross-resistance patterns against other fungicide classes and virulence (Becher et al. 2010). Analysis of the transcriptome of *F. graminearum* after treatment with tebuconazole indicated that the most prominently enriched functional transcript classes had predicted functions in sterol and steroid biosynthesis. Interestingly, transcript abundances for certain ABC transporters and transcription factors were also increased (Becher et al. 2011). Deletion mutants created for four genes encoding ABC transporters showed that FgABC3 and FgABC4 contribute to basal levels of azole tolerance (Abou Ammar et al. 2013). In the current work, we investigate molecular events that caused the emergence of quantitative azole resistance in adapted P1 strains.

RESULTS

Mutations in *CYP51* encoding the target of azoles were shown to lead to resistance in several fungal species (Becher & Wirsel 2012). In contrast to most other fungi, *F. graminearum* harbours three *CYP51* genes. Sequence analysis of these genes and their upstream regions in several adapted strains did not indicate any variation from the reference strain they originate from. Furthermore, we did not find in the adapted strains any sequence divergence in *FgABC3* and *FgABC4*.

To determine to which extent the two ABC transporter genes may contribute to enhanced azole tolerance in the adapted strains, they were deleted in strain P1-11. Representative transformants were tested for vegetative fitness but they did not deviate from their progenitor in any property assessed, analogously to the results obtained for deletion mutants of *FgABC3* and *FgABC4* generated in the WT background (Abou Ammar et al. 2013). Whereas the Δ*Fgabc3* mutants had lost the quantitative azole-resistant phenotype that had been acquired by their progenitor strain P1-11, deletion of *FgABC4* did not significantly alter azole sensitivity. This suggested that of the two genes analysed, only *FgABC3* contributed to increased azole tolerance in P1-11.

We postulated that during the adaptation process genetic alterations may have arisen at other loci, which indirectly led to an *FgABC3*-mediated increase of azole tolerance. Therefore, we sequenced the genomes of adapted P1 strains utilising an Illumina NGS platform. Among several SNPs found, we focused on one SNP occurring in a gene, called *FgAZR1*, which encodes a transcription factor. The P1 strains analysed have a single SNP in this gene, which leads to an amino acid exchange.

We generated transgenic lines to investigate to what extent this particular SNP may explain quantitative resistance acquired by P1 strains. Using the WT reference strain (*F. graminearum* NRRL 13383), we introduced into *FgAZR1* the SNP existing in the adapted P1 strains, leading to SHN strains (SNP allele, Hyg-marker, NRRL 13383 background). Inversely, we exchanged in the azole-resistant strain P1-11 the altered *FgAZR1* allele with a WT allele, leading to WHP strains (WT allele, Hyg-marker, P1-11 background). We also transformed the WT with a construct carrying only the *HygR* expression cassette that integrated at the same genomic position as in the SHN and WHP strains, leading to WHN strains (WT allele, Hyg-marker,

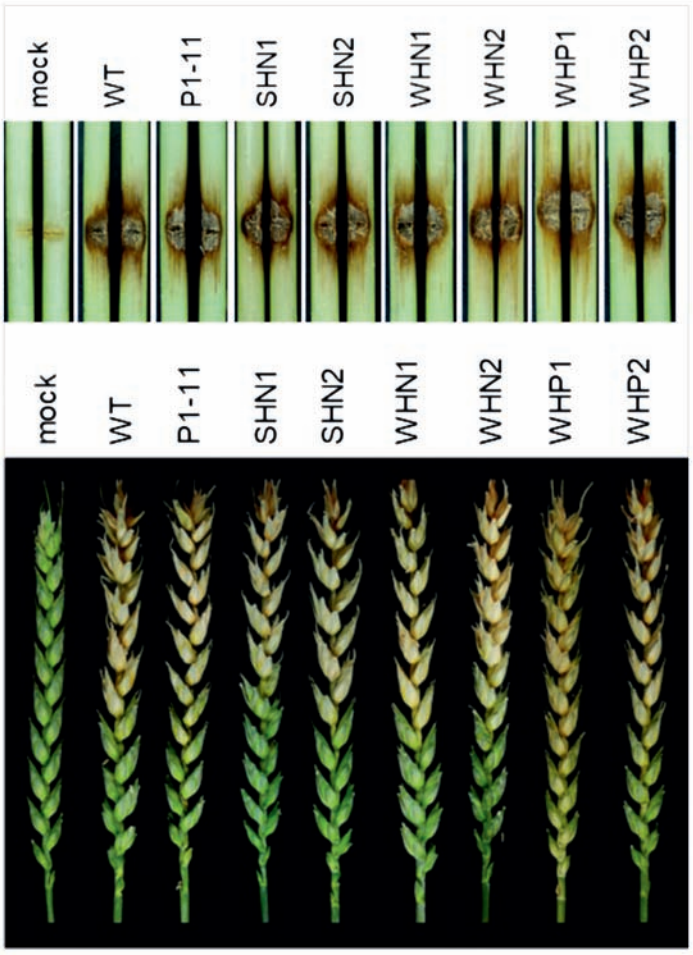


Figure 1 Virulence of mutants. P1-11 is an azole-adapted strain, SHN, WHN and WHP are transgenic strains. The upper panel shows maize stalks that were point-inoculated and split for photography at 14 dpi. The lower panel shows wheat heads with a single, central spikelet that was point-inoculated. The photograph was taken at 14 dpi.

NRRL 13383 background). These mutants served as controls for potential side-effects resulting only from integration of the resistance marker at the targeted locus. None of the transgenic strains generated differed significantly from the WT with respect to vegetative growth on media such as PDA (potato dextrose agar) and SNA (synthetic nutrient-poor agar). Likewise, the production and germination of macroconidia and ascospores did not differ between the mutants and the WT reference. Pathogenicity tests performed as point inoculations of single spikelets of wheat and of injured stalks of maize indicated that all transgenic strains maintained full virulence (Fig. 1). However, tolerance against triazoles differed considerably between the strains. As previously reported (Becher et al. 2010), adapted P1-11 was more tolerant to tebuconazole than the WT reference (Fig. 2). Remarkably, SHN strains showed similar degrees of quantitative resistance as P1-11. In contrast, WHP and WHN mutants remained as sensitive as the WT. The same result was obtained with additional triazoles tested but not with fungicides belonging to other FRAC classes. This confirmed that a single SNP in *FgAZR1* had enabled the P1 strain to acquire quantitative resistance specifically against triazoles, without affecting the vegetative and reproductive capabilities of the fungus.

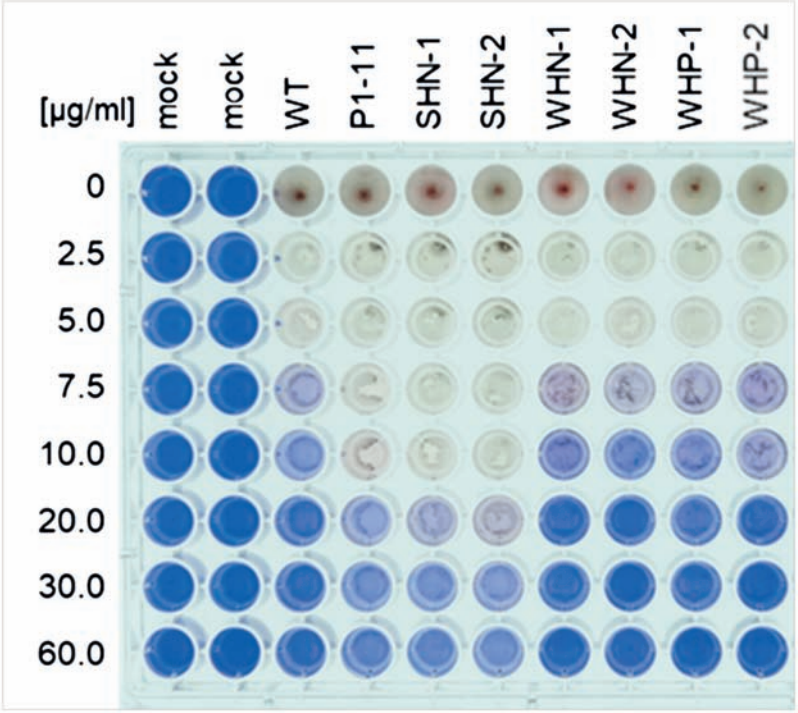


Figure 2 Sensitivity of mutants against tebuconazole. P1-11 is an azole-adapted strain, SHN, WHN and WHP are transgenic strains. The microtiter assay utilised resazurin as an indicator of cell viability. Blue colour displays inhibition by tebuconazole when amended at the indicated concentrations.

In addition, we assessed whether the introduction of the SNP in *FgAZR1* may influence the production of mycotoxins with detrimental effects to human and animal health, i.e. trichothecenes and zearalenone (ZEN). As the WT strain originally used for adaptation exhibits the NIV chemotype, nivalenol, as expected (Becher et al. 2010), was the only trichothecene detected at considerable amounts *in vitro*. There existed no significant variation between the WT and the SHN mutants or P1-11 (Fig. 3). In contrast, both the adapted strain and the SHN mutants produced significantly reduced amounts of ZEN on the rice bran medium used.

CONCLUSIONS

We detected a non-synonymous SNP in *FgAZR1*, encoding a Zn-finger transcription factor, in azole-adapted P1 strains of *F. graminearum*. The introduction of that SNP into the WT reference by genetic engineering recreated the same phenotypes observed in the adapted strain, i.e. quantitative resistance acquired specifically for triazoles and a reduced production of ZEN *in vitro*. WHN and WHP mutants were created in addition to the SHN mutants to assess the specificity of effects caused by the SNP in the SHN mutants. Since the WHN and WHP mutants are as sensitive to azoles as the WT, we conclude that the discovered SNP is responsible for the enhanced tolerance to triazoles in the P1 adaptants. Exposure to tebuconazole probably had led to the selection of this mutation during the adaptation experiment. This study proved that a single SNP in *FgAZR1* could increase azole tolerance if it would widely occur in field populations of *F. graminearum*.

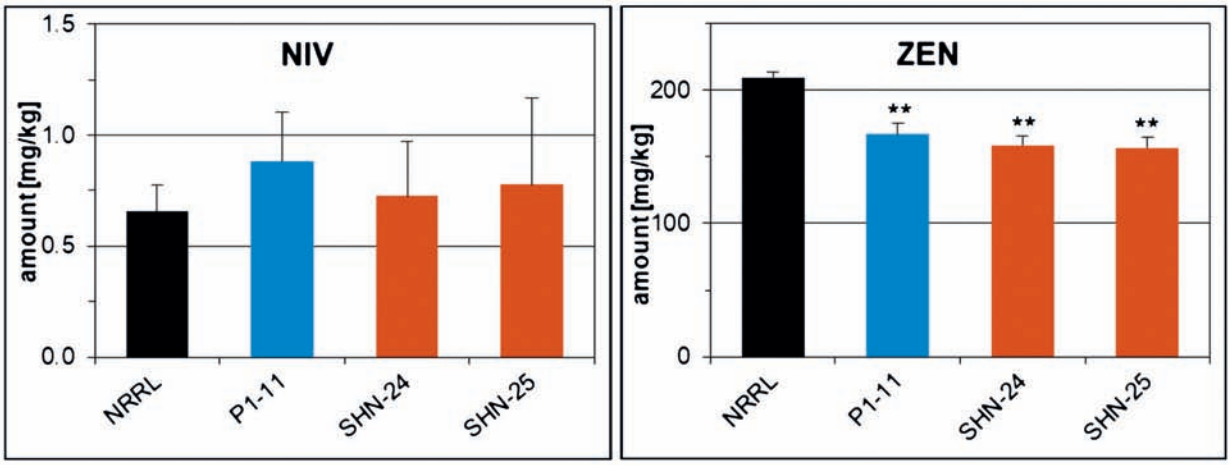


Figure 3 Production of mycotoxins of mutants *in vitro*. P1-11 is an azole-adapted strain and the SHN strains are transgenic strains. Rice bran medium was inoculated with conidia. ** indicates significance at $p = 0.005$ in t-test, n=5.

REFERENCES

Abou Ammar G; Tryono R; Döll K; Karlovsky P; Deising HB; Wirsel SGR (2013). Identification of ABC transporter genes of *Fusarium graminearum* with roles in azole tolerance and/or virulence. *PLoS ONE*, 8(11), e79042.

Becher R; Hettwer U; Karlovsky P; Deising HB; Wirsel SGR (2010). Adaptation of *Fusarium graminearum* to tebuconazole yielded descendants diverging for levels of fitness, fungicide resistance, virulence, and mycotoxin production. *Phytopathology*, 100(5), 444-453.

Becher R; Miedaner T; Wirsel SGR (2013). Biology, diversity and management of FHB-causing *Fusarium* species in small-grain cereals. In: *The Mycota XI, Agricultural Applications*, ed F Kempken, pp. 199-241. Springer: Berlin Heidelberg.

Becher R; Weihmann F; Deising HB; Wirsel SGR (2011). Development of a novel multiplex DNA microarray for *Fusarium graminearum* and analysis of azole fungicide responses. *BMC Genomics*, 12(1), 52.

Becher R; Wirsel SGR (2012). Fungal cytochrome P450 sterol 14alpha-demethylase (CYP51) and azole resistance in plant and human pathogens. Applied *Microbiology and Biotechnology*, 95(4), 825-840.

Klix MB; Verreet JA; Beyer M (2007). Comparison of the declining triazole sensitivity of *Gibberella zeae* and increased sensitivity achieved by advances in triazole fungicide development. *Crop Protection*, 26(4), 683-690.

Spolti P; Del Ponte EM; Dong Y; Cummings JA; Bergstrom GC (2014). Triazole sensitivity in a contemporary population of *Fusarium graminearum* from New York wheat and competitiveness of a tebuconazole-resistant isolate. *Plant Disease*, 98(5), 607-613.

Talas F; McDonald BA (2015). Significant variation in sensitivity to a DMI fungicide in field populations of *Fusarium graminearum*. *Plant Pathology*, 64(3), 664-670.

Yin Y; Liu X; Li B; Ma Z (2009). Characterization of sterol demethylation inhibitor-resistant isolates of *Fusarium asiaticum* and *F. graminearum* collected from wheat in China. *Phytopathology*, 99(5), 487-497.

Fehr M, Zito R, Meyer L, Stammler G, The mitochondrial *bc1* inhibitor Ametoctradin has an unusual binding mode. In: Deising HB; Fraaije B; Mehl A; Oerke EC; Sierotzki H; Stammler G (Eds), "Modern Fungicides and Antifungal Compounds", Vol. IX, pp. 111-116. © 2020. Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-16-7

The mitochondrial bc_1 complex inhibitor Ametoctradin has an unusual binding mode

Fehr M[1], Zito R[1], Meyer L[2], Stammler G[1]

[1]*BASF SE D-67117 Limburgerhof, Germany*

[2]*BASF France SAS, F-69130 Ecully, France*

Corresponding Author Email: marcus.fehr@basf.com

INTRODUCTION

Ametoctradin is a selective inhibitor of mitochondrial bc_1 complex of oomycetes. Previous studies using the isolated bc_1 complex from *Pythium* spp. argued that Ametoctradin binds to the Q_o-site of the enzyme. Since the chemical structure of Ametoctradin and the modeling studies suggested a binding mode like ubiquinol, the possibility for a dual Q_o- and Q_i-site binding mode was left open. Binding studies with mitochondrial membrane preparations from *Pythium* spp. and an *S. cerevisiae* strain with a modified Q_i-site were used to further investigate the binding mode of Ametoctradin. These studies argue that Ametoctradin has an unusual binding mode that is clearly different from those of the other oomycete-specific inhibitors Cyazofamid and Amisulbrom. The data suggested that Ametoctradin could bind to both the Q_o- and Q_i-sites of the mitochondrial bc_1 complex and that its position or binding pose in the Q_i-site differs from that of Cyazofamid and Amisulbrom. Furthermore, the data support the argument that Ametoctradin prefers binding to the reduced bc_1 complex.

MATERIAL AND METHODS

Isolation of mitochondrial membrane preparations from *Pythium tracheiphilum*:
A 500 ml pre-culture of *Pythium* spp. (10 g/l malt extract, 4 g/l yeast extract and 4 g/l glucose) was inoculated using ~20 mycelial plugs (~ 0.5 cm diameter) cut out from a culture dish and grown over night at 24 °C and shaking at 140 rpm. The next morning, 9 l of culture (9×1 l in 2 l flasks) were started from the pre-culture. *Pythium* was grown for 3 days at 24 °C and 140 rpm. The mycelium was harvested by filtering and was subsequently immersed in 500 ml buffer (650 mM mannitol, 5 mM EDTA, 2 g/l bovine serum albumin, 50 mM potassium phosphate, pH 7.4), yielding a total volume of ~1 l. After addition of 1 mM PMSF and 1 volume of glass beads (0.5 mm), the mycelium was homogenized in a glass mill (Desintegrator S, Bernd Euler Biotechnologie, Frankfurt, Germany) for 12 min at 3500 rpm. The glass beads were removed by filtering and cell debris was removed by centrifugation for 20 min at 10,000×g. The membrane fraction, including the mitochondrial membranes, was pelleted by centrifugation for 2 h at 50,000×g. The pellets were stored at −80°.

Isolation of mitochondrial membrane preparations from *Saccharomyces cerevisiae*

10 ml pre-cultures of the *S. cerevisiae* WT (AD1-9) and PFQi3 (AD1-9 that combines nine amino acid substitutions at the Qi-site, namely I190G, A191L, A192C, I195F, L198F, S207T, M221F, F225L and I226L within cytochrome *b* strains were inoculated from a culture dish and grown over night at 28°C and shaking at 160 rpm (10 g/l yeast extract, 20 g/l bacto tryptone and 10 g/l glucose). The next morning, 5 l of culture (5×1 l in 2 l flasks) were started for each yeast from the pre-cultures using ethanol as the carbon source (10 g/l yeast extract, 20 g/l bacto tryptone and 16 g/l ethanol). The yeasts were grown at 28 °C and 160 rpm for 44 h. Cells were harvested by centrifugation (15 minutes at 15,000×g and 4°C) and subsequently re-suspended in 250 ml buffer (650 mM mannitol, 5 mM EDTA, 2 g/l bovine serum albumin, 50 mM potassium phosphate, pH 7.4). After addition of 1 mM PMSF, cells were lysed using a high-pressure homogenizer (Emulsifier S, Avestin, Ottawa, ON, Candanda). Cell debris was removed by centrifugation for 25 min at 6,000×g. The membrane fraction, including the mitochondrial membranes was pelleted by centrifugation for 2 h at 50,000×g. The red-brown pellet was stored at −80 °C.

Photometric binding studies

The pellets containing the mitochondrial membranes (yeast ~1.5 g and Pythium ~3.5 g) were re-suspended in 60 ml of 50 mM K-phosphate buffer, pH 7.4, using gentle strokes with a potter until suspensions became homogenous. The suspension was directly used for photometric binding studies. Absorbance spectra were recorded by difference spectroscopy, using a spectrophotometer (Cary 4000, Agilent, Santa Clara, CA, USA) and ~ 0.1–0.5 µM of bc_1 complex as estimated by cytochrome *b* absorbance after reduction with dithionite (25.6 $mM^{-1}\times cm^{-1}$ for the average absorbance of the b_L and b_H hemes of cytochrome *b*). A baseline was recorded from 535 nm to 580 nm using 1 ml of fully oxidized mitochondrial membrane preparation generated by adding ~10 µM K-ferricyanide to sample and reference cuvettes. To record the dithionite reduced spectrum, a few grains of dithionite were added to 1 ml of the mitochondrial membrane preparation in a new sample cuvette. After 5 min of incubation, a difference spectrum against the fully oxidized complex in the reference cuvette was recorded from 535 nm to 580 nm. The spectrum was also recorded after the addition of ~10 µM decylubiquinol. To record the absorbance spectra in the presence of inhibitors, 50 µM of the inhibitors were added to 1 ml of mitochondrial membrane preparations 1 min prior to the addition of ~10 µM decylubiquinol. Spectra were recorded immediately after the addition of decylubiquinol, and 11 spectra were captured for each sample in intervals of 10 s. For samples that yielded a constant signal, the data were averaged to reduce noise. 100 µM KCN was used to inhibit cytochrome *c* oxidase. Baseline correction was applied to correct the drift of the spectra if necessary.

RESULTS

Previous work with the purified bc_1 complex argued that Ametoctradin binds to the Q_o-site of the *Pythium* spp.bc_1 complex. As the chemical structure of Ametoctradin and the binding pose in the Q_o-site suggested a binding mode like ubiquinol, the possibility of a dual site binding mode was left open (Fehr, *et al*., 2015). To identify the binding site of Ametoctradin, classical double-kill analyses were carried out and the reduction of the Pythium bc_1 complex with decylubiquinol was analyzed in the presence of combinations of inhibitors (Figure 1). Double-kill studies make use of the observation that combinations of Q_i- and Q_o-site inhibitors fully block the reduction of the bc_1 complex. As expected, the Q_o-site inhibitor Azoxystrobin together with the Q_i-site inhibitors Antimycin A, Cyazofamid and Amisulbrom completely abolished the reduction of the bc_1 complex by preventing the oxidant-induced reduction (Figure 1 a - c). Thus, Cyazofamid and Amisulbrom are classical Q_i-site inhibitors like Antimycin A. By contrast, Ametoctradin cannot block the reduction of the bc_1 complex either in combination with Azoxystrobin or with Antimycin A (Figure 1 d, e), suggesting that Ametoctradin can only bind to the bc_1 complex after it was reduced by decylubiquinol, indicating a strong preference for the reduced complex.

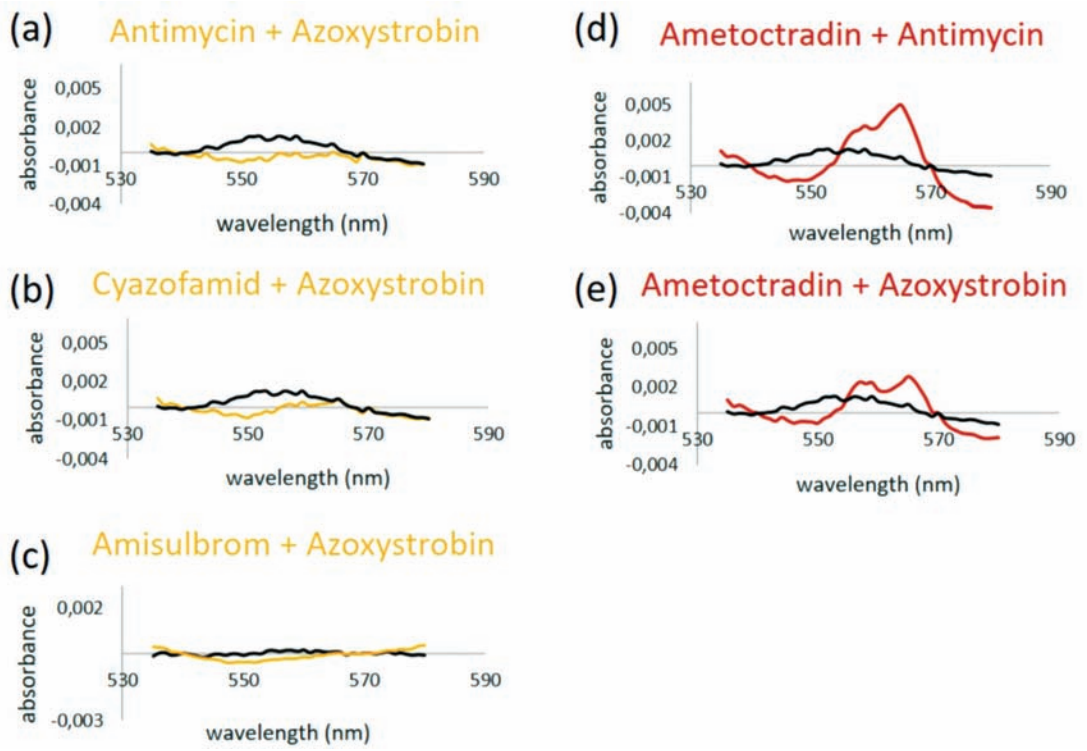


Fig. 1: Double-kill studies using mitochondrial membrane preparations from Pythium spp.: (a) Antimycin + Azoxystrobin, (b) Cyazofamid + Azoxystrobin and (c) Amisulbrom + Azoxystrobin fully block the reduction of the *Pythium* spp. bc_1 complex. In contrast, (d) Ametoctradin + Antimycin or (e) Ametoctradin + Azoxystrobin cannot block the reduction of the bc_1 complex by decylubiquinol. The reduction in the presence of decylubiquinol without inhibitor is shown in black. No reduction of the bc_1 complex is observed in the absence of inhibitor since the bc_1 complex is reduced by the functional respiratory chain in mitochondrial membrane preparations under these conditions.

The spectrum of the *Pythium* spp. bc_1 complex in the presence of Ametoctradin was similar to the spectra observed in the presence of the Q_i-site inhibitors Antimycin A, Cyazofamid and Amisulbrom (Figure 2). However, subtle differences were observed. The reduction of the bc_1 complex in the presence of Q_i-site inhibitors occurs via the Q_o-site and involves the reduction of the b_H and b_L hemes by the bifurcated reaction under conditions where cytochrome c_1 can be re-oxidized. In contrast, the reduction in the presence of Q_o-site inhibitors is thought to occur via the Q_i-site and to involve mainly the reduction of the b_H heme under conditions where decylubiquinol is in a highly reduced state when its oxidation via the high potential chain is blocked. Like the spectra observed in the presence of the Q_i-site inhibitors, the spectrum of the Ametoctradin-treated enzyme showed a strong contribution of the b_L heme. The spectrum in the presence of Ametoctradin showed a more deeply divided split b_L spectrum than in the presence of Q_i-site inhibitors that can be best explained by a higher spectral contribution of the b_L heme relative to the b_H heme. Hence, it is tempting to speculate that Ametoctradin raises the midpoint potential of the b_L heme but not that of the b_H heme. A raise of the b_L heme midpoint potential may also explain the preference of Ametoctradin for the reduced bc_1 complex.

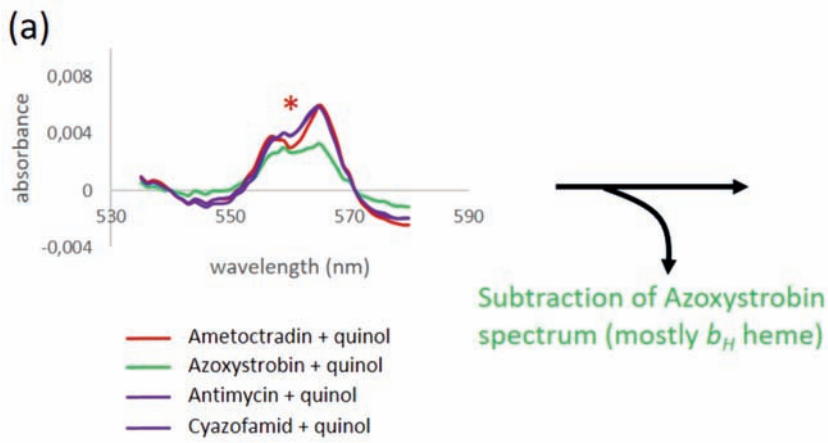
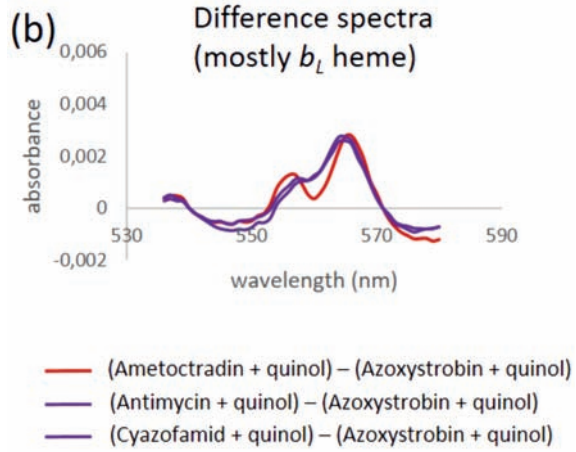


Fig. 2: Single-*kill studies using mitochondrial membrane preparations from Pythium* spp.:
(a) The spectra observed upon addition of Q_i-site inhibitors showed a shoulder at 558 nm and a peak at 566 nm (purple). In the presence of the Q_o-site inhibitors, a broader signal between 558 nm and 566 nm was observed (green). The shoulder at 558 nm and the peak at 566 nm can be attributed to the split alpha band of the b_L heme.



Accordingly, the broader signal in the presence of the Q_o-site inhibitors can be explained by the reduction of the b_H heme via the Q_i-site and its absorbance at 560 nm. Similar to the Q_i-site inhibitors, the spectrum of the Ametoctradin-treated enzyme showed a strong contribution of the b_L heme (red). However, the spectrum in the presence of Ametoctradin showed a more deeply divided split b_L spectrum than the Q_i-site inhibitors. (b) The effect on the b_L spectrum is better visible after subtraction of the Azoxystrobin spectrum (Q_o-site inhibitor) that is dominated by b_H heme absorbance.

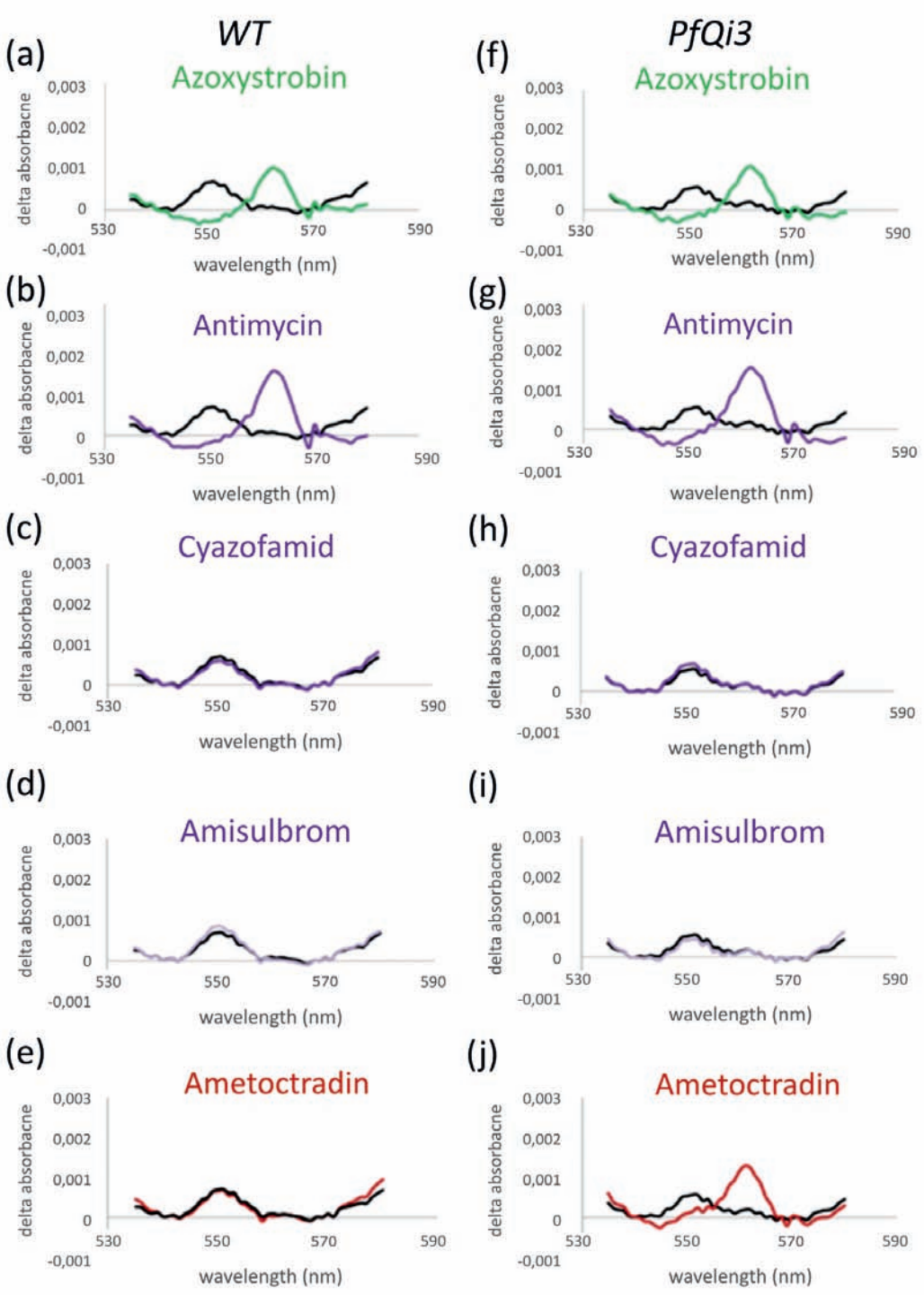


Fig. 3: Single-kill studies using mitochondrial membrane preparations from wildtype and PFQi3 yeast: Single-kill spectra of the wildtype yeast in the presence of (a) Azoxystrobin, (b) Antimycin, (c) Cyazofamid, (d) Amisulbrom, (e) Ametoctradin. Single-kill spectra of the PfQi3 yeast in the presence of (f) Azoxystrobin, (g) Antimycin), (h) Cyazofamid, (i) Amisulbrom and (j) Ametoctradin. The reduction in the presence of decylubiquinol without inhibitor is shown in black. No reduction of the bc_1 complex is observed in the absence of inhibitor since the bc_1 complex is reduced by the functional respiratory chain in mitochondrial membrane preparations under these conditions.

As no conclusion about Ametoctradin's binding site could be drawn from the double-kill studies with mitochondrial membrane preparationsfrom *Pythium* spp., binding studies with the bc_1 complex from the PFQi3 yeast were carried out (Figure 3). Ametoctradin is an oomycete-specific inhibitor that does not inhibit the bc_1 complex of wild type yeast. However, Ametoctradin inhibits the bc_1 complex of mutant PFQi3 yeast that was identified by susceptibility testing among several cytochrome *b* mutants (not shown). PFQi3 carries nine amino acid exchanges in the Q_i-site. Single-kill studies with Azoxystrobin and Antimycin A produced very similar spectra for the PFQi3 and wild type yeasts (Figure 3 a, b, f, g). In contrast to *Pythium* ssp., the contributions of the b_L and b_H hemes overlapped and added up to a single peak at 562 nm for both yeast strains. No reduction of cytochrome *b* from the wild type yeast was observed in binding studies with the oomycete-specific inhibitors Cyazofamid, Amisulbrom and Ametoctradin, confirming that these compounds do not bind to the bc_1 complex of the wild type yeast (Figures 3c, d, e). By contrast, Ametoctradin caused a reduction of cytochrome *b* of the PFQi3 strain indicating its binding to the bc_1 complex of this yeast (Figure 3j). Since the only difference between the two yeast strains lies in the Q_i-site, it can be concluded that Ametoctradin can also bind to Q_i-site of the PfQi3 yeast. Interestingly, neither Cyazofamid nor Amisulbrom bound to or inhibited the bc_1 complex of PFQi3 indicating that Ametoctradin has a different binding position and/or binding pose in the Q_i-site than Cyazofamid and Amisulbrom (Figures 3 h, i).

CONCLUSIONS

The data presented here argue that the anti-oomycete compound Ametoctradin binds to both the Q_o- and Q_i-sites of the bc_1 complex and support the argument that Ametoctradin has a different position or binding pose in the Q_i-site than the two other commercially available anti-oomycete compounds Cyazofamid and Amisulbrom. Furthermore, the data suggest that Ametoctradin preferentially binds to the reduced complex and may raise the midpoint potential of the b_L heme. Thus, Ametoctradin has an unusual binding mode and it is tempting to speculate, that it binds and traps the bc_1 complex in a different conformation of the catalytic cycle than most other inhibitors.

REFERENCES

Dreinert A; Wolf A; Mentzel T; Meunier B; Fehr M (2018). The cytochrome bc_1 complex inhibitor ametoctradin has an unusual binding mode. *Biochim Biophys Acta* 1859, 567-576.

Fehr M; Wolf A; Stammler G (2014). Binding of the respiratory chain inhibitor ametoctradin to the mitochondrial bc1 complex. *Pest Manag Sci* 72, 591-602.

Zito R et al., State of knowledge on molecular mechanisms leading to a reduced sensitivity of P. viticola towards ametoctradin and complex III inhibitors. In: Deising HB; Fraaije B; Mehl A; Oerke EC; Sierotzki H; Stammler G (Eds), "Modern Fungicides and Antifungal Compounds", Vol. IX, pp. 117-118. © 2020. Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-16-7

State of knowledge on molecular mechanisms leading to a reduced sensitivity of *P. viticola* towards ametoctradin and complex III inhibitors

Raffaello Zito[1], Lucie Meyer[2], Christophe Aumont[2], Marcus Fehr[1], Gerd Stammler[1]

[1]BASF SE D-67117 Limburgerhof, Germany; [2]BASF France SAS, F-69130 Ecully, France; Corresponding Author Email: raffaello.zito@basf.com

INTRODUCTION

Inhibitors of the mitochondrial complex III are very effective tools to control *Plasmopara viticola*, the causal agent of grape downy mildew. At the molecular level, they can bind at various positions: the Qo-site, the Qi-site or to both sites, like the naturally occurring coenzyme: ubiquinol. All active ingredients targeting the complex III address the ubiquinol binding sites and single amino acid exchanges within these sites can cause specific resistance. The molecular mechanisms leading to a reduced sensitivity of *P. viticola* towards Qo-inhibitors (QoI) are well understood, while the knowledge on the substitutions leading to Qi-inhibitors (QiI) and ametoctradin resistance is more recent. BASF monitors on a regular base the sensitivity of the European *P. viticola* populations. This publication summarizes the current knowledge on molecular mechanisms that can lead to the evolution of resistance towards complex IIIinhibitors.

MATERIAL AND METHODS

***In vivo* leaf disc test:** Leaf discs (19 mm) of the variety Riesling were treated with 2, 7 and 20 ppm of ametoctradin (200 g ai/L) or 1, 3 and 10 ppm of cyazofamid (160 g ai/L). The discs were placed in Petri dishes, 3 replicates per concentration, i.e. 9 discs per Petri dish. One day after fungicide application the discs were inoculated *via* airbrush with 1 mL of sporangia suspensions ($2*10^5$ sporangia/mL) from specific *P. viticola* isolates. The Petri dishes were incubated for 7 days at 20°C and 12 h light/dark per day. Subsequently, the sporulation on the leaf discs was visually assessed in a percentage of sporulating leaf area.

***In vitro* zoospore release test:** A zoospore release test was carried out with *P. viticola* isolates carrying a known mutation in the complex III gene, as described by Fehr et al. (2014). A $6*10^5$/mL sporangia suspension of each isolate was treated with 10 ppm of ametoctradin (200 g ai/L) or 10 ppm of cyazofamid (160 g ai/L). After 1.5h of incubation at 4°C followed by 15 min at room temperature, the percentage of empty sporangia in the suspension was visually assessed and the percentage of efficacy of the treatment was calculated.

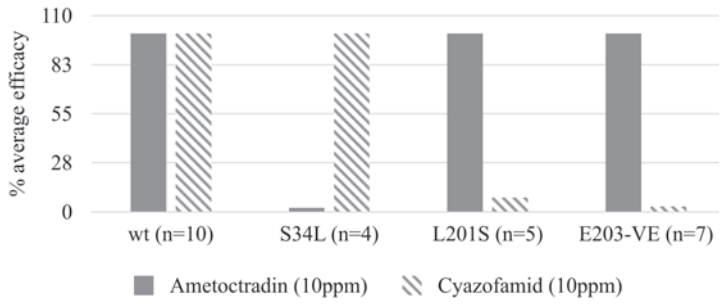
RESULTS

The well-known substitutions G143A and F129L are affecting the *P. viticola* sensitivity towards QoIs. New substitutions causing *P. viticola* resistance to QiIs or to ametoctradin were recently identified. The mutation S34L was identified in field isolates originating from south-western France. *In vivo* and *in vitro* tests have shown that the S34L has a specific negative impact on the activity of ametoctradin and no impact on QiIs, like cyazofamid or amisulbrom. The mutation L201S and the insertion E203-DE-V204 were identified as well in France. *In vivo* and *in vitro* tests have shown that these mutations affects specifically QiIs and have no impact on ametoctradin. In addition, a second type of insertion, the E203-VE-V204, was detected. Also in this case, *in vivo* and *in vitro* tests have shown that this insertion has no impact on ametoctradin, while it has an impact on QiIs. Besides these mutations, a general mechanism linked to the overexpression of the alternative oxidase (AOX) enzyme was observed. *In vivo* and *in vitro* tests performed confirmed that AOX overexpression affects the fungicidal activity of all the active ingredients that inhibit the complex III. The results of the cross-resistance tests performed are presented in Table 1 and Figure 1.

Table 1: *In vivo* detached leaf tests results (+ = sporulation, - = no sporulation)

	Ametoctradin 20 ppm	Cyazofamid 10 ppm
Wild-type isolate	-	-
S34L isolate	+	-
L201S isolate	-	+
E203-DE-V204	-	+
E203-VE-V204	-	+
AOX isolate	+	+

Figure 1: *In vitro* zoospore release test results (n= number of tested isolates)



CONCLUSIONS

The mutations recently found in the complex III of *P. viticola* resistant isolates confirm the specificity of the ametoctradin mode of action – which is able to bind to both the Qo- and Qi-site of the complex III (Dreinert et al. 2018), as well as the absence of cross-resistance between ametoctradin and Qi- or Qo-inhibitors. This knowledge could be used to set up and recommend effective anti-resistance management strategies for the control of *P. viticola*.

REFERENCES

Dreinert A; Wolf A; Mentzel T; Meunier B; Fehr M (2018). The cytochrome bc_1 complex inhibitor ametoctradin has an unusual binding mode. *Biochim Biophys Acta* 1859, 567-576.

Fehr M; Wolf A; Stammler G (2014). Binding of the respiratory chain inhibitor ametoctradin to the mitochondrial bc1 complex. *Pest Manag Sci* 72, 591-602.

Jacob S et al., Modulation of a phosphatase-gene as a novel molecular mechanism of fungicide resistance towards fludioxonil. In: Deising HB; Fraaije B; Mehl A; Oerke EC; Sierotzki H; Stammler G (Eds), "Modern Fungicides and Antifungal Compounds", Vol. IX, pp. 119-120. © 2020. Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-16-7

Modulation of a phosphatase-gene as a novel molecular mechanism of fungicide resistance towards fludioxonil

Jacob S[1,2], Bohnert S[1], Heck L[2], Gruber C[1], Neumann H[2], Yemelin A[1], Distler U[3], Tenzer S[3]

[1]*Institut für Biotechnologie und Wirkstoff-Forschung gGmbH (IBWF), Erwin-Schrödinger-Str. 56, D-67663 Kaiserslautern*

[2]*Mikrobiologie und Weinforschung am Institut für Molekulare Physiologie, Johannes Gutenberg-Universität Mainz, Johann-Joachim-Becherweg 15, D-55128 Mainz*

[3]*Institut für Immunologie, Universitätsmedizin Mainz, Langenbeck-Str. 1, D-55101 Mainz*

Corresponding Author Email: jacob@ibwf.de

The fungicide fludioxonil causes hyperactivation of the Hog1p MAPK essential for osmoregulation in pathogenic fungi (Jacob et al. 2015). The molecular regulation of MoHog1p phosphorylation is not completely understood. We identified and characterized a novel MoHog1p-interacting phosphatase gene *MoPTP2* in the filamentous rice pathogen *Magnaporthe oryzae* (Bohnert et al., 2019). Overexpression of the *MoPTP2*-gene conferred fludioxonil resistance, whereas the "loss of function" (lof) mutant *ΔMoptp2* was more susceptible towards the fungicide (Fig.1).

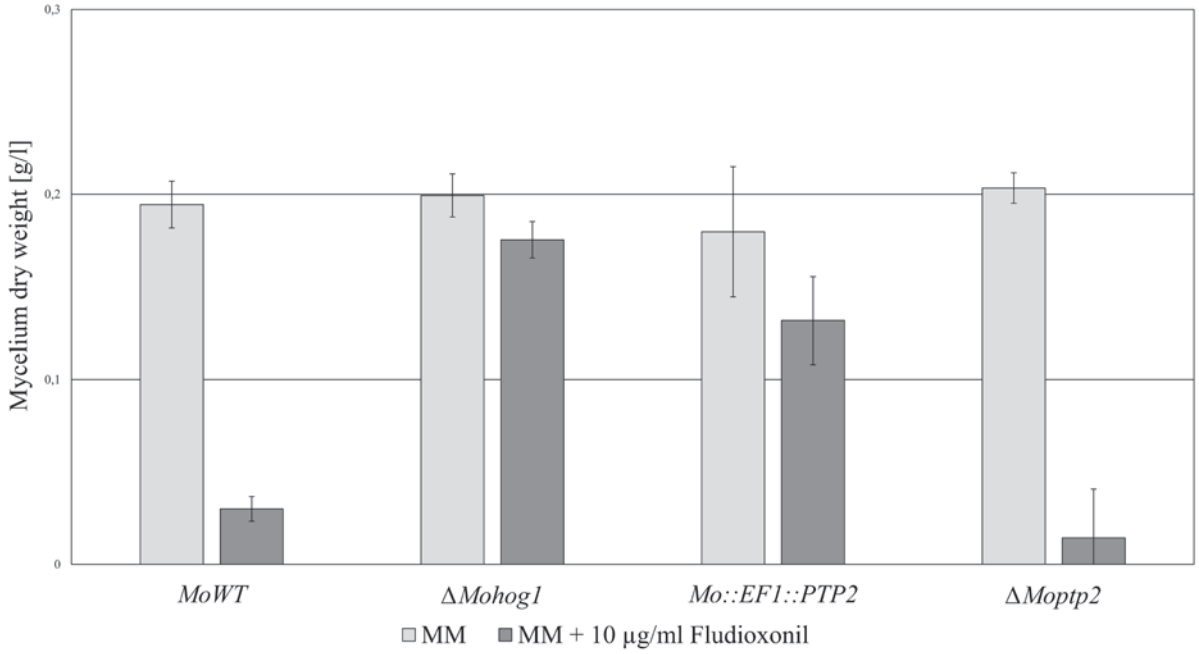


Figure 1 Mycelium dry weight of the *MoWT*, the overexpression mutant *MoEF1::PTP2* and the "lof" mutants *ΔMohog1* and *ΔMoptp2* after growth in liquid culture upon treatment with fludioxonil. The fungal cultures were grown in liquid MM and in MM including 10 µg ml-1 fludioxonil at 100 rpm and 26 °C. The error bars represent the standard deviation of three experiments.

In accordance with this, *in vivo* quantitative phosphoproteome profiling of MoHog1p-phosphorylation under pathway activation revealed lower phosphorylation levels of MoHog1p in the MoPtp2-overexpression mutant compared to the wildtype strain, whereas MoHog1p phosphorylation increased in the *ΔMoptp2* mutant (Bohnert et al. 2019).

These findings illustrate, that the phosphatase MoPtp2p is involved in the regulation of MoHog1p phosphorylation and that modulation of the gene *MoPTP2* is a molecular mechanism of fungicide resistance (Fig.2).

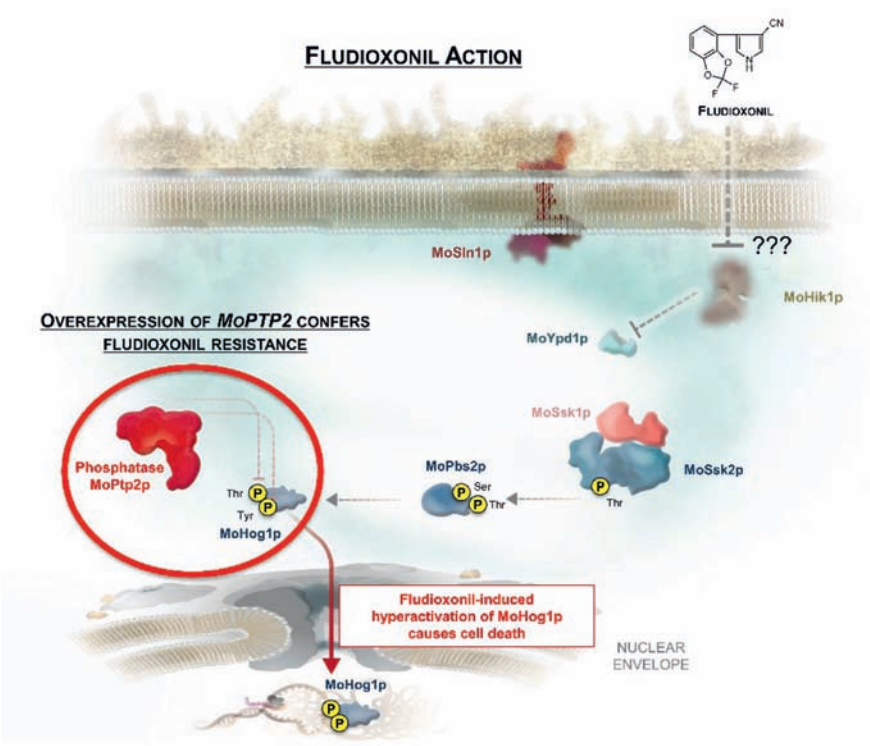


Figure 2 The mode of action of fludioxonil and the impact of MoPtp2p in *Magnaporthe oryzae*. Fludioxonil treatment results in dephosphorylation of the phosphorelay system MoHik1p-MoYpd1p-MoSsk1p. As a consequence, the MAPK cascade MoSsk2p-MoPbs2p-MoHog1p is hyperactivated. Overexpression of *MoPTP2* confers fludioxonil resistance, indicating that the phosphorylation-triggered hyperactivation of the MAPK MoHog1p is prevented by overexpression of *MoPTP2*.

REFERENCES

Bohnert S; Heck L; Gruber C; Neumann H; Distler U; Tenzer S; Yemelin A; Thines E; Jacob S (2019). Fungicide resistance toward fludioxonil conferred by overexpression of the phosphatase gene *MoPTP2* in *Magnaporthe oryzae*. *Molecular microbiology*, 111(3), 662-677.

Jacob S; Foster AJ; Yemelin A; Thines E (2015). High osmolarity glycerol (HOG) signalling in *Magnaporthe oryzae*: Identification of *MoYPD1* and its role in osmoregulation, fungicide action, and pathogenicity. *Fungal Biology,*119(7), 580–94.

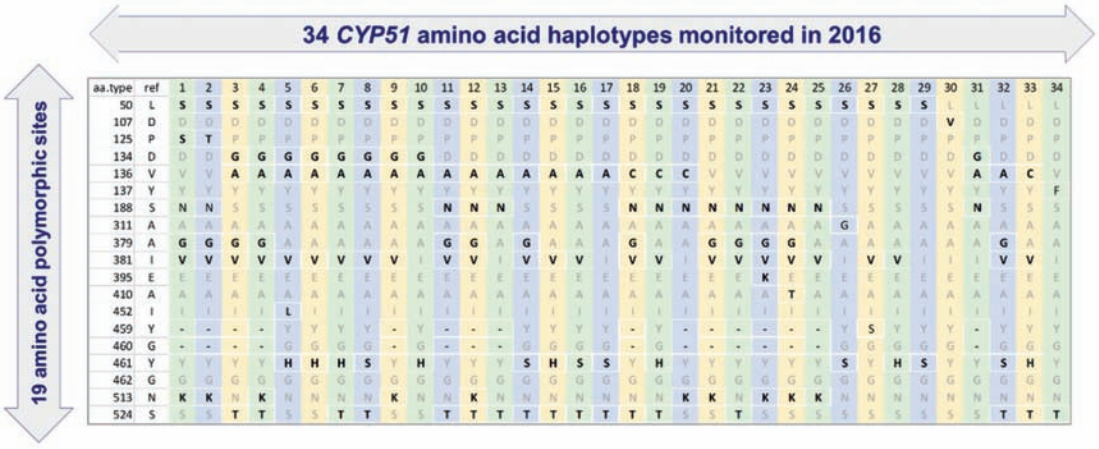
Frey R et al., The evolution of Zymoseptoria tritici sensitivity to triazole fungicides. In: Deising HB; Fraaije B; Mehl A; Oerke EC; Sierotzki H; Stammler G (Eds), "Modern Fungicides and Antifungal Compounds", Vol. IX, pp. 121-122. © 2020. Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-16-7

The Evolution of *Zymoseptoria Tritici* Sensitivity to Triazole Fungicides

Frey R[1], Lindenberger N[1], Brunner PC[2], Walder F[3], Scalliet G[1], Torriani SFF[1]

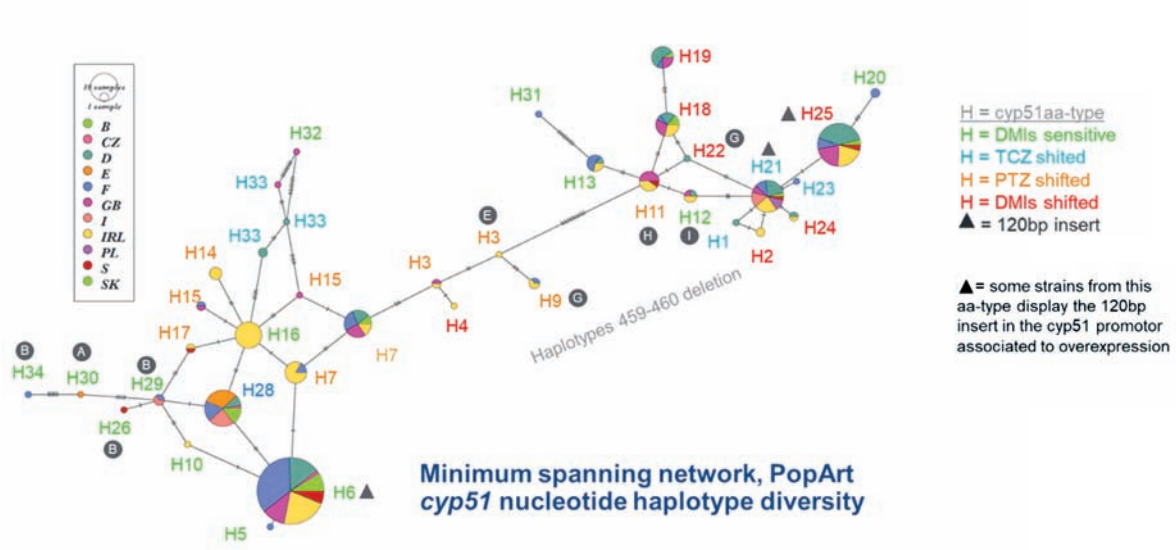
[1]*Syngenta Crop Protection Münchwilen AG, Schaffhauserstrasse 215, 4332 Stein, Switzerland*
ETH-Zurich, Universitätstrasse 2, 8092 Zürich, Switzerland
[3]*Agrocope Reckenholz, Reckenholzstrasse 191, 8046 Zürich, Schweiz*
Corresponding Author Email: regula.frey@syngenta.com

Zymoseptoria tritici, the causal agent of Septoria tritici blotch is the most severe pathogen of wheat worldwide (Torriani et al. 2015). Sustainable management of *Z. tritici* is mainly relying on fungicide spray programs. The DeMethylation Inhibitor (DMI) fungicides have been routinely used to control *Z. tritici* in Europe for more than 30 years and are classified by Fungicide Resistance Action Committee (www.frac.info) as moderate risk for resis¬tance development. Three mechanisms of resistance contribute to DMI sensitivity. A range of mutations in target gene cyp51 have been associated with the sensitivity shift (Huf et al. 2018; Zhan et al. 2006). This mechanism is associated to the higher resis¬tance factors. Additional mechanisms associated with lower resistance factors are cyp51 over expression (Cools et al. 2012) and cell detoxification through drug efflux mediated by Mfs1, a mem¬brane transporter belonging to the Major Facilitator Superfamily (Leroux & Walker 2011). The first *Z. tritici* sensitivity shift to DMIs was reported in Europe in the late 1990s, followed by shifts during2002-2004, 2009 and to a lower extend in 2015. Since 2015 the overall sensitivity level of European population stabilized with regional fluctuations. In this paper we present the cyp51 genotypes of 384 *Z. tritici* strains collected in 2016 from different European countries. The 384 strains collected in 2016 belonged to 34 distinct cyp51 amino acid haplotypes (cyp51 aa-types), determined by the combination of 19 polymorphic amino acid sites (Figure 1). The 34 distinct cyp51 aa-types were obtained from 40 nucleotide haplotypes cyp51 nt-types (Figure 2).



The 120 bp insertion in cyp51 promotor associated to high levels of overexpression was observed in only 3 aa-types. Other insertions in cyp51 promotor have been described in literature. Incomplete cross resistance exists between members of the DMI class. Strains

harboring mutation V136A results in a phenotype with higher sensitivity to tebuconazole compared with the strains without this mutation. Each of the 34 cyp51 aa-types shows a specific DMI cross resistance profile which can be summarized here as DMI sensitive, predominantly shifted to PTZ, predominantly shifted to TCZ or generally shifted to both PTZ and TCZ (Figure 2). Pattern of DMI incomplete cross resistance and different geographic distribution of *cyp51* genotypes were monitored. The DMI target gene *cyp51* evolved following selection imposed by a different use of DMIs applied in distinct regions and through recurring cycles of recombination. The more recent *cyp51* genotypes tends to show patterns of complete cross resistance to both tested DMIs. Additional DMI resistance mechanisms have been reported, including overexpression of *cyp51* gene or Mfs1-mediated multidrug resistance. Currently, high levels of *cyp51* overexpression is only restricted to a few haplotypes. The introduction of new DMIs might reshape *cyp51* evolution. Sound anti-resistance strategies need to be implemented to protect this fungicide class (www.frac.info).



REFERENCES

Cools HJ; Bayon C; Atkins S; Lucas JA; Fraaije BA (2012). Overexpression of the sterol 14α-demethylase gene (MgCYP51) in *Mycosphaerella graminicola* isolates confers a novel azole fungicide sensitivity phenotype. *Pest Management Science* 68, 1034-40.

Huf A; Rehfus A; Lorenz KH; Bryson R; Voegele RT; Stammler G (2018). Proposal for a new nomenclature for CYP51 haplotypes in *Zymoseptoria tritici* and analysis of their distribution in Europe. *Plant Pathology* 67, 1706-1712.

Leroux P; Walker AS (2011). Multiple mechanisms account for resistance to sterol 14α-demethylation inhibitors in field isolates of *Mycosphaerella graminicola*. *Pest Management Science* 67, 44-59.

Torriani SFF; Melichar JPE; Mills C; Pain N; Sierotzki H; Courbot M (2015). *Zymoseptoria tritici*: A major threat to wheat production, integrated approaches to control. *Fungal Genetics and Biology* 79, 8-12.

Zhan J; Stefanato FL; McDonald BA (2006). Selection for increased cyproconazole tolerance in *Mycosphaerella graminicola* through local adaptation and in response to host resistance. *Molecular Plant Pathology* 7:259–68.

FUNGICIDE RESISTANCE / RESISTANCE MANAGEMENT

Torriani SFF et al., Fungicide resistance evolution in soybean, a threat for disease control. In: Deising HB; Fraaije B; Mehl A; Oerke EC; Sierotzki H; Stammler G (Eds), "Modern Fungicides and Antifungal Compounds", Vol. IX, pp. 125-130. © 2020. Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-16-7

Fungicide resistance evolution in soybean, a threat for disease control

Scalliet G[1], Chagas N[2], Rosa DD[2], Frare VC[2], Mathioni SM[2], Appertet M[1], Sierotzki H[1], Torriani SFF[1]

[1]*Syngenta Crop Protection Münchwilen AG, Schaffhauserstrasse 215, 4332 Stein, Switzerland*
[2]*Syngenta Protecao de Cultivos Ltda Av. Nacoes Unidas 18.001 04795-900 Sao Paulo, Brazil*
Corresponding Author Email: stefano.torriani@syngenta.com

INTRODUCTION

In the past 15 years, soybean cultivation massively increased in Brazil. Recently, Brazil became the second largest producer worldwide with 114 million tons, after the USA (117 million tons). Soybean is the most relevant crop in Brazil (34 million ha) and the total cultivated area is comparable to that of the other seven major crops together (Cattelan and Dall'Agnol 2018).

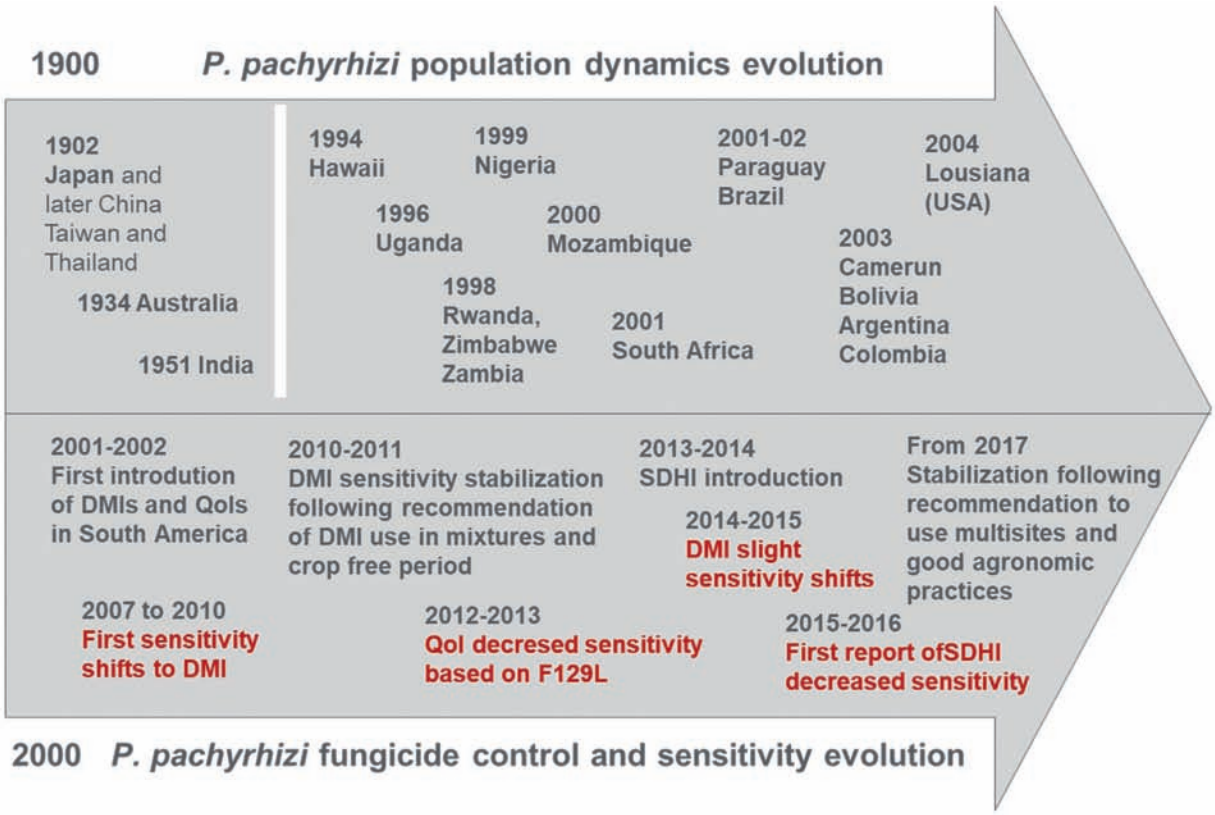


Figure 1: Asian soybean rust population dynamics and evolution (upper part) and introduction of fungicide classes and reports of decreased fungicide sensitivity (lower part)

Fungal diseases can significantly reduce yield, with up to 80% losses and, consequently, severe economic impact. *Phakopsora pachyrhizi,* the causal agent of Asian Soybean Rust (ASR), is the most devastating pathogen in Brazil. In 2001, ASR was described for the first time on the

American continent, i.e. in Paraguay in a field close to the Brazilian border (Yorinori et al. 2005). In 2002, *P. pachyrhizi* was detected in Brazil and in 2003 it was found in Bolivia, Argentina and Colombia (Rossi 2003). Despite previous reports, there are reports of an early ASR colonization of the American continent before 2000-01 (Freire et al. 2008). Possibly favored by the Hurricane Ivan (ISARD et al. 2005), ASR reached the continental USA in 2004, colonizing fields in the State of Louisiana (Schneider et al. 2005) (Figure 1). Urediniospores have a long dispersal potential, mainly favored by winds and human activities (Hartman and Haudenshield 2009). Agronomic strategies to control *P. pachyrhizi* have been adopted, such as sowing of resistant cultivars, elimination of secondary hosts and the introduction of a 60 to 90 days soybean free period. However, the most effective measure to control *P. pachyrhizi* remains the use of fungicides. Succinate-Dehydrogenase Inhibitors (SDHIs), DeMethylation Inhibitors (DMIs), Multisite inhibitors, Morpholines and Quinone Outside Inhibitors (QoIs) can currently be included in a spray program. After the first appearance of ASR in Brazil (2001) fungicide resistance monitoring programs have been initiated in 2004. A first sensitivity shift to DMIs was recognized from 2007 to 2010. Resistance to QoI was first noticed in 2012-2013 and it became quickly established in the Brazilian population. From 2015-16 the first ASR populations showing decreased sensitivity to SDHI were monitored (Figure 1).

Beside the ubiquitous presence of ASR, other fungal diseases with regional relevance are target spot and anthracnose caused by *Corynespora cassicola* and *Colletotrichum truncatum*, respectively. *C. cassicola* the causal agent of target spot can infect a range of different hosts, including cotton (Teramoto et al. 2017), and was first reported in Brazil in 1976 (Edwards Molina et al. 2019). Target spot control is mainly achieved by the cultivation of partially resistant cultivars, crop rotation, seed treatment and foliar application of different fungicide classes including DMI, SDHI and multisite fungicides (Soares, Godoy, and de Oliveira 2009). Resistance to MBC and QoI fungicides (FRAC, www.frac.info) was monitored and field efficacy was found impaired (Teramoto et al. 2017). *C. cassicola* field populations are today characterized by high frequency of G143A mutations conferring a disruptive resistance to QoI. Recently, single isolates showing high EC_{50} to SDHI fungicides have been found in Brazil, as reported by FRAC. Mainly as a result of adaptation to fungicides of different key soybean diseases the overall average number of needed applications increased in Brazil from an average of 3.9 treatments in 2015-16 season to 4.6 treatments in 2017-18. The largest average increase was observed in Rio Grande do Sul with an average increase from 4.4 to 5.6 applications in three seasons. The principal aim of this contribution is 1) to better characterize the different fungicide mechanisms related to fungicide resistance evolution, 2) to map the geographic distribution of fungicide sensitivity of the major soybean diseases and 3) to propose anti-resistance strategies aiming at delaying the development and progression of resistance.

MATERIALS AND METHODS

A total of 716 field populations of ASR were considered in this report (Figure 2). Infected soybean leaves from mainly commercial fields were collected and propagated on detached

leaves. The spore suspensions obtained from sporulating leaves were applied to trifoliate soybean leaves, previously treated with a range of fungicide concentrations. The trifoliate leaves were then placed on water agar and incubated at 22°C with an alternation of 12h light and 12 h of darkness. After 14 days the percentage of diseased leaf surface was estimated for individual fungicide concentrations compare to the untreated check, and the relative EC_{50} value inferred by regression analysis. The fungicide sensitivity to a commercially available DMI and SDHI was assessed for 102 *C. cassiicola* strains collected from infected soybean in Brazil during 2016-17 and 2017-18 seasons. Fungicide sensitivity was assessed using an *in vitro* test. To test the sensitivity of *C. cassiicola* to these selected fungicides, 50 µl fungicide solution and 50 µl spore suspension are mixed in 96-well microtiter plates. The following final concentrations of all fungicides used in the microtiter assays were 0; 0.001; 0.01; 0.1; 1; 10 and 100 ppm active ingredient. For each isolate and fungicide concentration, three replicate wells were used. Three replicate wells were also used per fungicide concentration as blanks (fungicide solution + YBA medium). The microtiter plates were put into plastic bags to avoid evaporation and incubated at 25°C in darkness. Five days after inoculation growth was measured in a photometer at λ 405 nm. The OD value were used calculate the relative EC_{50} values inferred by regression analysis

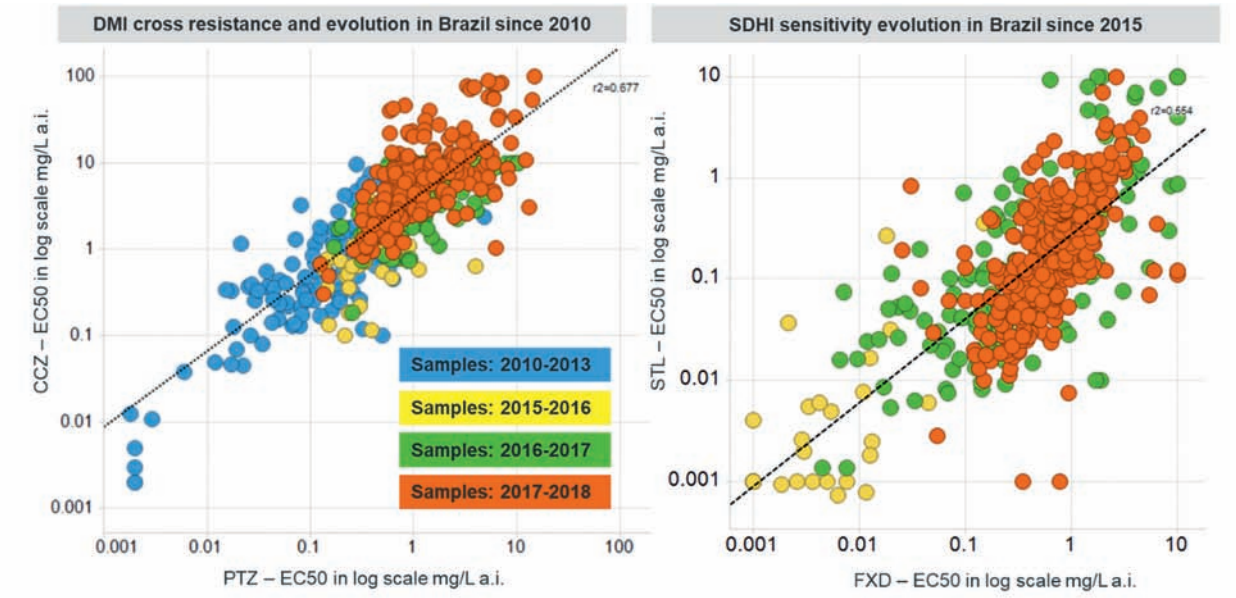


Figure 2: Left panel shows the DMI cross resistance profile and sensitivity evolution of 716 ASR field populations collected from different Brazilian states from 2010 to 2018 to cyproconazole (CCZ) and prothioconazole (PTZ). The right panel shows the SDHI cross resistance profile and sensitivity evolution of 571 ASR field populations collected from different Brazilian states from 2015 to 2018 to SOLATENOL™ (STL) and fluxapyroxad (FXD).

RESULTS AND DISCUSSION

DMI fungicides have been used since the first introduction of ASR in South America in 2001. A first sensitivity shift was observed between 2007 and 2010 following the selection pressure imposed by the recurrent use of this fungicide class. Sensitivity has been observed to stabilize

from season 2010-11. This phase of stabilization is most likely connected to the recommendation of an exclusive use of azole only in mixtures with a fungicide with a different mode of action. The introduction of a crop-free period could have contributed to decrease the overall agronomic risk. The trend of stable sensitivity lasted in the following seasons until season 2013-14. From season 2014-15 slight shifts in sensitivity have been observed. The 2017-18 monitoring showed in general a stable situation as in the previous years, but locally some slight shift was observable in western parts of Brazil. All DMIs should be considered as cross resistant (Figure 2 left panel), since they belong to the same fungicide class and are affected by the same resistance mechanisms. However, a variation of the intrinsic potency to control ASR can be observed between triazoles. Currently the major recommendations to protect DMIs from further shifts while ensuring a robust disease control, consist of their preventative use in mixture with other fungicide classes. Reduced rates below 75% of the full rate should be avoided and the proper coverage of the treated crop by appropriate and well calibrated application technology should be ensured. Restriction to the recommended spray intervals and adjustment to the disease epidemics will contribute to the overall resistance management for DMI.

The QoI fungicide sensitivity shift based on the F129L mutation in the target gene cytochrome b, was first reported at low to moderate frequency in Brazil during season 2013-14. The resistance allele F129L was rapidly selected and one year later this mutation was found in most of the samples collected throughout Brazil and Paraguay. Today, the F129L QoI resistance allele is homogenously distributed across Brazil at high frequency, as reported by independent monitoring programs reported at the FRAC web site.

Since 2011-12, SDHI fungicides have been introduced in Brazil for ASR control (Godoy et al. 2016) and no field efficacy reduction was observed before 2015-16. The first reports of reduced sensitivity to and reduced efficacy of SDHI have been reported in 2015-16. The first isolation of a less SDHI-sensitive phenotype occurred by the detection of survivors (single pustules) on leaves treated with SDHI concentrations, at which fully sensitive populations were completely inhibited. Such survivors were selected by propagation of single pustules over several cycles on detached leaves, and these strains were analyzed for the mechanism responsible for the lower SDHI sensitivity, identifying the sdhC-I86F mutation as the major determinant (Simões et al. 2017). However, other mutations might be present at lower frequency in natural populations and other mechanisms might contribute to shape the overall SDHI sensitivity (FRAC-SDHI working group). Until the 2016-17 season an increase in the frequency and distribution of populations showing decreased sensitivity to SDHI was observed. The frequency of less sensitive populations to SDHI stabilized in Brazil during 2017-18 season. In some areas, even a reduction in the frequency of less sensitive populations was noticed. If these findings are related to improved resistance management practices and/or to a certain fitness cost associated to resistance still needs to be further clarified. SOLATENOL™ and fluxapyroxad sensitivity results highlighted clear patterns of cross-resistance (Figure 2, right panel). As all SDHIs are cross-resistant, resistance management strategies must be cmparable for all SDHIs. Among the most important specific recommendations for SDHI are the application of this fungicide class always in mixture with a partner with a different mode of action and limiting the maximum of

SDHI containing sprays to two applications per soybean crop (under the assumption no soy after soy / double cropping occurs). SDHI should be applied preventively or as early as possible in the disease epidemic cycle, avoiding to reduce the rates strongly.

Good agricultural practices to reduce the inoculum source and disease progression are considered anti-resistance tool to protect DMI, QoI and SDHI. No multiple cropping, implementation and establishing soybean-free periods, cultivation of partially resistant soybean varieties, reduction of the planting window with preference to early-cycle varieties and destruction of volunteer plants can contribute to manage disease control and fungicide resistance evolution (Godoy et al. 2016). More information about ASR anti-resistance management is accessible at www.frac.info.

The intensiv use of QoI in Brazilian soybean fields has contributed to the evolution of resistance in *Cercospora kikuchii, Colletotricum truncatum* and *C. cassiicola* populations (De Mello et al. 2018). In 2015 and 2016, resistance due to the G143A mutation was detected in a significant number of *C. cassiicola* samples collected from Brazil (www.frac.info).

First DMI sensitivity studies were reported by FRAC in 2013-14 and 2014-15 seasons. These reports showed that the Brazilian *C. cassiicola* population is generally highly sensitive to DMIs. The same conclusion arose from a monitoring study including a total of 102 strains collected between 2016 and 2018 in Brazil (Figure 3 left panel). The range of sensitivity to DMI was comparable between the two seasons with no major regional variation in the distribution of sensitivity between Brazilian regions.

Decreased SDHI sensitivity was monitored in both 2016-17 and 2017-18 seasons in field isolates collected in Brazil. Molecular analysis confirmed the presence of the target site mutations sdhB-H278Y and sdhC-N75S in isolates with reduced sensitivity. These isolates were detected in Mato Grosso and at single sites in Rio Grande do Sul and in Bolivia. All sites analyzed from Goias, Minas Gerais and Parana were sensitive.

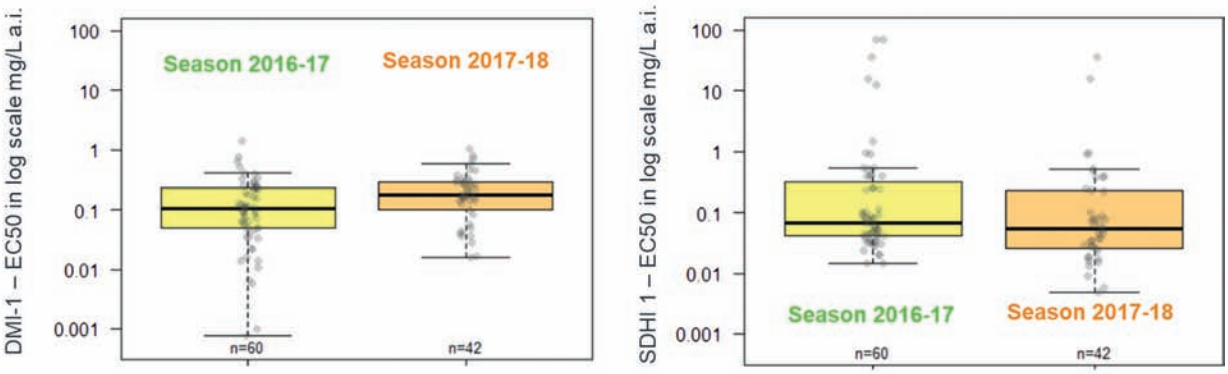


Figure 3: DMI (left) and SDHI (right) sensitivity of 102 *C. cassiicola* field strains collected from different Brazilian states in 2016-17 (n=60) and 2017-18 (n=42).

DMI and SDHI should be currently considered the backbone of soybean disease control. Cases of decreased sensitivity have been reported for both fungicide classes among relevant soybean pathogens. Widespread resistance to QoI in most relevant soybean diseases reduced the efficacy of this fungicide class especially in the presence of mutation cytb-G143A.

The soybean fungicide spray program should have as principal aim the mutual protection of both DMI and SDHI fungicide classes. Good agricultural practices and the use of multisite inhibitors and Amine fungicides must be considered as an integrative tool to manage soybean diseases.

REFERENCES

Cattelan AJ; Dall'Agnol A (2018) The Rapid Soybean Growth in Brazil. *Ocl* 25(1):D102.

Edwards Molina JP; Paul PA; Amorim A; da Silva LHCP; Siqueri FV; Borges EP; Campos HD; Nunes Junior J; Meyer MC; Martins MC; Balardin RS, Carlin VJ; Grigolli JFJ; Belufi LMdR; Godoy CV (2019) Meta-Analysis of Fungicide Efficacy on Soybean Target Spot and Cost–Benefit Assessment. *Plant Pathology* 68(1), 94-106.

Freire MCM; de Oliveira LO; de Almeida AMR; Schuster I; Moreira MA; Liebenberg MM; Mienie CMS (2008) Evolutionary History of Phakopsora Pachyrhizi (the Asian Soybean Rust) in Brazil Based on Nucleotide Sequences of the Internal Transcribed Spacer Region of the Nuclear Ribosomal DNA. *Genetics and Molecular Biology* 31, 920-931.

Godoy CV; Seixas CDS; Soares RF; Marcelino-Guimarães FC; Meyer MC; Costamilan LM (2016) Asian Soybean Rust in Brazil: Past, Present, and Future. *Pesquisa Agropecuaria Brasileira* 51(5), 407–21.

Hartman GL; Haudenshield JS (2009) Movement of Phakopsora Pachyrhizi (Soybean Rust) Spores by Non-Conventional Means. *Europ. Journal of Plant Pathology* 123, 225-228.

Isard SA; Gage SH; Comtois P; Russo JM (2005) Principles of the atmospheric pathway for invasive species applied to soybean rust. *Bioscience* 55, 851-861.

De Mello FE; Lopes-Caitar VS; Xavier SA; Mehl A; Marcelino-Guimaraes FC; VerreetJA; Balbi-Peña MI; Godoy CV (2018) Identification of QoI Mutation in Soybean Pathogens in Brazil." in *Conference: International Congress of Plant Pathology.* Boston, USA.

Rossi RL (2003) First Report of Phakopsora Pachyrhizi , the Causal Organism of Soybean Rust in the Province of Misiones, Argentina. *Plant Disease* 87(1),102.

Schneider RW; Hollier CA; Whitam HK: Palm ME; McKemy JM; Hernández JR; Levy L; DeVries-Paterson R (2005) First Report of Soybean Rust Caused by Phakopsora Pachyrhizi in the Continental United States. *Plant Disease* 89 (7), 774.

Simões K; Hawlik A; Rehfus A; Gava F; Stammler G (2017) First Detection of a SDH Variant with Reduced SDHI Sensitivity in Phakopsora Pachyrhizi. *Journal of Plant Diseases and Protection* 125 (1), 21-26.

Soares RF; Godoy CV; de Oliveira MCN (2009) Escala Diagramática Para Avaliação Da Severidade Da Mancha Alvo Da Soja. *Tropical Plant Pathology* 34(5),333-338.

Teramoto A; Meyer MC; Suassuna ND; da Cunha MG (2017). "In Vitro Sensitivity of Corynespora Cassiicola Isolated from Soybean to Fungicides and Field Chemical Control of Target Spot." *Summa Phytopathologica* 43(4), 281-89.

Yorinori JT; Paiva WM; Frederick RD; Costamilan LM; Bertagnolli PF; Hartman GE; Godoy CV; Nunes J (2005) Epidemics of Soybean Rust (Phakopsora Pachyrhizi) in Brazil and Paraguay from 2001 to 2003." in *Plant Disease* 89 (6), 675-677

Cherrad S et al., Resistance of *Plasmopara viticola* to complex III inhibitors: an overview on phenotypic and genotypic characterization of resistant strains. In: Deising HB; Fraaije B; Mehl A; Oerke EC; Sierotzki H; Stammler G (Eds), "Modern Fungicides and Antifungal Compounds", Vol. IX, pp. 131-136. © 2020 Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-16-7

Resistance of *Plasmopara viticola* to complex III inhibitors: an overview on phenotypic and genotypic characterization of resistant strains

Cherrad S[1], Hernandez C[2], Steva H[3], Vacher S [1]

[1]*CONIDIA, Parc d'activités en Chuel. Route de Chasselay. 69650 Quincieux, FRANCE*
[2]*CONIPHY, Parc d'activités en Chuel. Route de Chasselay. 69650 Quincieux, FRANCE*
[3]*CJH SAS, 21 C Chemin de la Girotte. 33650 La Brède, FRANCE*
Email: s.cherrad@conidia.fr

INTRODUCTION

The mitochondrial respiratory chain consists of multifunctional, oligomeric membrane enzyme complexes. Cytochrome *bc1* (complex III) is a key enzyme in the mitochondrial electron transport chain. Cytochrome *b* is a subunit of complex III and it catalyses the transfer of electrons from ubiquinol to cytochrome c, leading to proton translocation and energy transduction. The cytochrome *b* protein contains eight transmembrane helices encoded by *Cytb*, located in the mitochondrial genome. Theses helices contain target sites of widely used complex III inhibitor fungicides. The fungicides known as QoIs (Quinone outside inhibitors) block mitochondrial respiration by binding to the Qo (quinol oxidation) site (Grasso et al. 2006; Fisher & Meunier 2008). QoI fungicide-resistant isolates were detected in field plant pathogen populations of a large range of species. The use of this single-target fungicide group in grape downy mildew (*Plasmopara viticola)* management was restricted after widespread of resistance in French vineyards. Fungicides known as QiIs (Quinone inside inhibitors) inhibit the reduction of quinol in the Qi site close to the mitochondrial matrix. These fungicides are highly effective against oomycetes and play an important role in downy mildew management programs.

Downy mildew populations have developed resistance to QoIs fungicides by single amino acid substitution G143A in cytochrome *b*. More recently, less sensitive *P. viticola* populations to other complex III inhibitors, like Ametoctradin and Cyazofamid, were detected in French vineyards as well. In order to understand the molecular mechanisms of resistance to these molecules, *Cytb* of single-sporangia strains have been sequenced and analysed. Finally, a leaf-disc sensitivity bioassay was performed on isolated strains to investigate the cross-resistance pattern between the different complex III inhibitors.

MATERIAL AND METHODS

P. viticola populations and culture conditions

Downy mildew infected leaves were collected in vineyards of different French regions in 2016, 2017 and 2018 (a total of 27 populations were analysed). Sampling was carried out in vineyards with fungicide spray programs including or not complex III inhibitors. More than 50 foliar discs surrounding infected lesions (oil spots) per sample were prepared from the collected leaves. Foliar discs were placed onto Petri dishes, washed with distilled water and dried at room temperature. After 24h incubation, sporangia were collected in sterile water to inoculate decontaminated fungicide-free leaves from grape cultivar *Vitis vinifera* cv. Cabernet-Sauvignon. Inoculated leaves were incubated in Petri dishes for 7 days at 21°C with a 14:10 h light dark photoperiod. Freshly produced sporangia were harvested to inoculate leaf discs for sensitivity testing.

Chemical fungicides

Commercial formulations of Ametoctradin (Snooker®, concentrated solution containing 200g/l active ingredient, BASF, France), Cyazofamid (Ranman Top®, concentrated suspension containing 160g/l ai, ISK Biosciences Europe, France) and Amisulbrom (Leimay®, concentrated suspension containing 200g/l ai, Philagro, France) were tested. The fungicide formulations were dissolved in sterile distilled water. Stock solutions were stored at 4°C in the dark.

Resistant single-sporangia isolates and cross-resistance assay

Leaf discs with colonies sporulating at 1mg/l of Cyazofamid or Ametoctradin and in presence of 100mg/l SHAM (salicylhydroxamic acid) were used to isolate resistant strains of *P. viticola*, independent from potential presence or activity of alternative oxidase (AOX). Strains showing a high level of AOX-activity were also isolated in absence of SHAM. To investigate cross-resistance to other QiI fungicides, *in vitro* sensitivity of these strains to Ametoctradin, Cyazofamid and Amisulbrom, applied with or without SHAM, was measured with increasing concentrations of each fungicide (0.01-100mg/l). 10 discs were analysed for each condition, and assays for each isolate were repeated three times per fungicide concentration. After 7 days, individual leaf discs were evaluated for disease incidence and sporulation rate.

Total DNA extraction and PCR

Single sporangia strains of *P. viticola* growing leaf discs 7 days post inoculation were used as starting material for DNA extraction and PCR amplification. Total DNA was extracted using the Nucleospin® plant II kit (Macherey-Nagel, France) according to the manufacturers´ recommendations. PCR amplification of 1kb fragment of *Cytb* was carried out in 25µL reaction mixtures containing 30ng of total genomic DNA, forward (5'-TGAACCTGTAA ATTTAGCACAACAA-3') and reverse (5'-ACAGGACATTGACCAACCCA-3') primers (0.3µM), and 1x premixed Phusion Flash High-Fidelity PCR Master Mix (Thermo Scientific,

France). Amplifications were carried out in a thermal cycler LifeEco (BIOER Technology, France) using the following PCR programme: initial denaturation at 94°C for 1 min followed by 35 cycles at 95°C for 30s, 60°C for 60s, 72°C for 1 min, and a final extension at 72°C for 10 min. Amplification products were directly sequenced with the same primers as used for PCR amplification (GATC Biotech, Germany). To analyse the relevant *Cytb* sequence and to investigate point mutations in resistant strains, the generated sequences were compared against putative *Cytb* (accession number DQ459459.1) and against sequences of sensitive strains using the MAFFT sequence alignment program.

RESULTS AND DISCUSSION

Sensitivity of field isolates to Ametoctradin, Cyazofamid and Amisulbrom

Many downy mildew field populations with low sensitivity to Ametoctradin and Cyazofamid at different levels were observed in French vineyards in 2016, 2017 and 2018. Resistant and sensitive populations were selected to generate single-sporangia isolates. More than 70 single-sporangia strains were isolated including 41 resistant to Cyazofamid (QiI), 5 to Ametoctradin (QoSI), 7 to Pyraclostrobin (QoI), 15 to Ametoctradin+Pyraclostrobin and 3 strains showing exclusively high AOX expression. All selected resistant strains are growing at the discriminatory dose of 1mg/l. Further investigation on cross-resistance and genotypic characterization was conducted on a panel of 22 strains -listed in Table 1.

Molecular analysis of *Cytb* of *P. viticola* field isolates

S34L substitution in cytochrome b confer resistance to Ametoctradin

The growth of 4 strains (CONI-6/13/20/22) was inhibited only at 100 mg/l of Ametoctradin, with or without SHAM, and thus exhibited a high level of resistance (RF=1000) compared to the sensitive strain CONI-01 (MIC <0.1 mg/l). These strains are sensitive to Cyazofamid (MIC<0.1 mg/l) and Amisulbrom (MIC<0.1 mg/l), again with or without SHAM (Table 1).

Analysis of *Cytb* gene sequences of these strains reveals the presence of a single nucleotide mutation from cytosine to thymine at position 101 (TCA --> TTA, Figure 1A). This gene modification leads to substitution of the amino acid serine with leucine at position 34 (S34L) and obviously to specific Ametoctradin resistance in *P. viticola*. Serine S34 of Cytochrome b is located in the quinone inner site, suggesting that Ametoctradin inhibit mitochondrial respiration by interacting with complex III at the QiI site. On the other hand, full sensitivity was observed for Cyazofamid and Amisulbrom on S34L mutants. These results suggest that the mode of action of Ametoctradin at the QiI site is different from that of Cyazofamid and Amisulbrom. Recent investigation on the binding mode of Ametoctradin with its target site in the cytochrome *bc1* complex showed that Ametoctradin is able to interact with both, the Qo- and the Qi-site (Fehr et al. 2016; Dreinert et al. 2018).

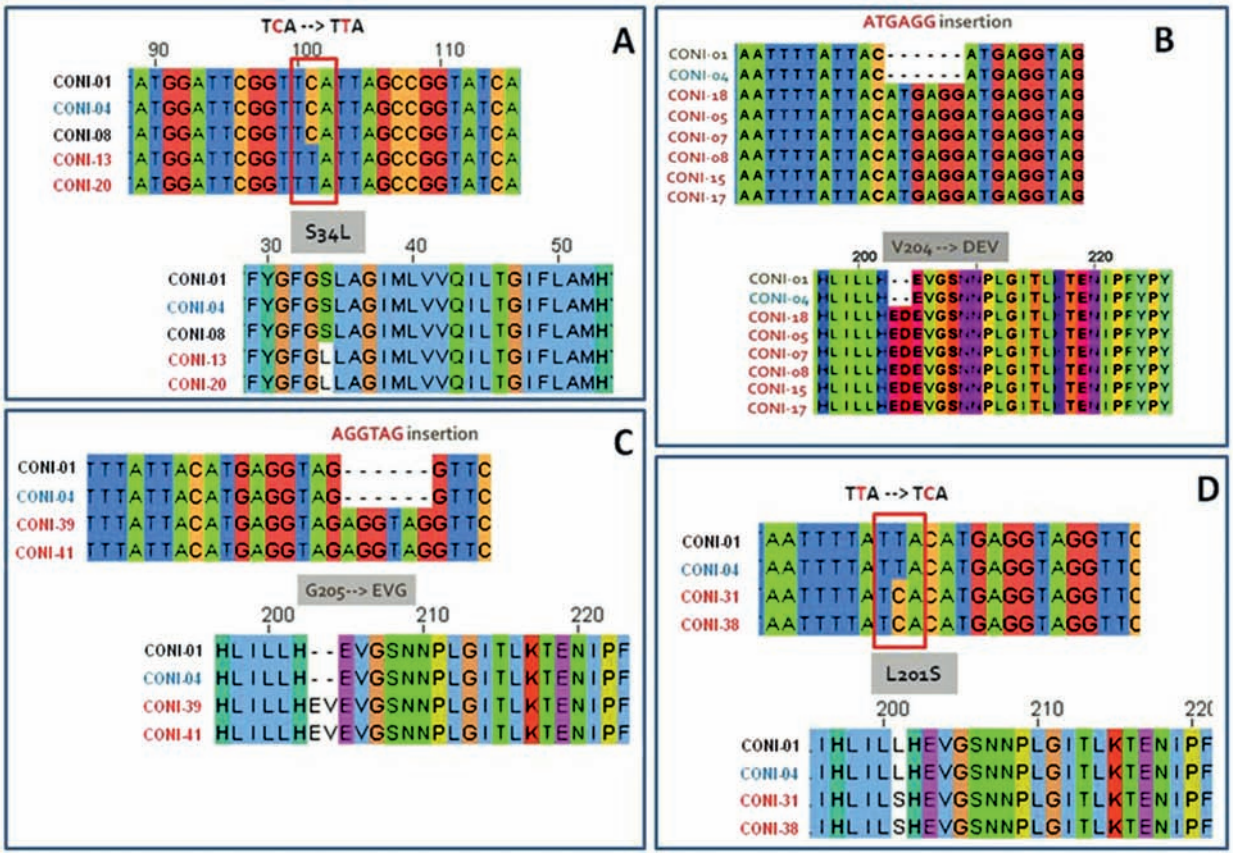


Figure 1 Alignment of *Cytb* partial sequences of Cyazofamid and Ametoctradin *P. viticola* resistant's isolates. In black: sequence of sensitive strain. In red: sequences of strains resistant to Cyazofamid or Ametoctradin. In blue: AOX strain. Sequences of strains with S34L substitution **(A)**, ATGAGG insertion **(B)**, AGGTAG insertion **(C)** and L201S substitution **(D).**

L201S substitution and two different insertions in the cytochrome b enconding gene confer resistance to Cyazofamid

According to the bioassays, 41 isolates of *P. viticola* are resistant to Cyazofamid applied alone or mixed with SHAM at a discriminate dose of 1 mg/l. Further analysis of 12 strains reveals that *P. viticola* development of 10 isolates was inhibited at 30 mg/l and 2 isolates (CONI-39/41) at 10 mg/l of Cyazofamid+SHAM compared to the sensitive strain CONI-01 (MIC<0.1 mg/l, Table 1). *Cytb* gene analysis of these strains showed that almost all isolated strains (31 isolates) carry a 6-nucleotides insertion, ATGAGG, compared to *Cytb* of Cyazofamid-sensitive strains. This short sequence insertion leads to protein modification with two additional amino acids (V204 --> DEV, Figure 1B). A second 6-nucleotides insertion, AGGTAG, was observed in other strains leading to modification G205 --> EVG (Figure 1C). In addition, *Cytb* gene sequences of 2 strains show a single nucleotide mutation at position 602 (TTA --> TCA), with amino acid substitution L201S in the Cytochrome b protein (Figure 1D). The described modifications in *Cytb* confer resistance to Cyazofamid but not to Ametoctradin. Sensitivity of some strains carrying insertion ATGAGG (CONI-07/09/10) and L201S (CONI-31) to Amisulbrom is slightly affected compared to sensitive strain (CONI-01: MIC<0.1mg/l), suggesting different level of cross-resistance to Amisulbrom (Table 1). Until now, fungicide

resistance caused by target modification occurred only with single nucleotide modification in all described plant pathogen and fungicide single-site mode of action. Insertion of short sequence in gene coding fungicide-target and probably protein conformational modification seems to be a new way to bypass to fungicide action.

CONCLUSION

This study provides an overview on the structure of collected grape downy mildew populations in French vineyards. In addition to the well-known QoI resistance of *P. viticola*, resistant strains to other complex III inhibitors were detected. *P. viticola* showed resistance to Ametoctradin due to target site mutation S34L, to Cyazofamid based on mutation L201S, and new target modifications by short nucleotides-insertions were detected in Cyazofamid-resistant population as well. No cross-resistance was observed between Ametoctradin and the other complex III inhibitors in strains tested in this study. Cyazofamid-resistant isolates show low sensitivity to Amisulbrom compared to sensitive strain and S34L mutants. These results suggest a different level of intrinsic activity of Amisulbrom and low cross-resistance with Cyazofamid. The analysis of collected populations shows, that many resistant phenotypes with different genotypes can co-exist in the same population.

REFERENCES

Dreinert A, Wolf A, Mentzel T, Meunier B, Fehr M (2018). The cytochrome bc1 complex inhibitor Ametoctradin has unusual binding mode. *Biochimica et Biophysica Acta* 1859 (8), 567-576.

Fehr M, Wolf A, Stammler G (2016). Binding of the respiratory chain inhibitor ametoctradin to the mitochondrial bc1 complex. *Pest Management Science* 72, 591-602.

Fisher N. and Meunier B., 2008 - Molecular basis of resistance to cytochrome bc 1 inhibitors. *FEMS Yeast Research* 8 (2),183–192.

Grasso V, Palermo S, Sierotzki H, Garibaldi A, Gisi U (2006). Cytochrome *b* structure and consequences for resistance to Qo inhibitor fungicides in plant pathogens. *Pest Management Science* 62 (6), 465-472.

Table 1 MIC (minimum inhibitory concentration) values for Ametoctradin, Cyazofamid and Amisulbrom of *P. viticola* strains and detected mutations or insertions in the *Cytb* gene

***P. viticola* strains**	**Ametoctradin (mg/l)**		**Cyazofamid (mg/l)**		**Amisulbrom (mg/l)**		**Substitution/Insertion**				
		+SHAM		+SHAM		+SHAM	**G143A**	**S34L**	**V204 --> DEV**	**G205 --> EVG**	**L201S**
CONI-01	0,3	<0,1	<0,1	<0,1	<0,1	<0,1	-	-	-	-	-
CONI-04	>100	0,3	30	<0,1	100	0,3	-	-	-	-	-
CONI-02	0,3	0,3	<0,1	<0,1	<0,1	<0,1	**X**	-	-	-	-
CONI-03	0,3	0,3	<0,1	<0,1	<0,1	<0,1	**X**	-	-	-	-
CONI-11	>100	0,3	30	<0,1	100	1	**X**	-	-	-	-
CONI-12	>100	0,3	30	<0,1	100	1	**X**	-	-	-	-
CONI-20	>100	100	<0,1	<0,1	<0,1	<0,1	-	**X**	-	-	-
CONI-22	>100	100	<0,1	<0,1	<0,1	<0,1	-	**X**	-	-	-
CONI-06	>100	100	<0,1	<0,1	0,3	<0,1	**X**	**X**	-	-	-
CONI-13	100	100	<0,1	<0,1	<0,1	<0,1	**X**	**X**	-	-	-
CONI-05	1	1	100	30	30	1	-	-	**X**	-	-
CONI-07	1	0,3	100	30	10	3	-	-	**X**	-	-
CONI-15	1	0,3	100	30	3	1	-	-	**X**	-	-
CONI-16	1	0,3	100	30	3	1	-	-	**X**	-	-
CONI-08	>100	0,3	100	30	30	1	-	-	**X**	-	-
CONI-09	>100	0,3	100	30	30	3	-	-	**X**	-	-
CONI-10	>100	0,3	100	30	30	3	-	-	**X**	-	-
CONI-17	1	0,3	100	30	3	1	-	-	**X**	-	-
CONI-31	>100	0,3	30	30	3	3	-	-	-	-	**X**
CONI-38	10	0,3	30	30	3	1	-	-	-	-	**X**
CONI-39	>100	0,3	30	10	100	1	-	-	-	**X**	-
CONI-41	0,3	0,3	30	10	1	1	-	-	-	**X**	-

Nanni IM et al., Sensitivity of Plasmopara viticola Populations and presence of Specific and Non-specific Resistance Mechanisms.
In: Deising HB; Fraaije B; Mehl A; Oerke EC; Sierotzki H; Stammler G (Eds), "Modern Fungicides and Antifungal Compounds",
Vol. IX, pp. 137-138. © 2020. Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-16-7

Sensitivity of *Plasmopara viticola* Populations and presence of Specific and Non-specific Resistance Mechanisms

Nanni IM, Taccioli M, Burgio SA, Collina M

Department of Agricultural and Food Sciences (DiSTAL), University of Bologna - Viale G. Fanin 40-42, 40127 Bologna, Italy

Corresponding Author Email: irenemaja.nanni2@unibo.it

INTRODUCTION

The Oomycete *Plasmopara viticola* is controlled by fungicides with different modes of action, which include cyazofamid and ametoctradin. Both fungicides are inhibitors of the mitochondrial complex III, with different binding behaviour. Cyazofamid (Mitani et al. 1998) is classified in the QiI group and ametoctradin (Gold et al. 2010) in the QoSI group. Compounds with a site-specific mode of action are more prone to cause resistance due to the occurrence of target site mutations. However, the first identified resistance mechanism for these two modes of action was a non-specific adaptation phenomenon, that affected all the inhibitors of the complex III. This mechanism is the overexpression of the alternative oxidase (AOX), which forms a bypass of the complex III. The aim of this work was to test the sensitivity of *P. viticola* populations from two North Italian regions towards cyazofamid and ametoctradin underlying resistance mechanisms.

MATERIALS AND METHODS

In order to evaluate the sensitivity of *P. viticola* to cyazofamid and ametoctradin, 25-30 downy-mildew-infected leaves from forty-two vineyards located in Northern Italy were collected during the seasons 2016-2017. Bioassays on leaf discs were carried out and some samples were selected to be processed for molecular analyses to evaluate the presence of the mutations correlated to QiI and QoSI resistance.

Bioassays

Six concentrations (from 0 to 100 mg/L) of ametoctradin (Enervin, BASF) and cyazofamid (Belchim) were applied in bioassays. For each concentration (including an untreated one), a total of 15 leaf discs (22 mm ∅) were soaked in formulated products. After 45 minutes, the leaf material was removed and dried on a grate at room temperature, then transferred to survival agar medium (1.5%) complemented with 2 mg/L of kinetin, in Petri dishes in triplicates.The inoculation was done by spraying the sporangial suspension ($5x10^4$ spores/ml) onto the adaxial face of each leaf disc. The Petri dishes were incubated under controlled conditions (20°C) with

a 12-h photoperiod. The assessment is done 8-10 days after the treatment evaluating the presence of sporulated leaf surface, then elaborated by PROBIT analysis to obtain EC_{50} values.

Molecular assays

Some samples were selected to be tested with molecular analysis in order to be able to discover a site specific mutations. Molecular assays were performed by BASF laboratories.

RESULTS AND DISCUSSION

Bioassays on leaf discs were carried out on forty – two populations collected in 2016 and 2017 seasons and performed with both fungicides with and without propylgallate (PPG) for Trentino Alto Adige (TN) and Friuli Venezia Giulia (FVG) regions. Populations coming from FVG showed EC_{50} values ranging from 0.00 to 1.27 mg/L for ametoctradin and from 0.00 to 1.26 mg/L for ametoctradin with PPG, cyazofamid values were from 0.01 to 13.85 mg/L and cyazofamid with PPG from 0.00 to 5.92 mg/L. Populations coming from TN region in 2016 showed for ametoctradin solo values ranging from 0.00 to >100 mg/L and ametoctradin with PPG values were 0.01 to 12.17 mg/L, regarding to cyazofamid the values were from 9.6 to >100 mg/L and cyazofamid with PPG were from 0.00 to >100 mg/L. In 2017 for the TN region EC_{50} values for ametoctradin solo and ametoctradin with PPG were from 0.00 to 37.90 mg/L and from 0.00 to 71 mg/L respectively. Cyazofamid values were ranging from 0.05 to 54 mg/L and cyazofamid with PPG were from 0.00 to 20.60 mg/L.

In our samples the mutation L201S or the insertions of two amino acids (E203 VE V204) were detected as mechanisms for specific target site resistance to cyazofamid. In particular, sample 439 that showed EC_{50} values for cyazofamid 13.18 and 15.39 mg/L without and with PPG respectively showed 8% of L201S mutation. In the samples selected for molecular analyses none mutations correlated to ametoctradin resistance were found.

Overexpression of AOX was also found in our samples, which affected the sensitivity to all complex III inhibitors. If the described resistance types lead to fitness penalties needs further investigation. Additional molecular analyses are still ongoing in order to evaluate the potential presence of the mutations in all samples carried out for biological assays.

REFERENCES

Gold RE; Schiffer H; Speakman J; Stammler G;, Klappach K;, Brix HD; Schlehuber S (2011). Initium a new innovative fungicide to the control of Oomycete in speciality crops. In: *Modern Fungicides and Antifungal Compounds VI*, eds HW Dehne, U Gisi, KH Kuck, PE Russell, H Lyr, pp. 55-62. DPG-Verlag, Braunschweig.

Mitani S., Araki S; Matsuo N; Camblin P (1998) IKF-916 a novel systmemic fungicide for the control of oomycete plant diseases In: *Proceedings of Brighton Crop Protection Conference: Pests& Diseases*, pp. 351–358.

Young D et al., Characterization of the Benzamide Binding Site on β-Tubulin by Analysis of Resistant Mutants in *Aspergillus nidulans* and Protein Modeling. In: Deising HB; Fraaije B; Mehl A; Oerke EC; Sierotzki H; Stammler G (Eds), "Modern Fungicides and Antifungal Compounds", Vol. IX, pp. 139-140. © 2020. Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-16-7

Characterization of the Benzamide Binding Site on β-Tubulin by Analysis of Resistant Mutants in *Aspergillus nidulans* and Protein Modeling

Young D, Wang N, Delgado J, Siegel C

Corteva Agriscience, 9330 Zionsville Road, Indianapolis, IN 46268, USA
Email: david.young@corteva.com

The anti-tubulin benzamide (BA) fungicide zoxamide is used to control Oomycete diseases. Zoxamide binds covalently to Cys-239 of β-tubulin in the colchicine binding domain and its binding site is presumed to overlap with that of methyl benzimidazole carbamate (MBC) fungicides based on positive or negative cross-resistance to MBCs (Young 2015). To elucidate details of the BA binding site and explore the basis for high Oomycete sensitivity, we have isolated resistant mutants in *Aspergillus nidulans* (ASPNID) to identify target site mutations and combined mutation sensitivity data with protein modeling and ligand docking approaches using tubulin homology models based on the structure of mammalian tubulin with the bound MBC, nocodazole (PDB: 5CA1; Wang et al. 2015). Whereas past efforts to isolate BA-resistant mutants in Oomycetes have been largely unsuccessful, we isolated resistant mutants in the Ascomycete ASPNID using MNNG mutagenesis with mutant selection on medium containing RH-1716, a BA analog which is 18-fold more potent than zoxamide against ASPNID. Additional mutants, selected for resistance to the MBC benomyl, also showed altered sensitivity to BAs. Resistance factors against BAs and the MBC nocodazole were determined using fungitoxicity assays in 96-well plates, and the β-tubulin gene was sequenced in 44 isolates that showed resistance, and in one case hypersensitivity, to BAs (39 mutants obtained by selection on RH-1716 and 5 on benomyl). Single amino acid substitutions were found in all isolates, with 15 different amino acid substitutions that involved 12 residues (H6L/Y/F, F20L, Y50C, Q134K, E198K, V236F, T238I, C239G, L240F/H, P259L, S314C and I316F). In ASPNID and *Phytophthora infestans* (PHYTIN) tubulin homology models, all amino acids where resistance mutations occurred, with the exception of P259, were found to cluster around a pocket of the colchicine binding domain which contained the C239 site of covalent binding (Fig. 1A). Ligand docking at this site was then used to identify the likely binding poses for BAs in ASPNID (Fig. 1A) and PHYTIN (Fig. 1B), and for nocodazole in ASPNID.

The C239G exchange conferred high resistance to BAs, consistent with the role of C239 in covalent binding. Since I316, T238 and L240 are close to C239, the mutations involving these residues may interfere with covalent binding. Q134 appears to interact with the methyloxime of RH-1716 and the carbamate of nocodazole, and the Q134K exchange should disrupt these interactions. H6, F20 and Y50 form an interaction network with Q134, therefore changes at these sites may also impact the Q134-ligand interactions. Q134K had a lesser effect on zoxamide sensitivity, consistent with lack of the methyloxime. The E198K exchange increased ASPNID

sensitivity to RH-1716, but not zoxamide, possibly due to enhanced binding caused by interaction of Lys with the methyloxime of RH-1716. The S314C exchange may be sterically unfavorable for BA binding due to the larger Cys residue.

While binding poses for BAs appear similar in PHYTIN and ASPNID, differences in certain amino acids might explain their higher potency towards Oomycetes. C165 (PHYTIN), versus A165 (ASPNID), may allow H-bonding of C165 to E198 and lead to a different conformation for E198. Discovery of a C165Y mutation in *P. sojae* (Liu et al. 2018), that confers resistance to ethaboxam (same FRAC code #22 as zoxamide), is consistent with this important role for C165. A further difference in this region is the smaller M200 (PHYTIN) instead of F200 (ASPNID). Since mutations involving residues 198 and 200 are commonly associated with altered BA sensitivity in Ascomycetes, these differences in the binding site in PHYTIN could explain higher sensitivity. Further differences between PHYTIN and ASPNID are observed for 3 residues where benzamide resistance mutations were found in ASPNID: I236, A314 and M316. The residues at positions 236 and 316 may influence covalent binding to C239 while the smaller A314 appears sterically more favorable than S314 in ASPNID.

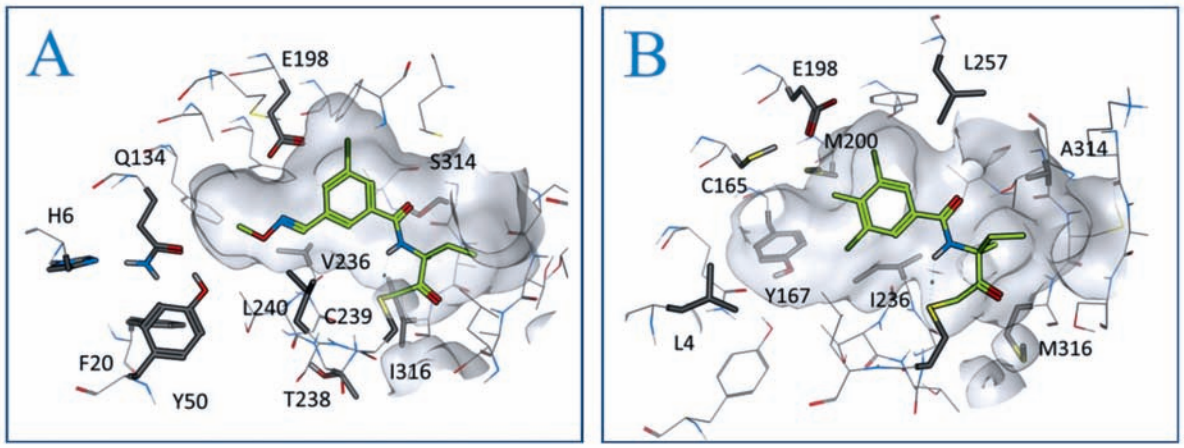


Figure 1. Binding of RH-1716 and zoxamide in homology models of ASPNID (A) and PHYTIN (B) β-tubulin, respectively.

REFERENCES

Young DH (2015). Anti-tubulin Agents. In: *Fungicide Resistance in Plant Pathogens*, eds H Ishii & D W Hollomon, pp. 93-103. Springer: Tokyo.

Wang Y; Zhang H; Gigant B; Yu Y; Wu Y; Chen X; Lai Q; Yang Z; Chen Q; Yang J (2015). Structures of a diverse set of colchicine binding site inhibitors in complex with tubulin provide a rationale for drug discovery. *FEBS J* 283, 102-111.

Liu X; Peng Q; Fang Y; Wang Z; Liu Y; Liu P; Huang Z; Xue Z; Li T (2018). Method for rapidly identifying beta-tubulin gene nucleotide point mutation of *Phytophthora sojae* and drug resistance of *Phytophthora sojae* to ethaboxam. Chinese Patent Application CN107746894A.

Paveley N et al., Managing resistance evolving concurrently against two modes of action. In: Deising HB; Fraaije B; Mehl A; Oerke EC; Sierotzki H; Stammler G (Eds), "Modern Fungicides and Antifungal Compounds", Vol. IX, pp. 141-146. © 2020. Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-16-7

Managing resistance evolving concurrently against two modes of action

Paveley N[1], Fraaije B[2], van den Bosch F[3], Kildea S[4], Burnett F[5], Clark W[6], Gosling P[7], Boor T[1], Corkley I[1], Young C[1]

[1]*ADAS, c/o ADAS High Mowthorpe, Duggleby, Malton, North Yorkshire, YO17 8BP, UK.*
[2]*Rothamsted Research, Plant Pathology and Microbiology Department, Harpenden, Hertfordshire, AL5 2JQ, UK*
[3]*Centre for Crop and Disease Management, Curtin University, Bentley 6102, Perth, Australia*
[4]*Teagasc, Crop Science Department, Oak Park Crops Research Centre, Carlow, Ireland*
[5]*SRUC, King's Buildings, West Mains Road, Edinburgh, EH9 3JG, UK*
[6]*NIAB Huntingdon Road, Cambridge, CB3 0LE, UK*
[7]*AHDB, Stoneleigh Park, Kenilworth, Warwickshire, CV8 2TL, UK*
Corresponding Author Email: neil.paveley@adas.co.uk

INTRODUCTION

Two or more single-site acting fungicides are applied in treatment programmes in many crops, creating selection pressure for resistance to evolve concurrently against more than one mode of action (MoA). Resistance has already occurred against multiple MoA in economically important pathogens. We know about these cases because the frequency of resistant strains has become sufficiently high to be detected and are monitored with appropriate technologies. There will be many more cases where resistance is in the process of evolving or present against more than one MoA, but is not yet detectable.

The current focus on managing resistance for each MoA individually needs to be broadened. If two or more single-site acting fungicides are being used to control a particular pathogen, the resistance management strategies implemented should be those which optimise the effective life of the MoA combination.

Trade-offs between resistance management strategies

Resistance management is simple in principle, but complex in practice. The complexity arises mainly from 'trade-offs'. Where the aim is to manage resistance against one MoA, there are sometimes trade-offs between the requirements of resistance management strategies and the need to obtain robust and cost-effective control. Where the aim is to manage resistance evolving concurrently against two or more single-site acting MoA, further trade-offs can occur. These are introduced below and are the focus of this paper.

Experimental and modelling studies have shown that three strategies are widely effective across diverse pathosystems (van den Bosch et al., 2014): limiting the number of applications of a MoA

per crop season, alternating MoA, and mixing MoA (alternation or mixing depend on there being little or no cross-resistance between MoA)

Consider a pathosystem where several fungicide applications are required to obtain effective control and a limited number of effective MoA are available. If the number of applications of fungicide A is limited in order to manage resistance against that MoA, then there will be treatments in the programme when fungicide A cannot be used as a mixture partner to protect fungicide B. The strategy of limiting treatments is therefore constraining the strategy of using mixtures. One MoA is, therefore, well protected against resistance (by limiting use) but other MoA are consequently less well protected (if other effective mixtures partners are not available). This situation is likely to become more common in Europe, where regulation is limiting the availability of products and is removing effective multi-site acting fungicides which are useful mixture partners.

In some circumstances, this trade-off between limiting treatments and use of mixtures could be overcome by using alternation rather than mixtures, but two problems arise. Firstly, mixtures are often the preferred practical approach, for example where a mixture is necessary to achieve a wider spectrum of activity or to ensure that resistance management is implemented by the use of formulated mixtures. Secondly, the evidence is often contradictory on whether alternation is as effective as mixtures for slowing selection. Better information is required on the circumstances when alternation or mixtures will be best.

Virtually all the experimental and modelling evidence on resistance management strategies comes from studies where selection was measured for strains resistant to one MoA. The few studies where resistance management against more than one MoA was considered do not answer the questions raised above. This paper reports preliminary results from an ongoing project measuring the effect of strategies to manage resistance evolving concurrently against two MoA.

METHODS

Although resistance management is more effective when implemented before resistance is detected, field experiments to compare strategies can only be conducted when the frequency of resistant strains is above detection thresholds for high-throughput genotyping. UK and Irish *Zymoseptoria tritici* populations are shifting for sensitivity against both succinate dehydrogenase inhibitor (SDHI) and demethylation inhibitor (DMI) fungicides (Blake et al., 2018). Hence septoria tritici blotch in wheat (STB) provides an appropriate, and important, experimental pathosystem for studying concurrent resistance.

Randomised and replicated field experiments, at a minimum of three locations per year, tested different aspects of resistance management, as described in the results section. Mean data across experiments are shown and are representative of the results in individual experiments.

The experimental treatments used DMI and SDHI fungicides. The evolution of DMI insensitivity is complex, with multiple mutations in the CYP51 target site, over-expression and enhanced efflux of fungicides from fungal cells, contributing to the sensitivity phenotype (Huf,

this volume). Despite this complexity, the presence or absence of a single amino acid substitution at codon position 524 (S524T) in the CYP51 binding site provides a discriminatory marker to quantify a group of newer, less sensitive, haplotypes which are being selected for currently across much of Europe (Jorgensen, this volume).

Multiple mutations in the sdh target site have been detected across Europe (Fraaije, this volume). The most practically relevant strains during the period of the experiments were those with moderate levels of SDHI insensitivity associated with amino acid substitutions SdhC-T79N, C-W80S and C-N86S, and highly resistant isolates (detected at low frequency in the UK) carrying the C-H152R substitution.

Samples were taken from one upper leaf layer across all plots of the experiments, at least one latent period after the last fungicide treatment. DNA was extracted from 25 STB-infected leaves per plot for SNP detection pyrosequencing assays developed according to the Assay Design Software (Version 1.0; Biotage). At the time of the experiments, the sdh mutations assayed had not been detected in combination within a strain, so the frequencies of the three moderately resistant genotypes were added to give a total frequency.

RESULTS

The results reported here are interpreted in relation to the governing principles (Milgroon and Fry, 1988) which determine the effect of changing fungicide treatments on selection for resistant strains. The effects predicted from the governing principles have been tested previously against evidence from a diverse set of pathogen/fungicide combinations (van den Bosch *et al*., 2014), including DMI resistance cases. Hence, we first consider whether the patterns of selection for SDHI resistance seen in the experiments agree with expectations from the governing principles.

Effect on selection of dose per application and number of applications

Data from field experiments in 2016 showed that selection for sdh mutants increased significantly with increasing SDHI dose per application (Figure 1). Information on field dose response curves for the activity of SDHI fungicides in the UK in 2016 is available from www.ahdb.org.uk. This agrees with the governing principle that a higher dose will have a greater effect on the *per capita* growth rate of sensitive strains than on the growth rate of resistant strains, thus increasing the difference in growth rates which determines selection rate. Experiments in 2018 showed that selection also increased significantly with increasing number of applications of a constant dose per application, i.e. increasing selection with increasing total dose (Figure 2). This agrees with the principle that the period over which selection occurs (and hence the amount of selection) increases with the exposure time of the pathogen population to the fungicide, during which the *per capita* growth rate of resistant strains will be higher than the growth rate of sensitive strains. In both figures, the increase above the dotted line provides an indication of the amount of selection during the season, as the untreated values approximate to the frequency that was present at the experimental sites prior to treatment. A doubling of

exposure time (from one application to two in Figure 2) approximately doubled selection over the untreated baseline. The difference in untreated frequency between Figure 1 and Figure 2 reflects the increase in frequency of mutants in the UK and Irish *Z. tritici* populations between 2016 and 2018.

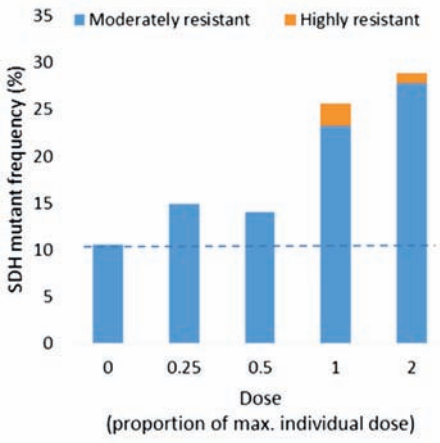


Figure 1: Frequency of SDHI insensitive strains following a single application of penthiopyrad at a range of doses (means from 3 experiments, 2016). P=0.006. Note: Double maximum individual dose is not permitted for commercial use.

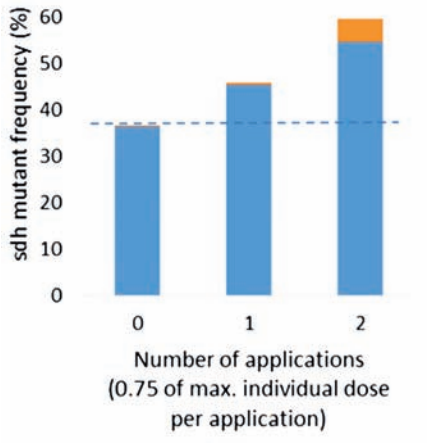


Figure 2: Frequency of SDHI insensitive strains following 0, 1 (at GS39) or 2 (at GS32 and 39) applications of isopyrazam in mixture with prothioconazole (means from 4 experiments, 2018). P<0.001.

Potential for managing resistance by limiting total dose rather than number of applications

It follows from the results above that increasing the number of applications, but decreasing the dose per application would have two opposing effects on selection. The net effect of 'dose splitting' cannot therefore be readily predicted, although the effect of increased exposure time from increased number of treatments might be expected to outweigh the decrease in selection due to lower dose per application. A modelling analysis by van den Berg *et al*. (2016) suggested that similar effective lives for a MoA could be obtained by limiting number of treatments or by limiting total dose. Limiting total dose, and allowing growers some flexibility on the number of applications, would resolve a trade-off by allowing effective mixtures to be used throughout the spray programme, even where there are few MoA available.

Four field experiments in 2018 tested the effect of splitting a fixed total dose of SDHI across 2, 3 or 4 applications (Figure 3). In each treatment, a DMI was applied four times, so where a SDHI was applied twice, two of those DMI applications were 'unprotected' by an effective mixture partner. Where the same SDHI total dose was split four ways, a DMI+SDHI mixture was used in all four applications.

All SDHI and DMI treatments significantly (P<0.001) increased the frequency of their respective mutants compared to the control. Dose splitting had no significant effect on the

frequency of sdh mutants, nor on the frequency of S524T strains. It is likely that the potential benefit of having fewer unprotected DMI treatments by allowing dose splitting was counteracted by the lower dose per treatment making the SDHI a less effective mixture partner.

The efficacy data gathered in the experiments could not be presented here due to limited space.

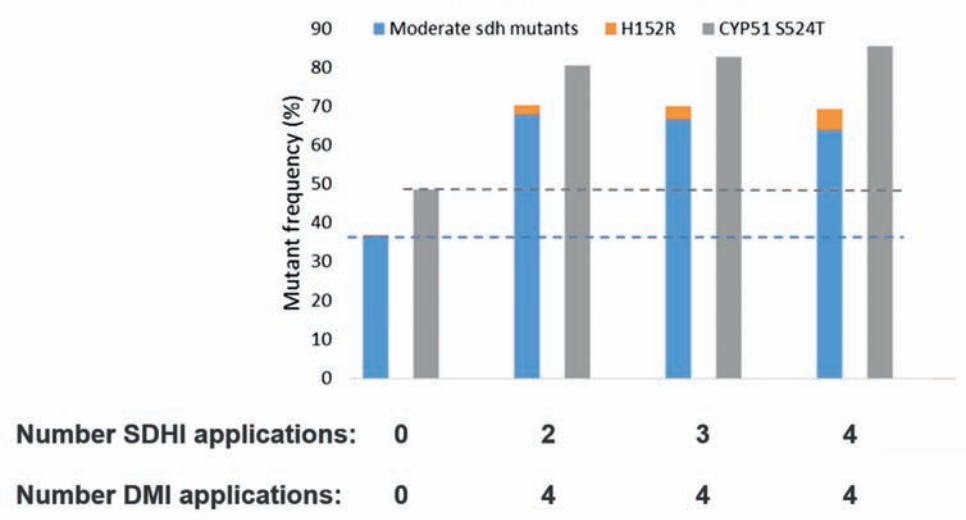


Figure 3 Frequency of SDHI and DMI insensitive strains after a four spray programme (means of 4 experiments, 2018). The same maximum total dose of isopyrazam was applied in all treatments (except the control) with the total dose split between 2 (GS32 and 39), 3 (GS32, 39 and 59) or 4 (GS30, 32, 39, 59) applications. All treated plots received 4 applications of prothioconazole at max. individual dose. DMI and sdh mutant frequencies in treated plots were significantly different from the untreated; $P<0.001$.

Implications of dose-splitting for whether mixtures or alternation are the best strategy

Consider two options for the simple case of a two-spray programme. The first option is to alternate fungicide A followed by fungicide B (where A and B are different MoA), each at the maximum permitted individual dose. The second option is to apply A at both spray timings, mixed with B. If both fungicides are of similar efficacy and there is no antagonism, then a mixture containing half the maximum individual dose of A and B will provide at least the same efficacy as the alternation programme and will apply the same total dose.

Effectively, the mixture progamme is dose-splitting (half the dose of A is applied twice as often). If, as in the case shown in Figure 3, dose splitting has no effect on selection, then the mixture strategy will be more effective at reducing selection than alternation. This is because the increase in exposure time is counteracted completely by the decreased dose per application, and the effect of mixture partner B reducing the *per capita* growth rates of strains resistant and sensitive to A will reduce selection further.

However, we must be careful not to generalize from the results shown here. Other cases reviewed by van den Bosch *et al*. (2014) showed increased selection with dose splitting. In such cases, depending on the size of the increase in selection, mixtures and alternation may be similarly effective at reducing selection or alternation may be more effective.

CONCLUSIONS

Experimental work continues, so these conclusions should be considered as preliminary.

- Concurrent resistance evolution is highly likely wherever two or more single-site acting fungicide MoA are used to control a pathogen.
- Reduced availability of multi-site acting fungicides in Europe, due to regulation, will increase dependence on single-site actives, increasing concurrent resistance evolution.
- Integrated Pest Management will become more important as it can reduce pathogen epidemics and dependence on fungicides. The former can slow fungicide resistance evolution by reducing *per capita* growth rates and the latter by reducing exposure time.
- Mixtures, alternation and limiting number of treatments are all effective resistance management strategies.
- Concurrent resistance evolution creates 'trade-offs' between these strategies.
- Circumstances in which different strategies are optimal are becoming better understood
- Alternation is only likely to be better than mixtures for reducing selection where: (i) dose splitting increases selection, and (ii) the mixture partner is relatively ineffective
- Where dose-splitting has little effect on selection, limiting total dose (rather than number of treatments) may be an effective resistance management strategy and allow more flexibility for growers to adjust their spray programmes.
- Work continues to test under what circumstances dose splitting does, or does not, have a substantial effect.
- While evidence is being gathered and interpreted, resistance management guidance should remain unchanged with respect to existing limitations on number of treatments and the use of MoA mixures or alternation.

ACKNOWLEDGEMENTS

Thanks are due for funding from Adama, AHDB, BASF, Bayer, Corteva, the Irish Government and Syngenta, and to colleagues of the authors for conducting the experiments.

REFERENCES

Blake JJ; Gosling P; Fraaije BA; Burnett FJ; Knight SM; Kildea S; Paveley ND (2018) Changes in field dose–response curves for demethylation inhibitor (DMI) and quinone outside inhibitor (QoI) fungicides against *Zymoseptoria tritici*, related to laboratory sensitivity phenotyping and genotyping assays. *Pest Management Science* 74, 302-313.

Milgroom MG; Fry WE (1988). A simulation analysis of the epidemiological principles for fungicide resistance management in pathogen populations. *Phytopathology* 78, 565–70

van den Berg F; Paveley N; van den Bosch F (2016). Dose and number of applications that maximise fungicide effective life exemplified by *Zymoseptoria tritici* on wheat – a model analysis. *Plant Pathology* Doi: 10.1111/ppa.12558

van den Bosch F; Oliver R; van den Berg F; Paveley N (2014). Governing principles can guide fungicide-resistance management tactics. *Annual Review Phytopathology* 52, 175-195.

Karaoglanidis G et al., Effects of Bacillus subtilis 713 on the management of SDHIs resistance in Botrytis cinerea. In: Deising HB; Fraaije B; Mehl A; Oerke EC; Sierotzki H; Stammler G (Eds), "Modern Fungicides and Antifungal Compounds", Vol. IX, pp. 147-153. © 2020. Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-16-7

Effects of Bacillus subtilis 713 on the management of SDHIs resistance in Botrytis cinerea

Samaras A, Chatzipetrou C, Karaoglanidis G

Plant Pathology Laboratory, Faculty of Agriculture, Aristotelian University of Thessaloniki, POB 269, 54124, Thessaloniki, Greece

Corresponding Author Email: gkarao@agro.auth.gr:

ABSTRACT

Resistance of *Botrytis cinerea,* causal agent of gray mold disease, to target-site inhibitors such as SDHIs, is widely distributed throughout the world. Resistance to SDHIs is associated with several point mutations located mainly in *sdh*B that differentially affect both the mutant` sensitivity to several SDHIs and the fitness of the mutated strains. The current study was initiated to test the hypothesis that *Bacillus subtilis* strain QST 713 (hereon called *B. subtilis* 713) can be utilized in IPM programs aiming to control *sdh*B mutants (H272R/Y, N230I and P225F/H/L) equally good compared to sensitive wt strains. The measurements of antagonistic activity and inhibitory effects on the mycelial growth of *B. cinerea sdh*B mutants by *B. subtilis* 713 *in vitro,* showed that for 31 out of 33 *B. cinerea* isolates tested (independently of the *sdh*B mutation that they were possessing), there was a strong antibiosis effect of the bacterial strain. Protective and curative applications of *B. subtilis* 713 on artificially inoculated plants with all the *sdh*B mutants showed that the Biological Control Agent (BCA) applications significantly reduced both disease incidence and severity. Competition experiments between *sdh*B mutants and wild-type (S) isolates conducted either in the absence of any fungicide selection pressure or in the presence of *B. subtilis* 713 showed a dominance of S isolates over the mutated strains suggesting that such treatments can contribute to a reduction in the frequency of *sdh*B mutants. Such results suggest that *B. subtilis* 713 can be an efficient tool for SDHIs resistance management in *B. cinerea*.

INTRODUCTION

Resistance of Botrytis *cinerea* to target-site inhibitors, including SDHIs, is widely distributed throughout the world, in crops treated with botryticides. Resistance to SDHIs is associated with several point mutations located mainly in *sdh*B that differentially affect both the mutant` sensitivity to particular SDHIs and the fitness of the mutated strains (Leroux et al. 2010; Veloukas et al. 2013). The development and introduction of novel SDHI molecules along with the widespread presence of *B. cinerea* strains with multiple resistance to several chemically

unrelated fungicides, makes it urgent to implementae anti-resistance strategies. Therefore, new modes of action are required or the use of alternatives to conventional chemical control has to be expanded. *Bacillus subtilis* strain QST 713 (hereon called *B. subtilis* 713) could be an attractive solution not only because it is a Biological Control Agent (BCA) and fits better to IPM solutions, but also because it may be more effective against pathogen` strains that are less fit such as most of the *sdh*B mutants (Laleve at al., 2014; Veloukas et al. 2014).

In the current study, following data will be provided and discussed:

1. Data related to the antagonistic activity and inhibitory effect on the mycelial growth of *B. cinerea sdh*B mutants by *B. subtilis* 713 *in vitro*.
2. Data related to the effects of protective and curative applications of the BCA on the development of gray mold caused by the *sdh*B mutants of *B. cinerea* on bean plants.
3. Data related to the competitive ability of *sdh*B mutants and the wild type strains in the presence or absence of the BCA.

MATERIALS AND METHODS

Fungal isolates. Thirty three (33) *B. cinerea* isolates that had been previously characterized phenotypically and genotypically for resistance to SDHIs were used in the study. Five (5) of the tested isolates were sensitive to SDHIs while the remaining were SDHIs-resistant and possessed the *sdh*B H272R, H272Y, P225F, P225H, P225L and N230I mutations (5, 5, 5, 3, 5 and 5 isolates, respectively).

Biocontrol agent. Pure cultures of Bacillus subtilis 713 were obtained after isolation from the formulated product Serenade ASO (1.34 SC), provided by Bayer AG, Division CropScience. Bacterial cells were stored in glycerol stocks (50%) at -80oC. The formulated product Serenade ASO was used in the in planta experiments.

***In vitro* assessment of *B. subtilis* 713 antagonistic activity against s*dh*B mutants.** Dual cultures consisted of the *B. subitilis* 713 or, as control, without BCAand the *Botrytis* isolates inoculated on opposite sides of PDA petri dishes were established and incubated at 25°C in darkness for 7 days.. Fungal growth was measured as colony diameter in mm and percentage of inhibition was calculated using the formula: % inhibition = (1 – (Fungal growth/Negative control growth)) x 100. The antagonistic activity was evaluated by measuring (in mm) the inhibition zones between the fungal isolate and the tested biocontrol agent. Five replicate plates were prepared for each isolate-biocontrol agent combination and the experiment was repeated three times.

Protective and curative activity of *Bacillus subtilis* 713 against *B. cinerea* s*dh*B mutants *in planta*. In planta experiments were conducted on bean plants (*Phaseolus vulgaris* L.) at the stage of 2 fully-expanded leaves. *B. subtilis* 713 (Serenade ASO, 1.34 SC) was applied (16 ml L^{-1}, f. p) to "run-off" with a hand sprayer at 24h before (protective treatment) and after (curative treatment) the inoculation of plants. Control plants were sprayed with sterile tap water. Artificial inoculation was conducted out by pippeting 10 µl of conidial suspension (2 x

10^5 spores ml^{-1}) at 2 different points of the adaxial surface of each leaf. After inoculation the plants incubated at 20ºC at 99% R.H. and a 12h photoperiod for 5 days. Then, the infection was scored by measuring disease incidence (number of rotting lesions that appeared on each plant) and disease severity (lesion diameter) on the artificially inoculated bean plants. The experiment was repeated in duplicate.

Competition experiments with mixed inocula of sensitive (S) and mutated *B. cinerea* strains at equal ratios. The competition experiment with mixed-genotype inoculum at equal ratio for all strains was conducted in the presence/absence of fungicideand, in the presence of *B. subtilis* 713 selection pressure and in the presence/absence of fluopyram (Luna Privilege 500 SC). Both *B. subtilis* 713 (16 ml L^{-1}) and fluopyram (0.35 ml L^{-1}) were applied to "run-off" at 24h before the inoculation. Control plants were sprayed with sterile tap water. The inoculum (2 x 10^5 spores ml^{-1}) was prepared by mixing appropriate volumes of conidial suspensions of all 6 isolates of each mutated genotype and of all the wild-type isolates in order to produce mixed inocula of S:H272R:H272Y:N230I:P225H:P225F:P225L at the ratio of 1:1:1:1:1:1:1. Five replicate plants per treatment (control, *B. subtilis* 713-treated, fluopyram-treated) were used. Following inoculation the plants were incubated at 20ºC for 5 days. The spores produced on each lesion were harvested in sterile distilled water and a new conidial suspension was prepared for the next disease cycle. The experiments were terminated after the 4^{th} disease cycle and repeated twice.

The measurement of SDHIs resistance frequency and the identification of the sdhB mutations was conducted in isolates obtained, from the infected leaves. The spores produced on each leaf after the end of the 4^{th} disease cycle were separately collected in distilled sterile water. From each leaf 5 single-spore isolates were obtained (10 isolates per replicate plant). In total 50 isolates were tested per treatment. The isolates were obtained after serial dilution plating of conidial suspensions on PDA amended with ampicillin at the concentration of 25 μg ml^{-1}. Then the pure *B. cinerea* single-spore isolates were allowed to sporulate on PDA and the conidia were used for the phenotypic characterization of the isolates. The phenotypic characterization was conducted using the discriminatory concentrations of boscalid and fluopyram, as described previously. Based on this procedure the isolates were grouped as sensitive to both fungicides (S isolates), isolates resistant to boscalid (B^Risolates, corresponding to H272R/Y mutants) and isolates resistant to both boscalid and fluopyram (B^RF^R isolates, corresponding to N230I, P225F, P225H or P225L mutants).The identification of sdhB mutations was conducted on isolates of B^RF^S and B^RF^R phenotypes, using a PIRA-PCR technique developed previously (Veloukas et al. 2011). Direct sequencing of the sdhB subunit was conducted for genotypes a PIRA-PCR technique (P225H and P225L) was not available (Leroux et al. 2010).

RESULTS AND DISCUSSION

In vitro assessment of *B. subtilis* 713 antagonistic activity against sdhB mutants. *B. subtilis* 713 reduced significantly the mycelial growth of all the *B. cinerea* isolates included in the study. For the isolates of wild-type sensitivity and the P225H mutants mean inhibition of mycelial growth had values of 57.7 and 55.2%, respectively, while for the remaining *B. cinerea* mutant groups the relative inhibition of mycelial growth was either higher or lower compared to that observed in isolates of wild-type sensitivity. In addition to the reduction of mycelial growth a strong inhibition zone was observed between the bacterial and the fungal colonies in most of the tested pairs. For 31 out of 33 *B. cinerea* isolates tested, the inhibition zone was greater than 10 mm suggesting a strong antibiosis effect of the bacterial strain. Such results confirm the ability of the BCA to exhibit antifungal activity against the entire spectrum of *B. cinerea* strains used in this study independently of their genetic background in terms of sdhB sequence variability.

B. cinerea sdhB mutants in planta.

Artificial inoculations with the selected *B. cinerea* strains showed that all strains were pathogenic on bean leaves. The mean disease incidence and severity values for each sdhB mutant group are shown in Table 2. For all the isolate groups disease incidence on the untreated (control) plants ranged from 73.6 to 94.6% (Table 1). Both the protective and curative application of the BCA resulted in a lower ($P<0.05$) disease incidence compared to that observed in the control plants for all the mutant groups included in the study (Table 1). Similarly, the protective application of the BCA resulted in a significant reduction of disease severity compared to that observed in the control plants for all the mutant groups, with values ranging from 0.24 to 0.49 and 0.76 to 1.23 cm, repectively. However, the curative application of the BCA did not resulted in a significant reduction of disease severity compared to that in the control plants for the H272R, H272Y and N230I mutants (Table 1). These results confirm the ability of the BCA to control the pathogen in planta and is in agreement with the findings of previous studies suggesting that Bacillus spp. can exhibit not only protective activity but also curative one (Zamir et al. 2016; Calvo et al. 2017).

Competition experiments with mixed inocula of sensitive (S) and mutated *B. cinerea* strains at equal ratios.

The competition experiment with mixed isolate inocula of all the genotypes at equal frequencies was conducted in 4 disease cycles. In the absence of any selection pressure after 4 disease cycles the sensitive isolates were found at a frequency of 92.5%, significantly higher ($P<0.05$) than the initial frequency of 14.3% sensitive spores prepared by mixing the inoculum (Table 2). At the same time both the fractions of the B^R and B^RF^R isolates were reduced at very low frequencies (Table 2). Identification of the sdhB mutations obtained from the diseased leaves after the 4^{th} cycle showed that all the B^R isolates were possessing the H272R mutation, while all the B^RF^R were possessing the N230I mutation (Fig. 1). The same pattern of an increase in frequency of sensitive strains was observed on the *B. subtilis* 713-treated plants. After 4 disease cycles the sensitive isolates increased from the initial frequency of 14.2% to a frequency of 69.3%. On the

other hand, both B^R and B^RF^R isolates decreased in frequency. The B^R isolates from an initial frequency of 28.4% were detected in a frequency of 9.6%, while the B^RF^R isolates from an initial frequency of 56.8% decreased at a frequency of 21.1% (Table 2).

Table 1 Disease incidence and severity caused by *B. cinerea sdh*B mutants on bean plants treated with *B. subtilis* 713 in protective and curative application.

Genotype	Treatment	Protective application[a]		Curative application	
		Disease Incidence[b]	Disease Severity[c]	Disease Incidence	Disease Severity
Wild type	Control	94.6[d]	0.89	94.6	0.89
	B. subtilis 713	46.0*[e]	0.26*	48.5*	0.47*
H272R	Control	86.2	0.76	86.2	0.76
	B. subtilis 713	44.0*	0.24*	57,0*	0.64
H272Y	Control	73.6	0.89	73.6	0.89
	B. subtilis 713	41.0*	0.35*	42.6*	0.42
P225F	Control	91.2	1.23	91.2	1.23
	B. subtilis 713	57.4*	0.33*	53.4*	0.24*
P225H	Control	81.6	1.23	81.6	1.23
	B. subtilis 713	58.6*	0.49*	59.3*	0.38*
P225L	Control	87.2	0.81	87.2	0.81
	B. subtilis 713	46.8*	0.38	49,8*	0.22*
N230I	Control	92.0	0.84	92.0	0.84
	B. subtilis 713	44.4*	0.25*	57.4*	0.33

[a] Protective and curative applications of *B. subtilis* 713 were conducted 24h before or after the artificial inoculation of the plants, respectively

[b] Disease incidence was calculated based on the symptoms appearance on the plants as follows, 0: no disease symptom in any inoculation point, 25: disease symptoms in 1 out of 4 inoculation points per plant, 50: disease symptoms in 2 out of 4 inoculation points per plant, 75: disease symptoms in 3 out of 4 inoculation points per plant and 100: disease symptoms in 4 out of 4 inoculation points per plant.

[c] Disease severity was measured as lesion diameter in cm

[d] Mean isolate group values of 2 independent replications

[e] asterisk indicates significant difference between control and BCA-treated plants according to a *t*-test at P=0.05

All the B^R isolates were found to possess the H272R mutation, while all the B^RF^R isolates were found to possess the N230I mutation (Fig. 1). In contrast, when competition was conducted on bean leaves treated with fluopyram, the frequency of the S and B^R isolates declined to complete disappearance, while, as expected the B^RF^R isolates dominated the population. Identification of the sdhB mutations in B^RF^R isolates revealed that the P225L isolates were increased in frequency with values ranging from 53.8 τo 71.2%, with a mean value of 62.5% (Fig. 1). The remaining sdhB mutants associated with the B^RF^R phenotype remained either stable or slightly increased, while the N230I mutants decreased significantly. The N230I mutants were detected only in the 1st replication at the very low frequency of 4.4%. The remaining two mutations (P225F and P225H) survived in the population but at frequencies that were variable (Fig. 1). The above

mentioned results suggest that the discontinued use of SDHIs in the field and their replacement by *B. subtilis* 713 treatments may contribute to a decrease of resistance frequency.

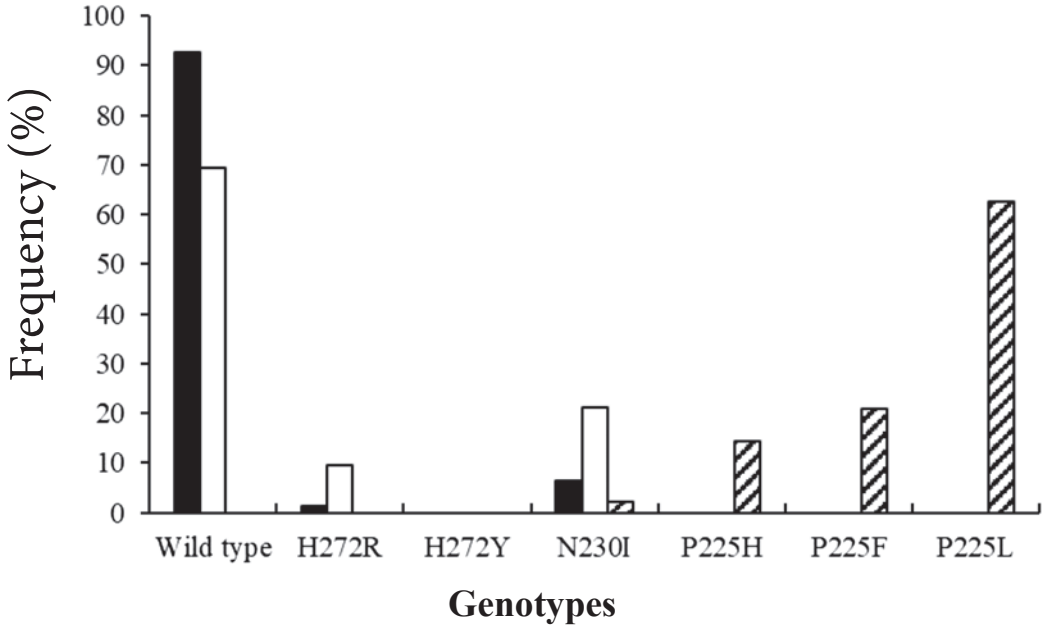


Figure 1 Frequency of *sdh*B mutations in isolates of *Botrytis cinerea* obtained after 4 disease cycles from bean plants treated with *B. subtilis* 713, fluopyram and untreated control and inoculated with mixed inocula of sensitive (S) and mutated *B. cinerea* strains at the ratio of 1:1:1:1:1:1:1:1. Initial frequency of each genotype in the initial inoculum was 14.3%. Plants were incubated at 20ºC.

Table 2 Frequency (%) of *B. cinerea* isolate` phenotypes, obtained from bean plants artificially-inoculated with conidial mixtures of all the wild-type isolates and all the mutant isolates at an initial ratio of 1:1:1:1:1:1:1:1 (WT:H272R:H272Y:N230I:P225H:P225F:P225L). The isolates were collected after 4 disease cycles on untreated control, Serenade-treated and fluopyram-treated bean plants incubated at **20ºC**.

Treatment	**Phenotype**		
	S[a]	**B^R**	**B^RF^R**
control	**92.5***	**1.2***	**6.3***
B. subtilis 713	**69.3***	**9.6***	**21.1***
fluopyram	**0***	**0***	**100***

[a] S= sensitive isolates, B^R=boscalid-resistant isolates, B^RF^R= boscalid- and fluopyram-resistant isolates

[b] * Indicates that the observed resistance frequency was significantly different from the initial according to χ^2 test at P=0.05. Initial frequencies S=14,2%, B^R=28.4%, B^RF^R= 57.4%.

CONCLUSION

In conclusion, the reported data suggest that *B. subtilis* 713 can contribute to a satisfactory control of gray mold in crops suffering from heavy attacks by the pathogen. More importantly, the use of this BCA may contribute to a reduction of selection pressure on the fungal population and in consequence, to a significant decrease in the frequencies of resistance to SDHIs. The combined use of *B. subtilis* 713 with conventional chemicals may provide a very high control efficacy along with a management of resistance to SDHIs.

ACKNOWLEDGMENTS

This work was funded by Bayer AG, Division CropScience.

REFERENCES

Calvo, H., Marco, P., Blanco, D., Oria, R., Venturini, M.E. (2017): Potential of a new strain of *Bacillus amyloliquefaciens* BUZ-14 as a biocontrol agent of postharvest fruit diseases. *Food Microbiology*, 63, 101-110.

Laleve, A., Gamet S.,Walker A-S., Debieu D., Toquin V., Fillinger, S. (2014): Site-directed mutagenesis of the P225, N230 and H272 residues of succinate dehydrogenase subunit B from *Botrytis cinerea* highlights different roles in enzyme activity and inhibitor binding. *Environmental Microbiology* 16, 2253-2266.

Leroux, P., Gredt, M., Leroch, M., Walker, A-S. (2010): Exploring mechanisms of resistance to respiratory inhibitors in field strains of *Botrytis cinerea*, the causal agent of gray mold. *Applied Environmental Microbiology,* 76, 6615- 6630.

Punja, Z.K., Rodriguez, G., Tirajoh, A. (2016): Effects of Bacillus subtilis strain QST 713 and storage temperatures on post-harvest disease development on greenhouse tomatoes, *Crop Protection*, 84, 98-104.

Veloukas, T., Leroch, M., Hahn, M., Karaoglanidis, G.S. (2011): Detection and molecular characterization of boscalid-resistant *Botrytis cinerea* isolates from strawberry. *Plant Disease* 95, 1302-1307.

Veloukas, T., Markoglou, A.N., Karaoglanidis, G.S. (2013): Differential effect of *SdhB* gene mutations on the sensitivity to SDHI fungicides in *Botrytis cinerea. Plant Disease* 97, 118-122.

Veloukas, T., Kalogeropoulou, P., Markoglou, A.N., Karaoglanidis, G.S. (2014): Fitness and competitive ability of *Botrytis cinerea* field-isolates with dual resistance to SDHI and QoI fungicides, associated with several *sdh*B and the *cyt*b G143A mutations. *Phytopathology* 104, 347-356.

FUNGICIDE RESISTANCE: MECHANISMS AND DIAGNOSIS IN DICOT PATHOGENS

Ishii H, Miyamoto T, Katayose K, Differential pattern of cross resistance to SDHI fungicides and association with *sdh* gene mutations in *Corynespora cassiicola*. In: Deising HB; Fraaije B; Mehl A; Oerke EC; Sierotzki H; Stammler G (Eds), "Modern Fungicides and Antifungal Compounds", Vol. IX, pp. 157-162. © 2020. Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-16-7

Differential pattern of cross resistance to SDHI fungicides and association with *sdh* gene mutations in *Corynespora cassiicola*

Ishii H[1,2], Miyamoto T[3], Katayose K[2]

[1]*University of Tsukuba, Tsukuba, Ibaraki, Japan;*

[2]*Kibi International University, Minami-awaji, Hyogo, Japan;*

[3]*Horticultural Research Institute, Ibaraki Agricultural Center, Kasama, Ibaraki, Japan*

Corresponding Author Email: hi481204@yahoo.co.jp

INTRODUCTION

In recent years, *Corynespora cassiicola* has become an important emerging pathogen on various crops such as cucumber, tomato, soybean, cotton and others in many countries. This fungus causes Corynespora leaf spot (= target leaf spot) on cucumber and strains resistant to benzimidazole (MBC), dicarboximide, quinone outside inhibitor (QoI), and more recently succinate dehydrogenase inhibitor (SDHI) fungicides have developed quickly in populations (Date et al. 2004; Ishii et al. 2007; Miyamoto et al. 2009; Duan et al. 2019; Zhu et al. 2019).

Very interestingly, however, the lack of cross resistance to fluopyram has been found earlier in very highly and highly boscalid-resistant isolates of this fungus strongly indicating that the pattern of cross resistance is not uniform among SDHI fungicides (Ishii et al. 2011). Similar phenomenon has also been reported in *Alternaria alternata* on pistachio (Avenot & Michailides 2010). These findings seem to have accerelated further development of new generation of fungicides carrying the same mode of action. In this study, some newer SDHI fungicides were included and differential pattern of cross resistance was examined.

MATERIALS AND METHODS

Fungal isolates and fungicides

Ten isolates of *C. cassiicola*, their sequences of *sdhB*, *sdhC*, and *sdhD* genes were analysed previously (Miyamoto et al. 2010), were chosen (Table 1). They are very highly boscalid-resistant (VHR), highly boscalid-resistant (HR), moderately boscalid-resistant (MR), or boscalid-sensitive (S) isolates (Miyamoto et al. 2010; Ishii et al. 2011). Formulations of eight SDHI fungicides (boscalid (50.0% dry flowable), penthiopyrad (20.0% flowable), fluopyram (41.7% flowable), isopyrazam (18.7% flowable), isofetamid (36.0% flowable), fluxapyroxad (26.5% flowable), pyraziflumid (20.0% flowable), and benzovindiflupyr (10.0% emulsion) were used. Fluopyram and benzovindiflupyr were supplied by Bayer CropScience and Syngenta, respectively, but all of the other fungicides were purchased.

Table 1 *Corynespora cassiicola* isolates used in this study

Isolate	Boscalid sensitivity*	Genotype of *sdh* genes
C6-2	S	Wild type
1658	S	Wild type
1361	MR	*sdhD*-S89P
1482	MR	*sdhC*-S73P
1679	MR	Wild type
Chikusei 2-4	MR	*sdhD*-G109V
3006	HR	*sdhB*-H278R
3011	HR	*sdhB*-H278R
Kara 1-1-1	VHR	*sdhB*-H278Y
Kara 2-1-1	VHR	*sdhB*-H278Y

*S, sensitive; MR, moderately resistant; HR, highly resistant; VHR, very highly resistant.

Mycelial growth tests on culture medium

Fungal isolates were precultured on PDA plates at 25ºC for five days in darkness, mycelial discs, 4 mm in diameter, were cut from actively growing colony margins and transferred onto YBA agar plates containing a fungicide at 0, 0.1, 1, 10 and 100 mg L^{-1} of active ingredient (a.i.). After incubation at 25ºC for three days in the dark, the colony diameter was measured, and EC_{50} values were calculated by regressing percentage growth inhibition against the log of fungicide concentration using a software (a gift from So K, ZEN-NOH).

Fungus inoculation tests on cucumber plants

Seedlings of cucumber in plastic pots were prepared in a greenhouse, and plants at the three-leaf stage were used. Distilled water (DW)-diluted boscalid or benzovindiflupyr suspensions, 334 mg a.i. L^{-1} and 200 mg a.i. L^{-1} for each, which is the recommended concentration of the former fungicide for commercial use, were sprayed onto whole plants of cucumber until run-off using a hand sprayer, and plants were kept until inoculation on the following day. DW was used as a control, and three replicate plants were served for each treatment. Experiments were conducted twice.

Eight isolates (two each of VHR, HR, MR and S isolates) of *C. cassiicola* were grown on vegetable agar plates at 25˚C in darkness for 1 week and under black light blue light for another 1 week. Conidia that formed were collected using a sterilised paint brush and washed by DW with centrifugation at 3,000 rpm for 5 min, and suspensions (ca. 1 to 2.5 x 10^5 conidia mL^{-1}) were spray inoculated onto potted cucumber plants previously treated with a fungicide or DW. After inoculation, plants were kept in a dew chamber for 36 h at 25˚C in the dark, and then transferred to a phytotron maintained at 25˚C under natural light conditions. Seven days after

inoculation, the development of Corynespora leaf spot disease was assessed according to the methods of Ishii et al. (2007). Experiments were performed twice.

RESULTS

Mycelial growth tests on culture medium

As reported before, fluopyram (Ishii et al. 2011) as well as isofetamid (Tsukuda 2015) showed high activity to inhibit the growth of isolates with very high or high boscalid resistance, carrying *sdhB*-H278Y and *sdhB*-H278R mutations, respectively (Table 2 & Figure 1). In contrast, cross resistance was clearly observed among boscalid, penthiopyrad, isopyrazam, fluxapyroxad, and pyraziflumid (Table 2 & Figure 2). However, benzovindiflupyr exhibited higher inhibitory activity than these fungicides (Figure 3).

Fungus inoculation tests on cucumber plants

As mentioned above, benzovindiflupyr exhibited high inhibitory activity against mycelial growth on culture medium. So the tests were focused on this fungicide. The results showed that the spray treatment of cucumber plants with benzovindiflupyr at 200 mg L^{-1} was highly effective against Corynespora leaf spot disease irrespective of the level of boscalid resistance of pathogen isolates inoculated (Table 3 & Figure 4). In contrast, the efficacy of boscalid clearly decreased when the level of boscalid resistance increased in inoculated isolates.

Table 2 Sensitivity of boscalid-sensitive and -resistant isolates to other SDHI fungicides in mycelial growth tests

Isolate		Average EC_{50} (mg L^{-1}):						
	Boscalid sensitivity*	Penthiopyrad	Isopyrazam	Fluxapyroxad	Benzovindiflupyr	Pyraziflumid	Fluopyram	Isofetamid
C6-2	S	0.196	0.067	0.006	0.161	0.027	0.744	0.386
1658	S	0.134	0.123	0.004	0.123	0.030	0.904	0.431
1361	MR	2.842	2.842	0.449	0.538	1.594	1.756	0.648
1482	MR	4.507	4.331	4.753	0.338	16.447	7.674	19.857
1679	MR	6.487	6.449	0.330	0.403	1.571	1.410	0.739
Chikusei2-4	MR	39.529	60.804	8.890	2.714	>100	6.722	1.867
3006	HR	13.506	20.668	0.790	1.217	1.085	0.563	0.244
3011	HR	3.279	3.428	0.788	0.710	0.380	0.580	0.294
Kara 1-1-1	VHR	>100	>100	3.869	1.547	12.619	0.452	0.066
Kara 2-1-1	VHR	>100	>100	5.644	2.445	30.785	0.380	0.082

*S, sensitive; MR, moderately resistant; HR, highly resistant; VHR, very highly resistant.

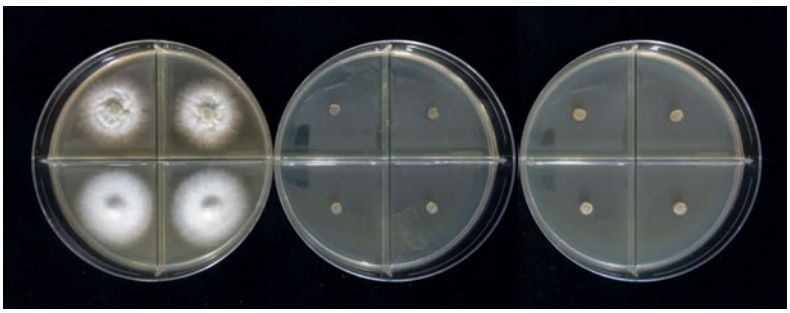


Figure 1 No cross-resistance in very highly boscalid-resistant isolates to fluopyram and isofetamid. Left to right: boscalid, fluopyram, and isofetamid at 100 mg L^{-1} each.

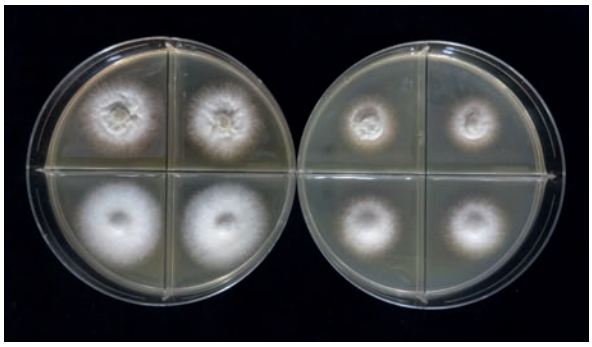


Figure 2 Cross-resistance of very highly boscalid-resistant isolates to boscalid (left) and pyraziflumid (right) at 100 mg L^{-1} each.

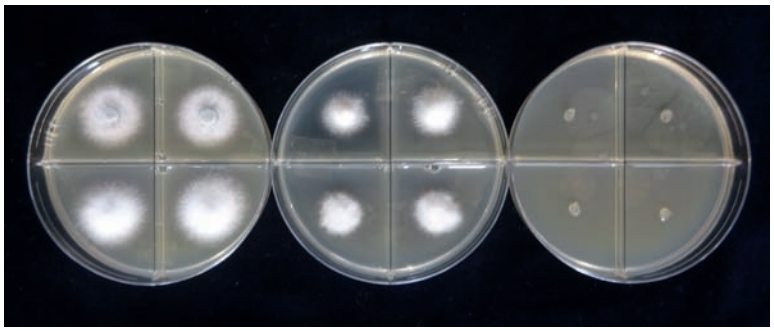


Figure 3 Differential sensitivity of very highly boscalid-resistant isolates to isopyrazam (left), fluxapyroxad (middle), and benzovindiflupyr (right) at 100 mg L^{-1} each.

Table 3 Control efficacy of boscalid and benzovindiflupyr against cucumber Corynespora leaf spot disease

Isolate*	% Control (average ± 95% CI) by: boscalid (334 mg L^{-1})	benzovindiflupyr (200 mg L^{-1})
S	91.4 ± 3.04	92.8 ± 7.55
MR	65.4 ± 42.15	95.3 ± 0.69
HR	6.2 ± 27.05	96.4 ± 1.57
VHR	0.5 ± 10.59	87.1 ± 18.72

*S, sensitive; MR, moderately resistant; HR, highly resistant; VHR, very highly resistant to boscalid.

DISCUSSION

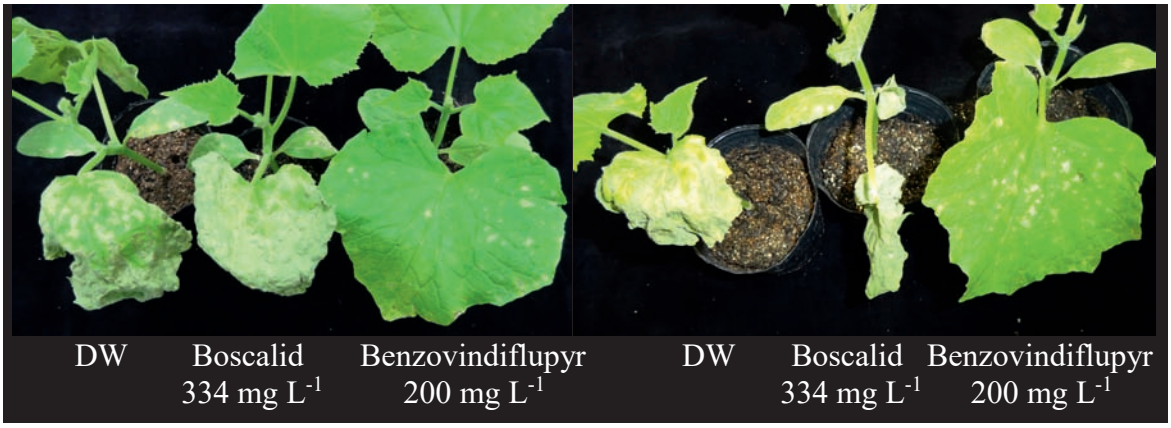


Figure 4 Efficacy of boscalid and benzovindiflupyr against very highly boscalid-resistant (left) and highly boscalid-resistant (right) isolates.

Due to the wide distribution of fungicide-resistant strains in *C. cassiicola* and failure of chemical control, cucumber cultivars tolerant to Corynespora leaf spot have been often introduced into commercial greenhouses resulting in the drastic decrease of this disease in Japan. However, the importance of this pathogen is rather increasing and fungicide treatment is still a major tool to control it in other countries. In this study, differential pattern of cross-resistance was examined using various SDHI fungicides. As reported before (Ishii et al. 2011; Tsukuda 2015), the lack of cross-resistance to both fluopyram and isofetamid was confirmed in very highly and highly boscalid-resistant isolates of *C. cassiicola*, carrying *sdhB*-H278Y and *sdhB*-H278R, respectively. On the contrary, high level of cross-resistance was observed to isopyrazam in very highly boscalid-resistant isolates. Cross-resistance was also confirmed for pyraziflumid.

Benzovindiflupyr belongs to SDHI fungicides, however, in mycelial growth tests conducted *in vitro*, the level of cross-resistance to this fungicide was particularly low in very highly bocalid-resistant isolates, the most important ones in practice. This low level of cross-resistance was further confirmed in pathogen inoculation tests performed on cucumber plants. Structural plasticity of benzovindiflupyr might explain such a difference of efficacy from boscalid. Ishii et al. (2016) found unique high antifungal activity of benzovindiflupyr against various *Colletotrichum* species, inherently insensitive to most of the other SDHI fungicides such as boscalid, fluxapyroxad, and fluopyram. No apparent resistance-relating mutations were found in the *sdhB*, *sdhC*, and *sdhD* genes encoding the subunits of fungicide-targeted succinate dehydrogenase in *Colletotrichum* species indicating that other mechanism(s) than fungicide target-site modification may be responsible for differential sensitivity of *Colletotrichum* species to SDHI fungicides. Very recently, high control efficacy of benzovindiflupyr against strawberry anthacnose disease has been confirmed in both laboratory tests and field experiments conducted in the United States (Oliveira et al. 2019; Rebello et al. 2019).

Although the risk of resistance development has to be taken in account, the SDHI fungicide benzovindiflupyr could serve as an alternative to control wide range of pathogens such as *C.*

cassiicola, *Colletotrichum* species on various crops when used properly in combination with other effective countermeasures.

REFERENCES

Avenot HF; Michailides TJ (2010). Progress in understanding molecular mechanisms and evolution of resistance to succinate dehydrogenase inhibiting (SDHI) fungicides in phytopathogenic fungi. *Crop Protection* 29, 643-651.

Date H; Kataoka E; Tanina K; Sasaki S; Inoue K; Nasu H; Kasuyamam S. (2004). Sensitivity of *Corynespora cassiicola*, causal agent of Corynespora leaf spot of cucumber, to thiophanate-methyl, diethofencarb and azoxystrobin. *Japanese Journal of Phytopathololology* 70, 10-13.

Duan Y, Xin W, Lu F, Li T, Li M, Wu J, Wang J, Zhou M. (2019). Benzimidazole- and QoI-resistance in *Corynespora cassiicola* populations from greenhouse-cultivated cucumber: An emerging problem in China. *Pesticide Biochemistry and Physiology* 153, 95-105.

Ishii H. Yano K, Date H, Furuta A, Sagehashi Y, Yamaguchi T, Sugiyama T, Nishimura K, Hasama W. (2007). Molecular characterization and diagnosis of QoI resistance in cucumber and eggplant pathogens. *Phytopathology* 97, 1458-1466.

Ishii H, Miyamoto T, Ushio S, Kakishima M. (2011). Lack of cross-resistance to a novel succinate dehydrogenase inhibitor, fluopyram, in highly boscalid-resistant isolates of *Corynespora cassiicola* and *Podosphaera xanthii*. *Pest Management Science* 67, 474-482.

Ishii H, Zhen F, Hu M, Li X, Schnabel G (2016). Efficacy of SDHI fungicides, including benzovindiflupyr, against *Colletotrichum* species. *Pest Management Science* 72, 1844-1853.

Miyamoto T, Ishii H, Seko T, Kobori S, Tomita Y (2009). Occurrence of *Corynespora cassiicola* isolates resistant to boscalid on cucumber in Ibaraki Prefecture, Japan. *Plant Pathology* 58, 1144-51.

Miyamoto T, Ishii H, Stammler G, Koch A, Ogawara T, Tomita Y, Fountaine JM, Ushio S, Seko T, Kobori S (2010). Distribution and molecular characterization of *Corynespora cassiicola* isolates resistant to boscalid. *Plant Pathology* 59, 873-881.

Oliveira M, Cordova L, Peres N (2019). Efficacy and baseline of succinate dehydrogenase inhibitor (SDHI) fungicides for management of Colletotrichum crown rot of strawberries. *APS Annual Meeting*, 134-P1.

Rebello CC, Forcelini BB, Baggio J, Mertely JC, Peres N (2019). Sensitivity of *Colletotrichum acutatum*, causal agent of anthracnose fruit rot of strawberry to fungicides alternative to quinone-outside inhibitors. *APS Annual Meeting*, 157-P1.

Tsukuda S (2015). Fungicidal properties and sensitivity study of a novel fungicide isofetamid (KENJA®). *Abstracts of the 25th Symposium of Research Committee on Fungicide Resistance, the Phytopathological Society of Japan*, 11-21.

Zhu F, Fadi Zhu, Shi Y, Xie X, Chai A, Li B (2019). Occurrence, distribution, and characteristics of boscalid-resistant *Corynespora cassiicola* in China. *Plant Disease* 103, 69-76.

Hu M-J et al., A 252-bp Deletion in *Bos1* was Consistently Found in Fludioxonil-Resistant Mutants from the Same *Botrytis* Isolate. In: Deising HB; Fraaije B; Mehl A; Oerke EC; Sierotzki H; Stammler G (Eds), "Modern Fungicides and Antifungal Compounds", Vol. IX, pp. 163-168. © 2020. Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-16-7

A 252-bp Deletion in *Bos1* was Consistently Found in Fludioxonil-Resistant Mutants from the Same *Botrytis* Isolate

Hu, M-J[1], Luo Q-C[1], Cosseboom S[1], Schnabel G[2]

[1]*Department of Plant Science and Landscape Architecture, University of Maryland, College Park, Maryland, USA*

[2]*Department of Plant and Environmental Sciences, Clemson University, Clemson, South Carolina, USA*

Corresponding Author Email: mjhu@umd.edu

ABSTRACT

Botrytis group S isolate 5d5 is sensitive to fludioxonil, but colonies started to grow after 3 days of incubation on MEA medium amended with 100 µg/ml fludioxonil. To examine the mutation rate, mycelial plugs of 5d5 isolate were placed on the fungicide-amended plates independently and the frequency of colonies growing from plugs was assessed. The experiment was repeated twice with the frequency of progeny growing on the selective medium ranging from 60% to 90%. While no variations were detected in *mrr1* gene sequences between the parental 5d5 isolate and the resistant mutants, a 252-bp deletion in the 4th exon of *bos1* gene was found in six of six resistant mutants and eight of eight mutants tested in experiments one and two, respectively. The deletion was never amplified from parental DNA. The agar plug test was conducted with 10 replicate plugs of each of twenty-two single spore progeny isolates obtained from the parental isolate 5d5. Resistant mycelium was recovered only from 1 of the 10 plugs of 2 of the 22 single spore isolates. The same 252-bp deletion was found in these mutants. Although a variety of different mutations in *bos1* have been linked to fludioxonil resistance in *Botrytis cinerea*, the variation described in this study is unique. We hypothesize either heterokaryosis or targeted mutagenesis in *bos1* of isolate 5d5 following exposure to fludioxonil *in vitro*.

INTRODUCTION

Gray mold (caused by *Botrytis* spp.) is an economically important disease on strawberries worldwide. Among *Botrytis* species, *Botrytis cinerea* attacks a wide range of plant hosts (Williamson et al. 2007) and represents the primary species affecting strawberries (Dowling et al. 2018; Rupp et al. 2017). *Botrytis* group S is currently considered a variant of *B. cinerea*, which has only been reported in German and U.S. strawberry fields (Fernández-Ortuño et al. 2015; Leroch et al. 2013). Although efforts have been continuously made in breeding programs

for resistant varieties, use of fungicides remains critical for gray mold control. However, *B. cinerea* has a strong ability to develop fungicide resistance. Widespread resistance to multiple chemical classes of fungicides in *B. cinerea* has been detected in strawberry fields (Fernández-Ortuño et al. 2014a; Weber & Hahn 2019). It is noteworthy that *Botrytis* group S was found to be predominately resistant to multiple fungicides in German strawberry fields (Leroch et al. 2013). Fludioxonil, a phenypyrrole, has widely been used against gray mold due to its high efficacy and infrequent resistance frequency (Avenot et al. 2018; Hu et al. 2016; Saito et al. 2019). Resistance to fludioxonil in *B. cinerea* (including *Botrytis* group S) is primarily caused by overexpression of the drug efflux pump *atrb* (Kretschmer et al. 2009). The overexpression of *atrb* is found to be partially caused by mutations in *mrr1*, which is a transcription factor regulating *atrb* expression (Fernández-Ortuño et al. 2015; Hu et al. 2019). Another mechanism conferring resistance in *B. cinerea*, though less common, is based on mutations in *bos1*, which typically lead to higher levels of resistance to fludioxonil (Ren et al. 2016).

Various mutations and/or duplications in *bos1* have been found to correlate with resistance to fludioxonil in *B. cinerea*. These mutations were found to be located in HAMP domains of the N-terminal region from the field isolates, and in both the HATPase_c domain and the HAMP domain of bos1 from laboratory mutants (Ren et al. 2016). As a histidine kinase, the bos1 serves as a regulator in the high-osmolarity glycerol (HOG) mitogen-activated protein kinase (MAPK) pathway (Bahn 2008). It appears that many mutations in *bos1* can reduce sensitivity to dicarboximide or phenylpyrrole fungicides, especially those occurring within the HAMP domain repeats (Duan et al. 2014; Fillinger et al. 2012).

Mutations are thought to occur randomly. Environmental factors may increase or decrease the rate of mutation but little evidence of their potential influence on the direction of mutation is available (Kimura 1967). Similarly, exposure of toxins such as fludioxonil may increase mutation rates of *bos1* or *mrr1* in *B. cinerea*, allowing for selection of laboratory resistant mutants. Given that many mutations in *bos1* or *mrr1* have been linked to fludioxonil resistance, it is worthwhile to characterize mutations in offspring of a given sensitive isolate to determine whether mutations are randomly occurring under fungicide exposure in isolates with identical or largely identical genetic background.

MATERIALS AND METHONDS

Fungal isolates and fungicide. A single spore *Botrytis* group S isolate 5d5, collected from a strawberry field in the US, was used in the study as parental isolate. The isolate was stored on filter papers at -20 °C. Potato dextrose agar (PDA) medium was used to initiate fungal culture from filter papers. The fungicide Scholar@ SC (Syngenta Crop Protection, USA) was used for sensitivity determination and mycelial adaptation experiment to induce resistant mutants. To make a stock solution, the fungicide was dissolved in sterile water and adjusted to a concentration of 10,000 µg a.i./mL and stored at 4°C for further use.

Induction of fludioxonil-resistant mutants in vitro. PDA plugs containing actively growing mycelia of 5d5 were placed on 1% malt extract agar (MEA) amended with 100 µg/mL of fludioxonil. The plates (each contained 9 to 10 mycelial plugs) were incubated at 22°C in darkness until new colonies emerged. Mycelia from the new colonies were then transferred to a new fludioxonil-amended MEA plate for resistance confirmation. The experiment was repeated twice independently. The same protocol was used to induce resistant mutants from single spore progenies of 5d5. The mutation rate was calculated by dividing the number of plugs forming mycelia by the number of plugs not forming mycelia on fludioxonil-amended plates times 100.

Sequencing analysis of bos1 and mrr1. To investigate possible mutations conferring resistance in the fludioxonil-resistant mutants, *bos1* and *mrr1* were amplified and sequenced from both parental and mutant isolates according to Hu et al. (2019). Primers Del_D-F (5′-3′: AATTGAGTCCGCTCGAAAGA) and Del_D-R (5′-3′: ATATTCATCCATTTGGGCTTG) were designed to specifically detect a 252-bp deletion in *bos1* found in this study. Additionally, primers Bos1-Cm4F (5′-3′: ATCATTGATGATATTCTC) and Bos1_Cm4R (5′-3′: ACATCGTATCGTTTCTCCT), located at flanking regions of the 252-bp fragment, were used to confirm the absence or presence of the 252 deletion.

RESULTS AND DISSCUSSION

Mutants with resistance to fludioxonil were obtained by adapting the mycelia of the 5d5 isolate to fludioxonil at 100 µg/ml *in vitro*. In total, 14 mutants were obtained, with the average mutation rate of 78% (Fig. 1). Resistance to fludioxonil is typically considered when a *Botrytis* isolate is able to grow on MEA plate amended with fludioxonil at 0.5 µg/ml (Fernández-Ortuño et al. 2014b). Thus, the mutants obtained in the study all possessed high levels of resistance to fludioxonil.

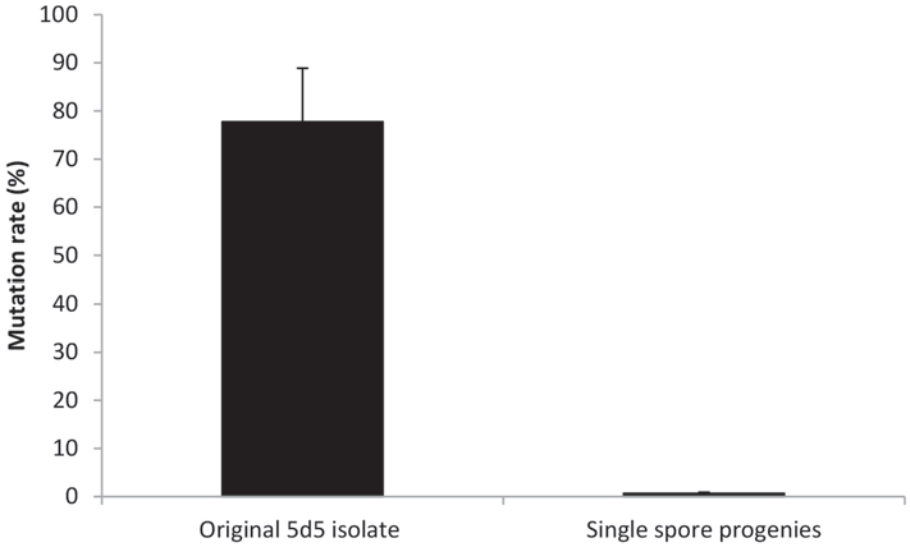


Figure 1 Frequency of resistance mutation in the parental isolate 5d5 and its single spore progenies.

Six mutants of 5d5, along with the parental isolates, were subject to *mrr1* and *bos1* sequencing analyses. Interestingly, a previously undescribed 252-bp deletion located in the fourth exon of *bos1* was consistently found in all six mutants of 5d5 (Fig. 2). No mutation was found in the sequences of *mrr1* between the mutants and parental isolate of 5d5 (data not shown).

Primer pair Del_D-F/Del_D-R yielded 581-bp fragment from the *bos1* genotype with the 252-bp deletion, whereas Bos1_Cm4F/Bos1_Cm4R yielded 1127-bp or 875-bp fragment from the wild or mutant genotype. The 252-bp deletion was detected in all mutants of 5d5, but not in the parental isolate. Subsequently, 22 single spore isolates were obtained from the wild isolate 5d5, and each singe spore isolate was used for mutation rate testing. Briefly, 10 mycelial plugs of each singe spore isolate were placed on the fungicide-amended media to test the mutation rate. Interestingly, among the 22 isolates, only 2 isolates produced mutants and each of those only produced one plug that produced a mutant (Fig. 1). The 252-bp deletion was detected from both of these mutants, but could not be detected in the parental 5d5 isolate despite several attempts.

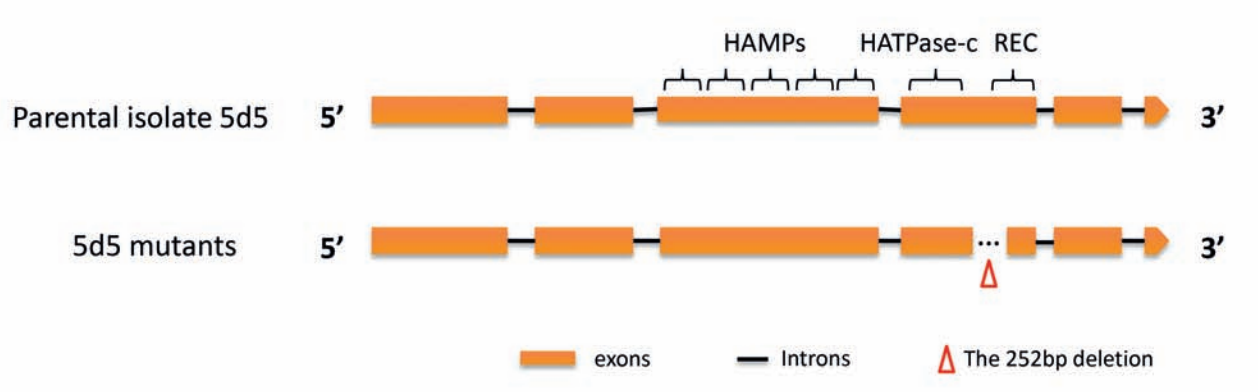


Figure 2 Schematic diagram of *bos1* gene from the parental and mutants of 5d5 isolate.

Although at much lower rate, single spore progenies of 5d5 produced resistant mutants with the same deletion in *bos1*. The most straightforward explanation for this phenomenon is that 5d5 resulted from a single spore that contained genetically different nuclei, namely a heterokaryon (Parmeter et al. 1963). The PCRs only detected the wild genotype of *bos1* from 5d5, which may indicate that the number of nuclei containing the mutant genotype of *bos1* is extremely low. It therefore stands to reason that many single spore progenies of the 5d5 isolate do not contain the mutant genotype, which would explain the low mutation rate of single spore progenies. Another explanation would be targeted mutagenesis assuming that the 252-bp deletion was not at all present (and was indeed not picked up by PCR) in the parental isolate. However, to the best of our knowledge, such phenomenon (i.e. targeted mutagenesis following the exposure to fludioxonil) has never been described in plant fungal pathogens.

REFERENCES

Avenot, H. F., Quattrini, J., Puckett, R., and Michailides, T. J. 2018. Different levels of resistance to cyprodinil and iprodione and lack of fludioxonil resistance in *Botrytis cinerea* isolates collected from pistachio, grape, and pomegranate fields in California. Crop Protection 112:274-281.

Bahn, Y.-S. 2008. Master and commander in fungal pathogens: the two-component system and the HOG signaling pathway. Eukaryot Cell 7:2017-2036.

Dowling, M. E., Hu, M.-J., and Schnabel, G. 2018. Fungicide resistance in *Botrytis fragariae* and species prevalence in the Mid-Atlantic United States. Plant Disease 102:964-969.

Duan, Y.-B., Ge, C.-Y., and Zhou, M.-G. 2014. Molecular and biochemical characterization of Sclerotinia sclerotiorum laboratory mutants resistant to dicarboximide and phenylpyrrole fungicides. Journal of Pest Science 87:221-230.

Fernández-Ortuño, D., Grabke, A., Li, X., and Schnabel, G. 2015. Independent emergence of resistance to seven chemical classes of fungicides in *Botrytis cinerea*. Phytopathology 105:424-432.

Fernández-Ortuño, D., Grabke, A., Bryson, P. K., Amiri, A., Peres, N. A., and Schnabel, G. 2014b. Fungicide resistance profiles in *Botrytis cinerea* from strawberry fields of seven Southern U.S. states. Plant Dis. 98:825-833.

Fillinger, S., Ajouz, S., Nicot, P. C., Leroux, P., and Bardin, M. 2012. Functional and structural comparison of pyrrolnitrin- and iprodione-induced modifications in the class III histidine-kinase *bos1* of *Botrytis cinerea*. PLOS One 7:e42520.

Hu, M.-J., Cox, K. D., and Schnabel, G. 2016. Resistance to increasing chemical classes of fungicides by virtue of "Selection by Association" in *Botrytis cinerea*. Phytopathology 106:1513-1520.

Hu, M.-J., Cosseboom, S., and Schnabel, G. 2019. AtrB-associated fludioxonil resistance in *Botrytis fragariae* not linked to mutations in transcription factor mrr1. Phytopathology 109:839-846.

Kimura, M. 1967. On the evolutionary adjustment of spontaneous mutation rates. Genetical Research 9:23-34.

Kretschmer, M., Leroch, M., Mosbach, A., Walker, A.-S., Fillinger, S., Mernke, D., Schoonbeek, H.-J., Pradier, J.-M., Leroux, P., De Waard, M. A., and Hahn, M. 2009. Fungicide-driven evolution and molecular basis of multidrug resistance in field populations of the grey mould fungus *Botrytis cinerea*. PLOS Pathogens 5:e1000696.

Leroch, M., Plesken, C., Weber, R. W. S., Kauff, F., Scalliet, G., and Hahn, M. 2013. Gray mold populations in German strawberry fields are resistant to multiple fungicides and dominated by a novel clade closely related to *Botrytis cinerea*. Applied and Environmental Microbiology 79:159-167.

Parmeter, J. R., Snyder, W. C., and Reichle, R. E. 1963. Heterokaryosis and variability in plant-pathogenic fungi. Annual Review of Phytopathology 1:51-76.

Ren, W., Shao, W., Han, X., Zhou, M., and Chen, C. 2016. Molecular and biochemical characterization of laboratory and field mutants of *Botrytis cinerea* resistant to fludioxonil. Plant Disease 100:1414-1423.

Rupp, S., Plesken, C., Rumsey, S., Dowling, M., Schnabel, G., Weber, R. W. S., and Hahn, M. 2017. *Botrytis fragariae*, a new species causing gray mold on strawberries, shows high frequencies of specific and efflux-based fungicide resistance. Applied and Environmental Microbiology 83: e00269-17.

Saito, S., Michailides, T. J., and Xiao, C. L. 2019. Fungicide-resistant phenotypes in *Botrytis cinerea* populations and their impact on control of gray mold on stored table grapes in California. European Journal of Plant Pathology 154:203-213.

Weber, R. W. S., and Hahn, M. 2019. Grey mould disease of strawberry in northern Germany: causal agents, fungicide resistance and management strategies. Applied Microbiology and Biotechnology 103:1589-1597.

Williamson, B., Tudzynski, B., Tudzynski, P., and Van Kan, J. A. L. 2007. *Botrytis cinerea*: the cause of grey mould disease. Molecular Plant Pathology 8:561-580.

Gazzetti K et al., Sensitivity of Stemphylium vesicarium of pear to SDHIs. In: Deising HB; Fraaije B; Mehl A; Oerke EC; Sierotzki H; Stammler G (Eds), "Modern Fungicides and Antifungal Compounds", Vol. IX, pp. 169-172. © 2020. Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-16-7

Sensitivity of *Stemphylium vesicarium* of pear to SDHIs

Gazzetti K, Ciriani A, Menghini M, Casagrandi F, Collina M

University of Bologna, Department of Agricultural and Food Science, Viale G. Fanin 42, 40127 Bologna, Italy

Corresponding Author Email: marina.collina@unibo.it

ABSTRACT

Stemphylium vesicarium is the causal agent of Brown Spot of Pear (BSP), producing high economical losses in several pear-growing areas in Europe.

In Italy, BSP control in field can also rely on the use of SDHI (Succinate Dehydrogenase Inhibitor) compounds such as boscalid, penthiopyrad, fluopyram and fluxapyroxad.

Efficacy field tests on these fungicides sprayed as solo product were conducted during 2016 and 2017 in a pear experimental orchard located in the Emilia-Romagna region. All the fungal strains isolated in both years from the plots were tested for their *in vitro* sensitivity to SDHI fungicides already authorized in Italy against BSP, together to bixafen and isopyrazam.

Overall, the results highlighted the onset of problems related to the efficacy of SDHIs. These observations were mirrored in EC_{50} values showed by the strains collected in both the years. Molecular analysis carried out in the laboratory, led to discover amminoacidic substitutions conferring SDHIs resistance in the *S. vesicarium* of pear.

INTRODUCTION

Brown spot of pear (*Pyrus communis* L.) is a disease caused by the fungus *Stemphylium vesicarium* (Wallr.) Simmons. BSP symptoms consist of necrotic lesions on pear fruits, leaves and twigs. The first outbreaks of the disease were first reported in 1975 in the Emilia-Romagna region in Italy on cv Abate Fétel (Ponti et al. 1982). Since the 80‘, new BSP outbreaks were observed in France, Spain, the Netherlands and Portugal, indicating that the spread of the disease of economic importance has taken place within the main pear-production areas in Europe (Blancard et al.1989; Heijne & Mourik 2001; Vilardell 1988).

Many fungicide applications are required from petal fall to fruit ripening to protect orchards from BSP (Ponti et al. 1996). In Italy, the fungus showed field resistance toward key products as dicarboximides and, in few cases, fludioxonil (Alberoni et al., 2005; Alberoni et al., 2010a) as well as strobilurins (Alberoni et al., 2010b). The introduction in field of new chemicals with different mode of action is thus fundamental to enlarge the range of effective fungicides against

BSP and to reduce the risk of further resistance issues. Boscalid was the first SDHI fungicide authorized in Italy against BSP, in 2007. To date, penthiopyrad, fluopyram, fluxapyroxad and isopyrazam, are also authorized for the BSP control in field (Brunelli et al., 2016). These compounds are classified by the FRAC SDHI Working Group as fungicides with medium to high risk of resistance, because of their specific mode of action. Since 2015, FRAC reports the existence of substitutions conferring SDHIs resistance occurring in the B subunit (B-P225L, B-H272Y/R), characterized in samples of *S. vesicarium* collected on asparagus in field.

In this study, the SDHI compounds currently authorized against BSP were applied during 2016 and 2017 in a pear experimental orchard, in order to perform efficacy tests, and disease incidence was evaluated. *In vitro* sensitivity tests to SDHI products were conducted on fungal strains isolated from fruits collected in the plots. Molecular investigations were then carried out to establish the probable presence of resistance mutations to these active ingredients.

MATERIAL AND METHODS

Both trials were conducted in an experimental orchard located in the Emilia-Romagna region, on the BSP susceptible cultivar Abate Fétel. Plots treated with boscalid, fluxapyroxad, penthiopyrad, and fluopyram were randomized, and cyprodinil + fludioxonil was used as reference treatment. Each plot was composed by 6-7 plants, compounds were tested as solo product, and four repetitions per treatments were performed. The plots received more than 10 applications in both the years. The disease assessments were performed on all fruits of the plots at the end of August. Results were statistically analized by ANOVA, and averages were compared with the Duncan test (p=0.05).

All fruits were collected from each plot and, for each fruit, was cut one fragment from the symptomatic area and transferred in a plate of V8 agar, amended with 50 mg/L streptomycin sulphate. Spore suspensions were obtained from 7 day-old colonies by adding a few millilitres of sterile water and gently scraping the colony surface with a spatula. This suspension was then filtered through a 100 µm filter. Sensitivity assays of the pathogen to boscalid, fluxapyroxad, penthiopyrad, fluopyram, bixafen, isopyrazam were carried out on spore suspension in microtiter plates using spectrophotometer. For each compound, 6 concentrations were tested on each strains, in four replicates. After two days of incubation EC_{50} (mg/L) values were calculated by probit analysis.

Standard PCRs were performed by specific primers designed in our lab to amplify the entire encoding sequences of the SDH B, C and D subunits, and amplification products were sequenced by Sanger method. Sequencing results were aligned by Sequencer 5.6.4 (Gene Codes Corporation, USA) against the SDHIs sensitive reference genome of the strain 173-1a13FI1M3 (Gazzetti et al. 2019), in order to identify SNPs candidated to confer SDHIs resistance.

RESULTS

In 2016, the plot treated with fluxapyroxad exhibited a statistically significant difference in disease incidence percentage versus other treatments and a no statistically significant difference versus the untreated. These results were confirmed in 2017 for all the tested SDHIs. Results of sensitivity assays conducted on conidia of all fungal strains showed the presence of resistance toward all SDHIs. Performing molecular analysis on the *S. vesicarium* resistants populations isolated in 2016 and 2017 from the plots of experimental orchard, made it possible to discover the existence of specific point mutations involved in SDHIs resistance.

CONCLUSIONS

These data describe the detection of the occurrence of a shift of the pathogen sensitivity to the SDHI fungicides, both in field and *in vitro*, and suggest that strict resistance management strategies are recommended to maintain SDHIs as effective tool for BSP control.

REFERENCES

Alberoni G, Collina M, Pancaldi D, Brunelli A. (2005) Resistance to dicarboximide fungicides in *Stemphylium vesicarium* of Italian pear orchards. *European Journal of Plant Pathology* 113, 211–219.

Alberoni G, Collina M, Lanen C, Leroux P, Brunelli A. (2010a) Field strains of *Stemphylium vesicarium* with a resistance to dicarboximide fungicides correlated with changes in a two-component histidine kinase. *European Journal of Plant Pathology* 128, 171–184

Alberoni G, Cavallini D, Collina M, Brunelli A. (2010b) Characterization of the first Stempylium vesicarium isolates resistant to strobilurins in Italian pear orchards. *European Journal of Plant Pathology* 126, 453–457.

Blancard D, Allard E, Brest P. (1989) La stemphyliose du poirier ou “macules brunes”. *Phytoma* 406, 35–37.

Brunelli A, Fabbri M, Casagrandi F, Geminiani E, Troiano PP. (2016) Attività di recenti fungicidi inibitori della Succinato Deidrogenasi (SDHI) contro la maculatura bruna del pero. *Atti Giornate Fitopatologiche* 2, 377-384.

FRAC (2015). www.frac.info/docs/default-source/sdhi-wg/sdhi-references/list-of-species-resistant-to-sdhis-april-2015.pdf?sfvrsn=2d144a9a_2**.**

Gazzetti K, Diaconu EL; Nanni IM; Ciriani A, Collina M. (2019). Genome Sequence Resource for Stemphylium vesicarium, causing Brown Spot Disease of pear. *Molecular Plant-Microbe Interactions* doi.org/10.1094/MPMI-11-18-0299-A.

Heijne B, Van Mourik J. (2001) Zwartvruchtrot op peer neemt toe. *Fruitteelt Den Haag* 91, 18-19.

Ponti I, Brunelli A, Tosi C, Cavallini G, Mazzini, F. (1996). Chemical control trials on pear brown spot (*Stemphylium vesicarium*). *Atti Giornate Fitopatologiche* 2, 165-172.

Ponti I, Cavanni P, Brunelli A (1982) Brown spot of pear: Aetiology and protection. *Informatore Fitopatologico* 32, 35–40.

Vilardell P. *Stemphylium vesicarium* en plantaciones de peral. (1988) *Fruticultura Profesional*. 18, 51–55.

Adaskaveg JE, Luo Y, Förster H, Characterization of Resistance to Five SDHI Sub-Groups in Alternaria Species Causing Leaf Spot of Almond in California. In: Deising HB; Fraaije B; Mehl A; Oerke EC; Sierotzki H; Stammler G (Eds), "Modern Fungicides and Antifungal Compounds", Vol. IX, pp. 173-180. © 2020. Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-16-7

Characterization of Resistance to Five SDHI Sub-Groups in *Alternaria* Species Causing Leaf Spot of Almond in California

Adaskaveg JE, Luo Y, Förster H

Department of Plant Pathology and Microbiology, University of California, Riverside, CA 92521, U.S.A.

Email: jim.adaskaveg@ucr.edu

ABSTRACT

Succinate dehydrogenase inhibitors (SDHIs) are a major component in chemical disease management of tree fruit crops in California and elsewhere. On almond, boscalid, fluopyram, fluxapyroxad, isofetamid, and penthiopyrad are currently registered, and others such as pydiflumetofen, and pyraziflumid are being developed for use on this crop. Together, these fungicides belong to five SDHI subgroups. After widespread resistance development against the QoIs in the Alternaria leaf spot pathogens *Alternaria alternata* and *A. arborescens*, SDHIs, polyoxin-D, and several demethylation inhibitors are commonly used to manage this disease. Reduced sensitivity with a wide range of resistance factors is now also present to SDHI compounds, with most resistance occurring to the pyridine-carboxamide (e.g., boscalid), pyrazole-4-carboxamide (e.g., fluxapyroxad, penthiopyrad), and pyrazine-carboxamide (e.g., pyraziflumid) subgroups. Reduced sensitivity to pydiflumetofen was only detected in one of the isolates of *Alternaria* spp. evaluated. We identified cross resistance patterns among SDHI fungicides that correlated with mutations B-H277Y, B-277L, and C-H134R. Most isolates carried the C-H134R mutation. Resistance to fungicides in newer SDHI sub-groups is being detected before commercial introduction. Thus, either less-sensitive variants pre-exist in the pathogen populations or resistance is an ongoing selection process related to fitness or non-detrimental changes in the pathogen.

INTRODUCTION

With currently over 500,000 hectares planted, almond ranks first followed closely by grapes in agricultural crop production area and value in California. The high market demand of this valuable crop has resulted in more intensive production systems that include high-density

plantings with increased irrigation and fertilization to increase yield. This has created more favorable conditions for some diseases of almond such as Alternaria leaf spot caused by *Alternaria alternata* and *A. arborescens*, scab caused by *Fusicladium carpophilum*, and hull rot caused by *Rhizopus stolonifer*, and species of *Monilinia*, *Aspergillus*, and other fungi.

Alternaria leaf spot symptoms develop as necrotic lesions in late spring and summer. At high relative humidity, lesions show black sporulation in the center. High disease severity may lead to early tree defoliation, and new mid- to late-season leaf flushes weaken the tree and cause reduced flowering next season. Several fungicide modes of action are available for management of Alternaria leaf spot, and the most effective ones belong to FRAC Codes 3 (demethylation inhibitors), 19 (polyoxins), 11 (quinone outside inhibitors - QoIs), and 7 (succinate dehydrogenase inhibitors - SDHIs). Two or three applications between mid-May and early July can reduce the disease to very low levels. Resistance to QoIs, however, is widespread in pathogen populations. Among SDHIs, boscalid (a pyridine-carboxamide; registered in 2004), fluopyram (a pyrazole-4-carboxamide) and penthiopyrad (a pyrazole-4-carboxamide) (both registered in 2012), fluxapyroxad (a pyrazole-4-carboxamide; registered in 2016), and isofetamid (a phenyl-oxo-ethyl-thiophene-amide; registered in 2018), are currently available, whereas pyraziflumid (a pyrazine-carboxamide) and pydiflumetofen (a N-methoxy-(phenylethyl)-pyrazolecarboxamide) are in development. These compounds belong to six SDHI subgroups, and they are used by themselves or in selected pre-mixtures with FRAC Codes 3 or 11.

Resistance to SDHI fungicides has been previously reported in *Alternaria* spp. including *A. solani* from potato and tomato (Gudmestad et al. 2013; Mallik et al. 2014; Miles et al. 2014; Sierotzki & Scalliet 2013) and *A. alternata* from pistachio (Avenot & Michailides 2010; Avenot et al. 2014; Lichtemberg et al. 2018) and peach (Yang et al. 2015). Resistance was associated with selected mutations in subunits B (H277Y, H277R, H277L), C (H134R, S135R), and D (T28A, A47T, D123E, H133R, H133P, H133T) of the SDH gene. With widespread use of SDHI fungicides in California almond production, we evaluated *Alternaria* spp. isolates from major production areas for their sensitivity to selected SDHI fungicides and we determined mutations in SDH subunits for resistant isolates.

MATERIALS AND METHODS

Collection of *Alternaria* sp. isolates and determination of *in vitro* sensitivities to six SDHI fungicides.

Diseased almond leaves were collected from orchards throughout the main almond growing areas in the Sacramento and San Joaquin Valleys of California between 2007 and 2018. Following standard isolation procedures, 144 single-spore isolates were obtained. *In vitro* sensitivities (i.e. EC_{50} values) to boscalid, fluopyram, fluxapyroxad, isofetamid, penthiopyrad, pydiflumetofen, and pyraziflumid were determined using the spiral gradient dilution method

(Förster et al. 2004). Scott's formula (Scott 1979) was used to calculate bin widths for the frequency distributions of EC_{50} values for all isolates for each fungicide except for penthiopyrad. Cross resistance among the fungicides was determined by regressing EC_{50} values for one of the fungicides against those of another one. R^2 values of >0.6, 0.4 to 0.6, or <0.4 were considered to indicate a relatively strong, weak, or poor positive correlation, respectively.

Sequencing of SDH subunits and resistance phenotypes associated with selected mutations.

Partial sequences of SDH-B, SDH-C, and SDH-D were obtained using previously published primers (Avenot & Michailides 2010). For this, 73 isolates were selected that included 14 isolates sensitive to the six SDHI fungicides and the remaining ones with different levels of resistance. For mutations identified, resistance phenotypes were graphically displayed in radar diagrams with EC_{50} concentrations on a $\log_{10}$ scale using MS PowerPoint.

RESULTS

***In vitro* sensitivities of *Alternaria* sp. isolates to six SDHI fungicides.**

For the 141 isolates collected from major almond growing regions, there was a wide range of sensitivities for mycelial growth inhibition for each fungicide with a continuum from very sensitive to moderately or highly resistant. Sensitivities ranged from 0.028 to >40 µg/ml for boscalid, 0.014 to 14.6 µg/ml for fluxapyroxad, 0.017 to 0.59 µg/ml for fluopyram, 0.011 to >40 µg/ml for pyraziflumid, 0.018 to 2.44 µg/ml for isofetamid, and 0.001 to 0.215 µg/ml for pydiflumetofen. For a subset of 33 isolates evaluated, an EC_{50} range of 0.02 to >20 µg/ml was determined for penthiopyrad. The highest incidence of reduced sensitivity was found for boscalid: 81 isolates had EC_{50} values higher than 10 µg/ml, and 35 isolates had values of less than 1 µg/ml. Histograms displaying the frequency of isolates in calculated sensitivity categoriesfor each fungicide showed a clear bimodal distribution only for fluopyram with populations of isolates either more or less sensitive (Fig. 1). For the other fungicides, frequencies of the isolates were distributed in less clear patterns over the sensitivity range, and distinct subpopulations were more difficult to distinguish.

Cross-resistance among five of the fungicides (pydiflumetofen was excluded because only a single isolate with an EC_{50} value >0.2 µg/ml was identified) was evaluated based on R^2 values of pairwise regressions of EC_{50} values for all 141 isolates. Cross-resistance was highest between fluopyram and isofetamid (R^2 = 0.616), lower between fluopyram and boscalid, between fluopyram and pyraziflumid, and between boscalid and pyraziflumid, whereas only poor correlations (R^2 = <0.4) were found for the remaining fungicide combinations (Fig. 2). Cross-resistance between two pyrazole-4-carboxamides, fluxapyroxad and penthiopyrad, was evaluated for a subset of 33 isolates, and a weak correlation with an R^2 value of 0.535 was obtained in the regression analysis.

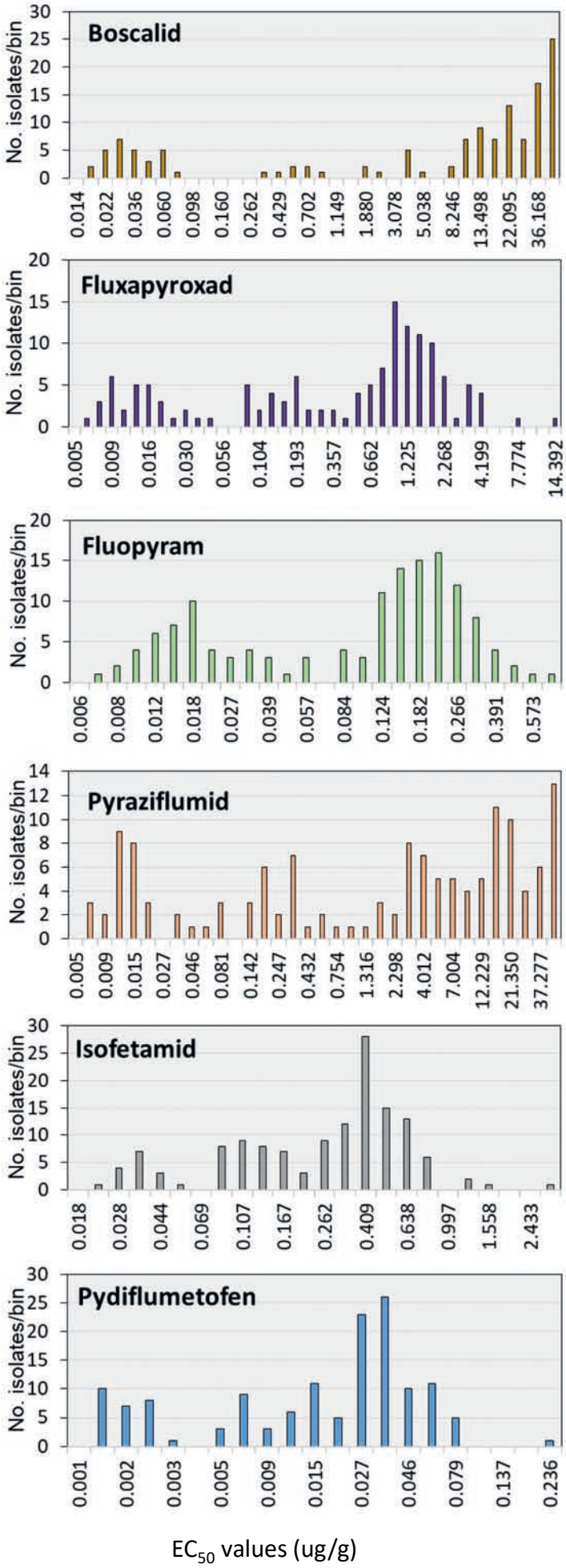


Figure 1
EC_{50} frequency distributions of 141 isolates of *Alternaria* spp. from almond collected between 2007 and 2018 showing their sensitivity to six SDHI fungicides. Bin width h was calculated using Scott's formula: $h = 3.49sn^{-1/3}$ (Scott 1979) Where s = estimate of the standard deviation and n = number of isolates.

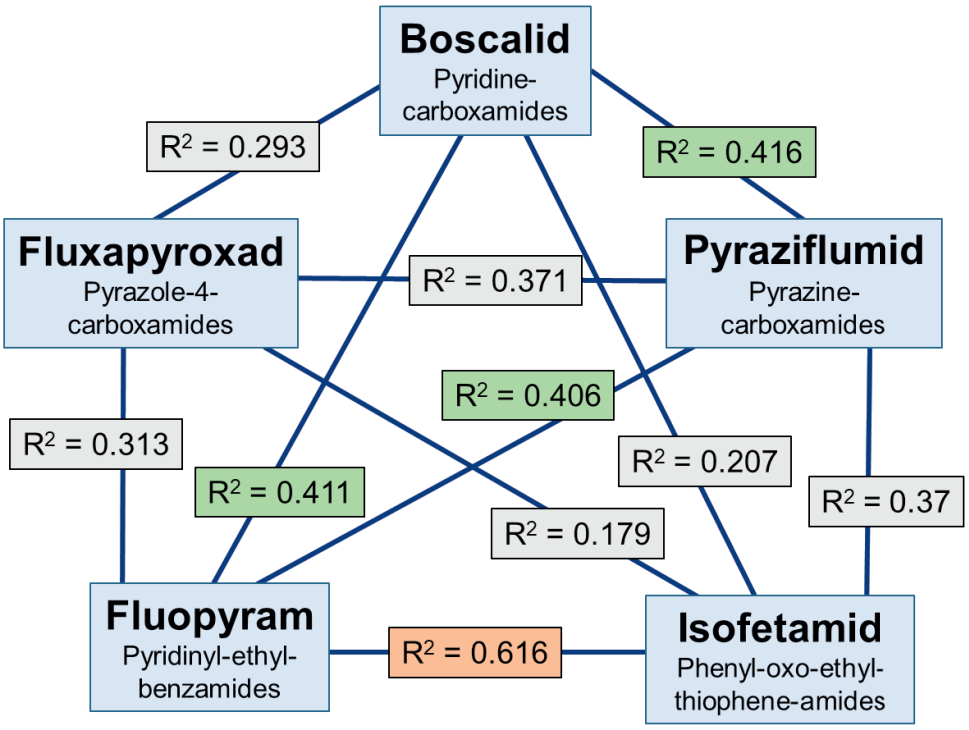


Figure 2 Cross-resistance among five SDHI fungicides in 141 *Alternaria* spp. isolates from almond based on pairwise regressions of EC_{50} values. R_2 values of >0.6, 0.4 to 0.6, and <0.4 indicate a relatively strong, weak, and poor positive correlation, respectively.

Sequencing of SDH subunits and resistance phenotypes associated with selected mutations.

Partial sequencing of the B, C, and D subunits of the SDH gene for 73 isolates demonstrated the presence of mutations H277Y (7 isolates) and H277L (2 isolates) in SDH-B and mutations H134R (46 isolates) and S135R (2 isolates) in SDH-C in isolates with reduced sensitivity to at least one of the six SDHI fungicides tested. Three mutations that always occurred together in subunit D (i.e., T28A, A47T, and M31I) of nine isolates with reduced sensitivities were also present in some isolates sensitive to all six fungicides. Sensitivity phenotypes for selected SDH mutations are graphically displayed and compared to sensitive isolates in Fig. 3. Seven isolates collected between 2009 and 2012 that carried the B-H277Y mutation mostly showed reduced sensitivity to boscalid whereas sensitivity to fluxapyroxad and pyraziflumid was only slightly reduced and that for the other fungicides was not affected as compared to isolates with no mutations (Fig. 3A, B). Among the 46 isolates with a H134R mutation in SDH-C, one was collected in 2009, six in 2012, and the remaining ones in 2018 and 2019. All isolates with this mutation showed moderate to high resistance to boscalid and pyraziflumid and moderate resistance to fluxapyroxad, whereas sensitivity to isofetamid and fluopyram was sometimes reduced as compared to isolates with no mutation (Fig. 3C). Sensitivity to pydiflumetofen was mostly not affected, but an isolate with the highest EC_{50} value of 0.22 µg/ml identified among

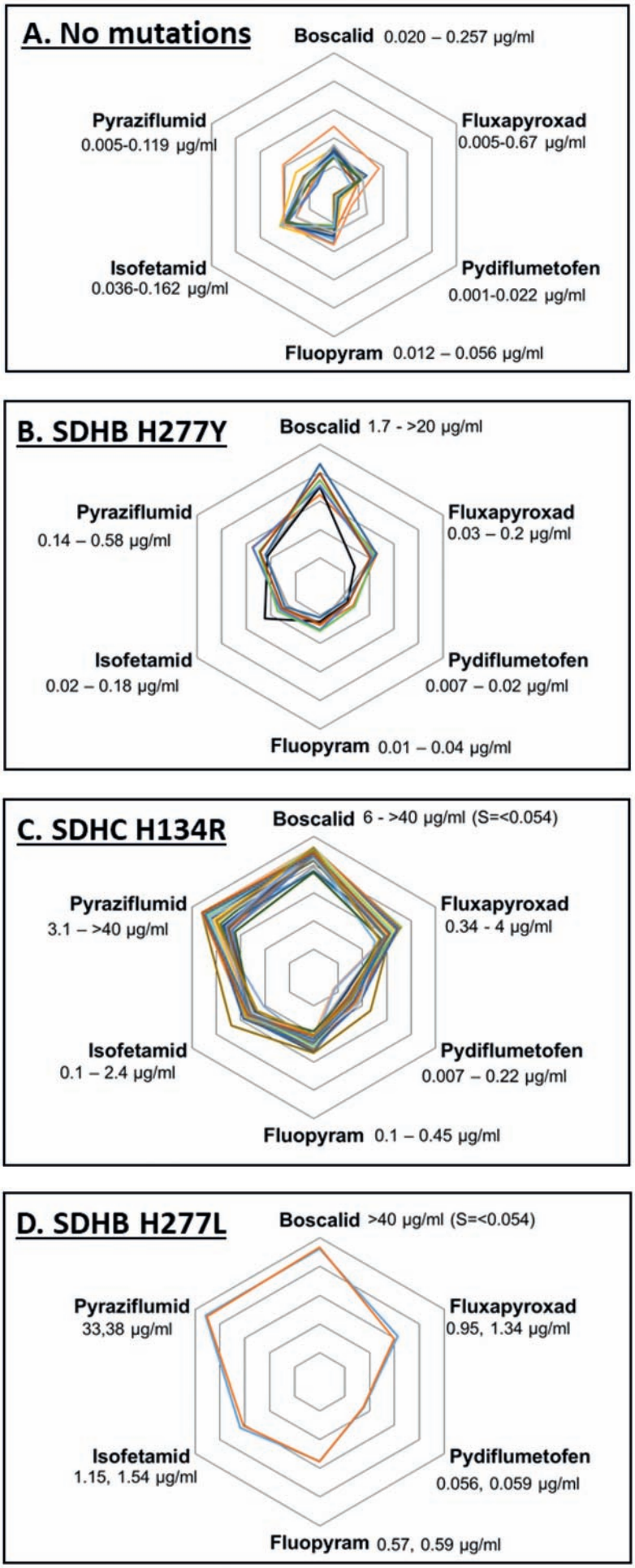


Figure 3

Fungicide sensitivity phenotypes of **A**, 15 isolates with no mutation, **B**, 7 isolates with mutation H277Y in SDHB, **C**, 46 isolates with mutation H134R in SDHC, and **D**, 2 isolates with mutation H277L in SDHB. EC_{50} values for each fungicide are on a $\log_{10}$ scale with 50 µg/ml at the edge of each diagram. The range of EC_{50} values for isolates with each mutation is indicated.

the 141 isolates also carried this mutation. Only two isolates, both collected in 2018, were found to have the H277L mutation in SDH-B. The sensitivity phenotype of these isolates was very similar to the H134R mutation in SDH-C described above (Fig. 3D). Thus, isolates had high resistance to boscalid and pyraziflumid, low levels of resistance to fluxapyroxad, isofetamid, and fluopyram, and sensitivity to pydiflumetofen was not affected.

DISCUSSION

SDHI fungicides are currently widely used for managing numerous diseases of almond in California, including brown rot, jacket rot, shot hole, scab, rust, Alternaria leaf spot, and hull rot. For this, four to six applications need to be done over the growing season. Although the importance of resistance management with rotations and mixtures of FRAC codes is widely recognized, resistance in almond fungal pathogens affecting flowers, leaves, and fruit has developed against methyl benzimidazole, QoI, as well as SDHI fungicides. Because fungicides of six SDHI subgroups are either available or are planned for registration on almond, their cross-resistance relationships and molecular mechanisms for resistance need to be investigated to possibly improve recommendations for best usage strategies. With SDHI resistance also reported in *Alternaria* spp. from almond (Adaskaveg unpublished), we investigated isolates that were obtained between 2007 and 2018 from main growing areas for their resistance characteristics.

Utilization of the spiral gradient dilution method allowed us to efficiently determine accurate EC_{50} values for mycelial growth inhibition for the 141 isolates collected. A wide range of sensitivities to SDHI fungicides ranging from high sensitivity to reduced sensitivity, moderate, and high resistance was detected, and only few of the isolates were highly sensitive to all of the fungicides tested. The highest incidence of reduced sensitivity was found for boscalid, whereas for pydiflumetofen all except one isolate had EC_{50} values of less than 0.1 µg/ml. Interestingly, less sensitive isolates were also identified for isofetamid (EC_{50} values $\leq$ 2.4 µg/ml), and resistance was detected against pyraziflumid (46 isolates with EC_{50} values $\geq$ 10 µg/ml). These isolates were never exposed to these two fungicides because they were collected prior to product registration on almond. Thus, less-sensitive variants may pre-exist in the pathogen populations or resistance is an ongoing selection process related to fitness or non-detrimental changes in the pathogen. Partial cross-resistance was found to be present among SDHI subgroups. The highest correlation in pairwise comparisons of EC_{50} values was found between fluopyram and isofetamid, two fungicides where only moderate resistance was detected. Strong cross resistance between fluopyram and isofetamid was previously established for *Zymoseptoria tritici* (Yamashita & Fraaije 2018).

In isolates of *Alternaria* spp. collected between 2009 and 2012 with reduced sensitivity to boscalid, only the B-H277Y mutation was detected. Isolates with this mutation seem to be less

fit because this mutation was not detected in later years. Instead, the majority of resistant isolates collected after 2012 carried the C-H134R mutation, and two isolates had the B-H277L mutation. Interestingly, these two mutations exhibited very similar sensitivity phenotypes with generally high resistance to boscalid and pyraziflumid, reduced sensitivity or moderate resistance to fluxapyroxad and isofetamid, and reduced sensitivity to fluopyram. The B-H277Y and C-H134R mutations were also reported to be common in isolates of *Alternaria* spp. from pistachio (Lichtemberg et al. 2018) and peach (Yang et al. 2015). Additionally, a similar shift from B-H277Y to C-H134R mutations over time was found in isolates from pistachio, and this was related to the use of new SDHI fungicides following the registration of boscalid (Lichtemberg et al. 2018; Sierotzki et al. 2010).

REFERENCES

Avenot HF; van den Biggelaar H; Morgan DP; Moral J; Joosten M; Michailides TJ (2014). Sensitivities of baseline isolates and boscalid-resistant mutants of *Alternaria alternata* from pistachio to fluopyram, penthiopyrad, and fluxapyroxad. *Plant Disease* 98,197-205.

Avenot HF; Michailides TJ (2010). Progress in understanding molecular mechanisms and evolution of resistance to succinate dehydrogenase inhibiting (SDHI) fungicides in phytopathogenic fungi. *Crop Protection* 29, 643-651.

Förster H; Kanetis L; Adaskaveg JE (2004). Spiral gradient dilution, a rapid method for determining growth responses and EC_{50} values in fungus-fungicide interactions. *Phytopathology* 94,163-170.

Gudmestad NC; Arabiat S; Miller JS; Pasche JS (2013). Prevalence and impact of SDHI fungicide resistance in *Alternaria solani. Plant Disease* 97, 952-960.

Lichtemberg P; Puckett RD; Felts D; Luo Y; Doster L; Rodriguez D; Michailides TJ (2018). Survey of the pathogen of Alternaria late blight reveals different levels of carboxamide fungicide resistance in the main pistachio producing regions of California. *California Agriculture* 72,170-178.

Mallik I, Arabiat S, Pasche JS, Bolton MD, Patel JS, Gudmestad NC (2014). Molecular characterization and detection of mutations associated with resistance to succinate dehydrogenase-inhibiting fungicides in *Alternaria solani*. *Phytopathology* 104, 40-49.

Miles TD; Miles LA; Fairchild KL; Wharton PS (2014). Screening and characterization of resistance to succinate dehydrogenase inhibitors in *Alternaria solani*. *Plant Pathology* 63, 155-164.

Scott DW (1979). On optimal and data-based histograms. *Biometrika* 66,605-610.

Sierotzki H; Scalliet G (2013). A review of current knowledge of resistance aspects for the next-generation succinate dehydrogenase inhibitor fungicides. *Phytopathology* 103, 880-887.

Yamashita M; Fraaije B (2018). Non-target site SDHI resistance is present as standing genetic variation in field populations of *Zymoseptoria tritici*. *Pest Management Science* 74, 672–681.

Yang JH; Brannen PM; Schnabel G (2015). Resistance in *Alternaria alternata* to SDHI fungicides causes rare disease outbreak in peach orchards. *Plant Disease* 99, 65-70.

Derpmann J et al., Characterization of fungicide resistant strains of *Sclerotinia sclerotiorum* and its´ implication on stem rot control in oilseed-rape. In: Deising HB; Fraaije B; Mehl A; Oerke EC; Sierotzki H; Stammler G (Eds), "Modern Fungicides and Antifungal Compounds", Vol. IX, pp. 181-186. © 2020. Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-16-7

Characterization of fungicide resistant strains of *Sclerotinia sclerotiorum* and its´ implication on stem rot control in oilseed-rape

Derpmann J, Kleemann J, Mehl A

Bayer AG, Division CropScience, Alfred-Nobel-Strasse 50, D-40789 Monheim, Germany
Email: juergen.derpmann@bayer.com

ABSTRACT

Sclerotinia sclerotiorum is a necrotrophic fungal pathogen infecting more than 400 hosts, under which economically important crops such as oilseed-rape. Since *S. sclerotiorum* is a monocyclic pathogen not possessing asexually produced conidia, its epidemic in oilseed-rape relies on ascospores, infecting susceptible floral parts. After dropping of colonized petals, mycelium can infect leafs and stems leading to the typical stem rot disease symptoms as observed in unprotected fields. Therefore, application timing as well as choice of fungicides has a significant impact on effective Sclerotinia stem rot control.

Microbiological and molecular biological experiments were conducted to identify resistance to fungicides with different modes of action such as SDHI and DMI fungicides. The impact of fungicide resistance is shown in *in vivo* experiments and its implication on fungicide resistance management is discussed.

INTRODUCTION

Sclerotinia sclerotiorum (Lib.) de Bary is the causal agent of stem rot, commonly known as white mold disease. It is an increasing threat to oilseed rape (OSR) production in European countries in years with favorable climatic conditions for disease development. Infestation levels of up to 40% have been reported in Germany and yield losses of greater than 20% may occur (Dunker & Tiedemann, 2004).

The life cycle of *S. sclerotiorum* is monocyclic, because the pathogen does not produce asexual conidia. The disease starts with apocarpous germination of sclerotia on the ground or on infected plant debris, which develops apothecia. Under favorable climatic conditions, ascospores are released from apothecia into the air. Conidia are not able to infect directly green plant tissue, such as leafs or stems, but need an additional nutrient source or dead tissue to germinate and colonize the plant. In case of OSR, the time of ascospore release can be simultaneous to the flowering stage of the crop, so that released ascospores can infect susceptible floral parts, especially when the flower is becoming senescent. After dropping of colonized petals and stamina, mycelium can utilize those additional nutrients to infect healthy leafs and stems of OSR

leading to the stem rot disease symptoms as observed in unprotected fields (Young & Werner, 2012).

Application timing and choice of fungicides has a significant impact on the efficacy of Sclerotinia stem rot control as well as high frequencies of fungicide resistant strains in a field population of *S. sclerotiorum* as reported for dicarboximides or benzimidazoles (Kaczmar et al. 2000). The first field isolate carrying resistance to Succinate Dehydrogenase Inhibitor (SDHI) fungicides conferred by a mutation in subunit D of the *sdh*-genes was reported by Stammler et al. (2011). In the following years, further mutations in field isolates were reported, but their frequency remained on a low level in all countries with the exception of France (FRAC, 2018; Walker et al. 2015). No cases of field resistance were reported in *S. sclerotiorum* for neither Quinone outside Inhibitor (QoI) fungicides nor for Demethylation Inhibitor (DMI) fungicides (FRAC, 2018; Walker et al. 2015).

In this work following experiments were conducted:

- *in vitro* SDHI cross-resistance tests with molecular biologically characterized genotypes
- *in vivo* studies to evaluate the impact of genotypes on efficacy of SDHI containing products

MATERIAL AND METHODS

SDHI cross-resistance study

Isolates with an EC_{50} value of more than 1 mg/l of boscalid or fluopyram were chosen from those isolates tested as part of the resistance monitoring studies conducted by Bayer AG from 2016 to 2018 as reported in FRAC (2018). DNA was extracted and *sdh*-genes were sequenced to identify single nucleotide polymorphisms (SNPs). Subsequently, the identified genotypes were tested together with ten isolates showing wild-type sensitivity in a microtiter assay as described by Hu et al. (2011) by using mycelium suspensions as inoculum as also described below. Two replicates were tested per isolate. EC_{50} values were calculated from the blank-corrected extinction values using ABASE software. The experiment was repeated twice.

Detached leaf assay

One isolate per SNP as detected in the SDHI cross-resistance study was selected for the detached leaf assay. Mycelium plugs of five isolates (four genotypes and a wild-type isolate) were transferred into falcon tubes containing 10 mL of Potato Dextrose Broth and incubated for 4 days in the dark while shaking at 120 rpm. Subsequently, mycelium suspensions were prepared by blending the liquid cultures using an ultra-thorax blender and adjusted to an optical density of 0.1 at 600 nm at the day of inoculation.

Winter oilseed rape plants (*Brassica napus*, var. PR46 W15 Z) were grown in a greenhouse at 15°C until growth stage four, showing fully expanded leaves. Subsequently, plants were treated with commercial fungicidal products as listed in Table 1 at a water rate of 300 l/ha in a calibrated spray cabin.

After further 24 hours in the greenhouse, mature leaves were cut and each two leaves were placed into one humid box lined with moist tissue. For inoculation, small filter paper discs were

soaked in mycelium suspension and two discs were placed upside down onto the adaxial surface of both halves of each leaf. Humid boxes were incubated at 20°C for four days in the dark. Lesion area was calculated as an ellipse based on measurement of two diameters in a 90° angle and subtracting the area of the filter paper discs. The mean of two lesions per leaf were recorded for six leafs per isolate and treatment. Results for each genotype were analyzed using one-factorial ANOVA and a posteriori Tukey's HSD test ($p \leq 0.05$). The efficacy expressed as percent ABBOTT was calculated for each product by using means of treatments and comparing it to the untreated control. The experiment was repeated twice.

Table 1 SDHI containing products used for protective treatment of oilseed rape plants applied one day prior to infection at 300 l/ha in a calibrated spray cabin.

Product name	Product rate	Fungicide(s)	Fungicide rate(s)
Cantus® WG 500 g/kg	0.5 kg/ha	Boscalid	250 g/ha
Seguris® EC 125 g/L	1.6 l/ha	Isopyrazam	200 g/ha
FLU SC 500 g/L	0.25 l /ha	Fluopyram	125 g/ha
Symetra® SC 200+125 g/L	1 l/ha	Isopyrazam + Azoxystrobin	200 g/ha + 125 g/ha
Propulse® SE 125+125 g/L	1 l/ha	Fluopyram + Prothioconazole	125 g/ha + 125 g/ha

RESULTS AND DISCUSSION

SDHI cross-resistance study

The *sdh*-genes of isolates with an individual EC_{50} of more than 1 mg/l of boscalid and/or fluopyram were sequenced and following SNPs were identified:

*sdh*B-H273Y, *sdh*C-G91R, *sdh*C-H146R and *sdh*D-H132R (Table 2).

With exception of *sdh*C-G91R, all genotypes were already reported in *S. sclerotiorum* by other researchers (FRAC, 2018; Walker et al. 2015). Genotype *sdh*C-G91R was reported in *Sclerotinia homoeocarpa* field isolates (Popko et al. 2018), but first time reported in *S. sclerotiorum* in this study. To characterize the impact of identified genotypes on the activity of SDHI fungicides, in total seven SDHI fungicides from different chemical classes were tested in an *in vitro* cross-resistance study.

The pyridine-carboxamide boscalid showed resistance factors of more than 300 for all genotypes studied, indicating strong *in vitro* resistance (Table 2). Interestingly, genotype *sdh*B-H273Y showed high sensitivity to fluopyram, whereas all pyrazole-4-carboxamides, such as bixafen, as well as the N-methoxy-(phenyl-ethyl)-pyrazole-carboxamide pydiflumetofen showed moderate resistance factors. For all other genotypes, low to moderate resistance factors were measured for the tested SDHI fungicides dependent on the chemical group and SNPs tested (Table 2). Such an incomplete cross-resistance between SDHIs studied *in vitro* was reported for *S. sclerotiorum* by Walker et al. (2015) and for other plant pathogens by Sierotzki & Scalliet (2013).

Table 2 Mean resistance factors of four genotypes of *S. sclerotiorum* towards seven different SDHI fungicides, determined in an *in vitro* cross-resistance study in relation to the mEC_{50} value of wild-type isolates.

		mean Resistance Factor [mEC_{50} mutation / mEC_{50} wildtype]						
		pyridine-carboxamide	pyridinyl-ethyl-benzamide	pyrazole-4-carboxamides				N-methoxy-(phenyl-ethyl)-pyraz.-carboxa.
Genotypes	n	Boscalid	Fluopyram	Bixafen	Benzovin-diflupyr	Isopyra-zam	Fluxa-pyroxad	Pydiflumetofen
B-H273Y	4	>568*	0.5	33	25	27	34	20
C-G91R	2	>442	11	2	8	7	10	4
C-H146R	4	>426	15	24	15	16	20	3
D-H132R	4	>352	9	12	4	4	6	6
mEC_{50} value of wild-type	10	0.05	0.07	0.06	0.11	0.25	0.08	0.04

* The highest concentration tested in the microtiter assay was 30 mg/l of active ingredient. In case the EC_{50} value of at least one isolates was higher than 30 mg/l, the mean Resistance Factor caused by the genotype is marked by the ">"-sign.

In vivo efficacy of SDHI-containing products

However, detection of mutations or *in vitro* measurement of increased EC_{50} values do not necessarily lead to field resistance. Therefore, *in vivo* studies were conducted to determine the impact of the described genotypes on the efficacy of a protective application of commercially available product concepts at their recommended field rates and comparably realistic spray conditions one day prior to inoculation in the greenhouse.

In the greenhouse, lesion size on untreated leaves was not significantly different between genotypes, therefore an interaction between aggressiveness of isolates and efficacy of products is improbable. A clear loss of efficacy of the full field rate of the solo boscalid containing product was observed for all genotypes tested compared to an efficacy of 94% in controlling the wild-type isolate (Figure 1). Thus, this result nicely demonstrates the impact of high resistance factors observed in the *in vitro* cross-resistance study (Table 2). Consequently, strong occurrence of such genotypes may lead to a loss of efficacy under field conditions, as observed for other genotypes showing a qualitative resistance phenotype with similarly high resistance factors (Kaczmar et al. 2000).

The application of a product containing solo isopyrazam showed a non-significant decrease in efficacy from 98% for the wild-type isolate to 79-89% for the tested genotypes, which reveals the impact of low to moderate resistance factors. The protective efficacy was increased to 87-98% by adding azoxystrobin in the product concept Symetra®. These findings highlight the benefit of using mixtures with non-cross-resistant partners (Figure 1). However, due to the generally lower *in situ* efficacy of QoIs for controlling *S. sclerotiorum*, especially in case of curative application timing (Bradley et al. 2006; Spitzer et al. 2017), it did not reach the level of control as observed for the wild-type (99%), where both mixture partners show full efficacy.

The solo application of fluopyram showed high efficacy in controlling genotype *sdh*B-H273Y, similar to the level of control of the wild-type isolate (98% vs. 99%). A significant decrease in efficacy to 50% was observed for genotype *sdh*C-H146R, which probably will have an impact on the field efficacy of a solo application of fluopyram. The other genotypes, *sdh*C-G91R and *sdh*D-H132R, showed a non-significant decrease in efficacy to 75% and 77%, respectively. However, in the mixture concept Propulse®, containing the DMI fungicide prothioconazole, the efficacy in controlling the tested genotypes was restored with 98-99% to the same level as observed for the wild-type (99%), thus fully controlling all genotypes detected in the monitoring program conducted in the last years.

These results highlight the need for product concepts containing SDHIs with a non-cross-resistant mixture partner being able to sufficiently control the disease on its own, as recommended in the resistance management guidelines annually published by the FRAC SDHI Working Group (FRAC, 2018). Field results as well as first results of *in vivo* studies indicate, that especially products concepts containing QoIs could lead to a lower efficacy in a curative application timing (Bradley et al. 2006; Spitzer et al. 2017). Therefore, further research is needed to elucidate the impact of application timing of different modes of action on different developmental stages of *S. sclerotiorum* during the infection process. These findings, together with disease prediction models for *S. sclerotiorum* infections as well as biocontrol agents, such as *Coniothyrium minitans* (Koch et al. 2007), can be utilized in integrated Sclerotinia stem rot disease- and effective fungicide resistance management programs to keep a sufficient number of effective disease management tools available for farmers in the future.

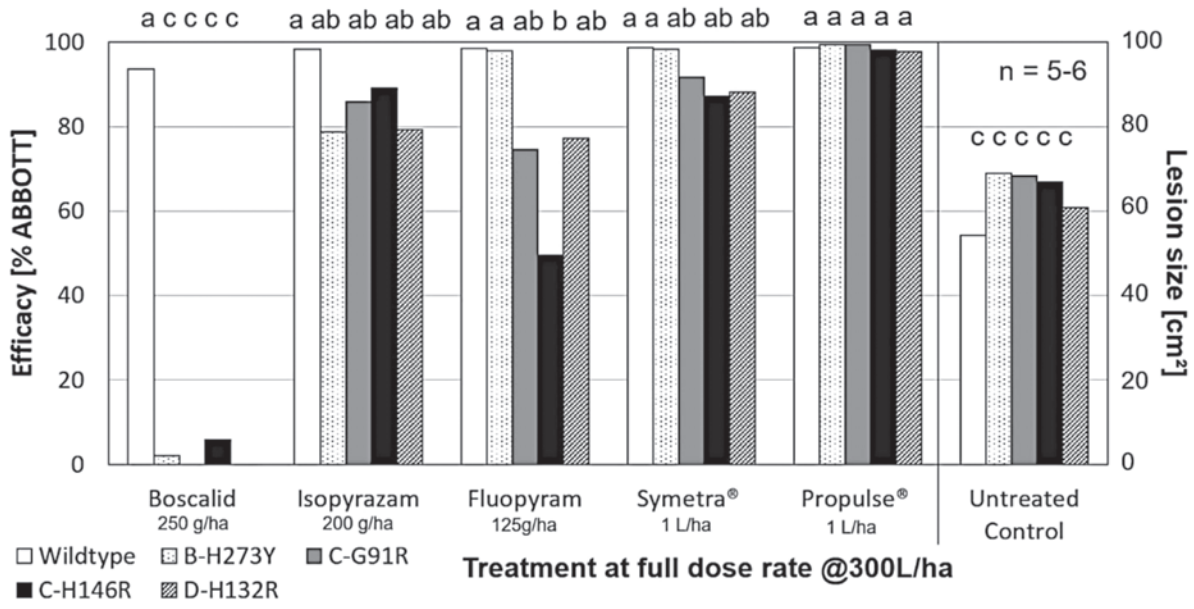


Figure 1 Efficacy expressed as % ABBOTT of different solo SDHIs and SDHI containing products in controlling four genotypes of *S. sclerotiorum* carrying different mutations in *sdh*-genes and the wild-type tested in a detached leaf assay. Lesion size of the isolates tested is given for the untreated control. Different letters indicate significant differences between treatments of one genotype according to Tukey's HSD test.

REFERENCES

Bradley CA; Lamey HA; Endres GJ; Henson RA; Hanson BK; McKay KR; Halvorson M; LeGare DG; Porter PM (2006). Efficacy of fungicides for control of *Sclerotinia* stem rot of Canola. *Plant Disease* 90, 1129-1134.

Dunker S; von Tiedemann A (2004). Disease/yield loss analysis for *Sclerotinia* stem rot in winter oilseed rape. In: *IOBC Bulletin 'Integrated Control in Oilseed Crops* 27, 59-66.

FRAC (2018). Minutes of the 2018 SDHI Working Group meeting in December and Telefone Conferences in March/June 2019 with Recommendations for 2019. In: *www.frac.info*

Hu MJ; Luo CX; Grabke A; Schnabel G (2011). Selection of a Suitable Medium to Determine Sensitivity of *Monilinia fructicola* Mycelium to SDHI Fungicides. *Journal of Phytopathology* 159, 616-620.

Kaczmar MJ; Wilson V; Leroux P (2000). Sclérotiniose du colza, le carbendazime en sursis. *Phytoma-LDV* 529, 31-33.

Koch S; Dunker S; Kleinhenz B; Röhrig M; von Tiedemann A (2007). A crop loss-related forecasting model for *Sclerotinia* stem rot in winter oilseed rape. *Phytopathology* 97,1186-1194.

Popko JT; Sang H; Lee J; Yamada T; Hoshino Y; Jung G (2018). Resistance of *Sclerotinia homoeocarpa* Field Isolates to Succinate Dehydrogenase Inhibitor Fungicides. *Plant Disease* 102, 2625-2631.

Sierotzki H; Scalliet G (2013). A Review of Current Knowledge of Resistance Aspects for the Next-Generation SDH Inhibitor Fungicides. *Phytopathology*103, 880-887.

Stammler, G; Glättli, A; Koch, A; Schlehuber, S (2011). Mutations in the target protein conferring resistance to SDHI fungicides. In: *Modern fungicides and antifungal compounds VI. Proceedings of the 16th International Reinhardsbrunn Symposium*, eds. HW Dehne; HB Deising; U Gisi; KH Kuck; PE Russell & H Lyr, pp. 195–198. DPG Selbstverlag: Braunschweig, Germany.

Spitzer T; Bílovský J; Kazda J (2017). Effect of Fungicide Application Timing on *S. sclerotiorum* Infection Rate and Yield inWinter Oilseed Rape. *Gesunde Pflanzen* 69, 67-72.

Walker AS; Auclair C; Remuson F; Micoud A; Moinard J; Carpezat J; Leflon M; Penaud A (2015). Statut de la Resistance aux SDHIs des Populations Francaises de *Sclerotinia sclerotiorum* et caracterisation des Souches Resistantes. In: *AFPP-Onzieme conference international sur les maladies des plantes tour*s *2015*. pp. 355-364.

Young CS; Werner CP; (2012). Infection routes for *Sclerotinia sclerotiorum* in apetalous and fully petalled winter oilseed rape. *Plant Pathology* 61, 730-738.

Gazzetti K et al., Sequence and properties of the draft genome of *Stemphylium vesicarium*. In: Deising HB; Fraaije B; Mehl A; Oerke EC; Sierotzki H; Stammler G (Eds), "Modern Fungicides and Antifungal Compounds", Vol. IX, pp. 187-188. © 2020. Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-16-7

Sequence and properties of the draft genome of *Stemphylium vesicarium*

Gazzetti K[1], Diaconu EL[2], Nanni IM[1], Ciriani A[1], Collina M[1]

[1]*Uni. of Bologna, Dep.of Agricult. and Food Science, Viale G. Fanin 42, 40127 Bologna, Italy*

[2] *Bio-Fab Research srl – Via Mario Beltrami, 5 – 00135 Roma*

Corresponding Author Email: marina.collina@unibo.it

ABSTRACT

Stemphylium vesicarium is the causal agent of several plant diseases as Brown Spot of Pear (BSP), which is one of the most economically important fungal diseases in European pear-production areas. Here the resource and the properties of the genome sequence of the *S. vesicarium* strain 173-1a13FI1M3 isolated from pear is announced. The strain is sensitive to the most used fungicide classes currently authorized in Europe against BSP. The availability of this draft genome could represent a further important step in elucidating the physiology and the infection mechanisms of this pathogen. Furthermore, this contribution helps indirectly improving integrated control of BSP and of other plant diseases and the investigation of *S. vesicarium* as a potential allergenic fungus in humans.

INTRODUCTION

Stemphylium vesicarium (Wallr.) E. Simmons can cause disease of non-crop plants, herbaceous crops, and fruit trees. BSP mainly starting from conidial airborn inoculum, can damage pear twigs, leaves and fruits, and many fungicide applications are thus required from petal fall to fruit ripening to protect orchards. Moreover, conidia widespread from plant material infected by the pathogen can trigger respiratory allergy. The aim of this work is to provide the first available genome sequence of *S. vesicarium*, accompanied by genome properties.

MATERIAL AND METHODS

The monoconidial *S. vesicarium* strain 173-1a13FI1M3 (CBS-KNAW accession number: CBS 45331) was isolated in August 2013 from Altedo (Emilia-Romagna region, Italy), from leaves of pear (cv. Abate Fétel) affected by BSP. The strain was sensitive to dicarboximides, fludioxonil, strobilurins and SDHI fungicides. The following steps were performed as described in Gazzetti et al. 2019: gDNA purification, Illumina shotgun sequencing, *de novo* assembly, gene finding, primary functional annotation, combined prediction of transmembrane topology and signal peptide, identification of biosynthetic gene clusters and search for possible orthologues of pathogenicity genes. Manual annotation was carried out by using DNAMAN v4.15 and NCBI BLAST, Clustal Omega and FGENESH free tools.

RESULTS

1,127 contigs were assembled, obtaining a total assembly length of 38.66 Mb. The genome sequence has been deposited in the GenBank under the accession number: QXCR00000000 (BioProject: PRJNA470620, BioSample: SAMN09098503). The gene finding process led to predict 12,309 genes. The functional annotation provided information about Orthologous Groups, Gene Ontology terms (GO terms), KEGG pathways, and SMART/Pfam domains for each group. Among predicted genes it was possible to identify orthologs of genes belonging to other plant pathogenic Ascomycota and required to carry out different functions (Table 1).

Table 1. Number of predicted genes considered, related to pathogenesis or full virulence on plants, host cell wall degradation, components of signaling and secondary metabolism pathways, fungicide resistance, trigging of human allergy.

Function	Number
CAZymes	521
Secreted CAZymes (GO 0005576)	77
Secondary metabolism	553
Signal transduction	444
Pathogenesis (GO 0009405)	40
Fungicide resistance (manually annotated)	4
Orthologs of Alternaria spp. allergens (manually annotated)	4

CONCLUSIONS

The genome sequence of the *S.* vesicarium strain 173-1a13FI1M3 has a similar size to those of *Stemphylium lycopersici* (Franco et al., 2015). The announced genome sequence is the second currently available for the *Stemphilium* genus, and its knowledge opens a new scenario in the investigation of fungal lifestyle, epidemiology and molecular plant/pathogen interaction. Moreover, it could be used in comparative studies, and as reference genome to discover mutations in fungicide target genes of resistant strains. This resource could be fundamental in order to design more effective and sustainable fungicide management strategies to control the disease on pear and on other hosts and make it possible to investigate *S. vesicarium* as a potential allergenic fungus in humans.

REFERENCES

Franco MEE; López S; Medina R; Saparrat MCN; Pedro Balatti P (2015). Draft Genome Sequence and Gene Annotation of *Stemphylium lycopersici* Strain CIDEFI-216. *Genome Announcements*. 3: e01069-15

Gazzetti K, Diaconu EL; Nanni IM; Ciriani A, Collina M (2019). Genome Sequence Resource for *Stemphylium vesicarium*, causing Brown Spot Disease of pear. *Molecular Plant-Microbe Interactions*. https://doi.org/10.1094/MPMI-11-18-0299-A

Mair W et al., The 2019 update to the unified nomenclature of target site mutations associated with resistance to fungicides. Volume. In: Deising HB; Fraaije B; Mehl A; Oerke EC; Sierotzki H; Stammler G (Eds), "Modern Fungicides and Antifungal Compounds", Vol. IX, pp. 189-198. © 2020. Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-16-7

The 2019 update to the unified nomenclature of target site mutations associated with resistance to fungicides

Mair W, Lopez-Ruiz F, Oliver, RP

Centre for Crop Disease Management, Curtin University, Bentley, WA 6102, Australia
Richard.oliver@curtin.edu.au

INTRODUCTION

Many cases of fungicide resistance are due to target site mutations affecting orthologous amino acids selected by fungicides with the same mode of action in different species. As the orthologous amino acids often have different numbers due to differences in the length of the protein, we proposed a system to unify the numbering by alignment to an archetype sequence. All changes are given an italicised "label" based on the number of the amino acid in the archetype. This system was published in 2016 and updated in the 2017 Reinhardsbrunn proceedings (Lopez-Ruiz et al., 2017; Mair et al., 2016a). We present here a further update using new information available in 2019. The tables here list the new species and the labels assigned to different amino acid changes. The complete table and alignments are available upon request to the authors.

TABLES

Table 1. CytB
Position number based on alignment to reference sequence from *Zymoseptoria tritici* (NCBI gene accession number AY247413).

Substitution in archetype	Homologous position in other species	Reference
G37V	NA	(Fehr et al., 2016)
F129L	F129L in COLLAC	(Forcelini et al., 2016)
	F129L in FULVFU	(Watanabe et al., 2017)
G137	G137R in PHYTCP	(Ma et al., 2018)
	G137S in CLADCA	(Standish et al., 2016)
G143A	G143A in COLLAC	(Forcelini et al., 2016)
	G143A in PODOXA	(Vielba-Fernandez et al., 2018)
D203-ins-T204	E203-insDE-V204 in PLASVI	(Mounkoro et al., 2018)

Table 2. Cyp51A

Position number based on alignment to reference sequence from *Aspergillus fumigatus* (Cyp51A) (NCBI gene accession number AF338659).

Substitution in archetype	Homologous position in other species	Reference
E105	E103Q in GIBBZE	(Duan et al., 2018)
L159	V157L in GIBBZE	(Duan et al., 2018)
M172I	NA	(Zhang et al., 2017a)
L206	L208Y in COLLDU	(Chen et al., 2018)
H236	H238R in COLLDU	(Chen et al., 2018)
D245	D243N in GIBBZE	(Duan et al., 2018)
S299	S302A in COLLDU	(Chen et al., 2018)
I367	I366L in COLLDU	(Chen et al., 2018)
G448S	G443S in GIBBZE	(Duan et al., 2018)
F495I	F489L in PYRNTE	(Mair et al., 2016b)

Table 3 Cyp51B

Position number based on alignment to reference sequence from *Zymoseptoria tritici* (NCBI gene accession number AY253234).

Substitution in archetype	Homologous position in other species	Reference
L110	F105L in CANDAL	(Keniya et al., 2018)
G112	A107T in CANDAL	(Keniya et al., 2018)
A119	A114S in CANDAL	(Keniya et al., 2018)
Y123	Y118A in CANDAL	(Keniya et al., 2018)
	Y123H in FUSAVR	(Fan et al., 2014)
F131	F126S in CANDAL	(Keniya et al., 2018)
Y137F	Y137H in GIBBZE	(Qian et al., 2018)
	Y137H in USTNVI	(Wang et al., 2015a)
	Y144F/H in LEPTNO	(Pereira et al., 2017)
K148	K143E/R/Q in CANDAL	(Keniya et al., 2018)
T234	T229A in CANDAL	(Keniya et al., 2018)
M258	I253V in CANDAL	(Keniya et al., 2018)
Q287	H283D in CANDAL	(Keniya et al., 2018)
A311G	G307S in CANDAL	(Keniya et al., 2018)
H382	H373N in COLLDU	(Chen et al., 2018)

L385	F380S in CANDAL	(Keniya et al., 2018)
	M376L in COLLDU	(Chen et al., 2018)
Y461D/H/S	F449V/M/S in CANDAL	(Keniya et al., 2018)
	Y464S in CERCBE	(Trkulja et al., 2017)
G476S	G461S in MONIFC	(Lichtemberg et al., 2017)
S524T	S511T in COLLDU	(Chen et al., 2018)

Table 4. *b*-Tubulin

Position number based on alignment to reference sequence from *Aspergillus nidulans* (benA) (NCBI gene accession number M17519).

Substitution in archetype	Homologous position in other species	Reference
N52	A52G in FUSAME	(Zhang et al., 2018)
Q134K	Q134L in FUSAVR β_2-tubulin	(Xu et al., 2019)
M163	M163I in CORYCA	(Duan et al., 2019)
F167	F167Y in ALTEAL	(Wang and Zhang, 2018)
	F167Y in CORYCA	(Duan et al., 2019)
E198D/K/Q	E198A in CORYCA	(Duan et al., 2019)
F200Y	F200Y in SCLEHO	(Hu et al., 2018)
	F200S in CORYCA	(Duan et al., 2019)
M233	M233I in BOTRCI	(Cai et al., 2015)
C239	C239S in PHYTMS	(Cai et al., 2016)
L240	L240F in SCLESC	(Lehner et al., 2015)
S351	T351I in BOTRCI	(Adnan et al., 2018)
	T351I in FUSAPF β_1-tubulin	(Xu et al., 2019)

Table 5. SdhB

Position number based on alignment to reference sequence from *Pyrenophora teres* f. sp. *teres* (NCBI gene accession number XM_003302513).

Substitution in archetype	Homologous position in other species	Reference
A11	A11V in SCLESC	(Wang et al., 2015b)
S228	S217L in RAMUCC	(Piotrowska et al., 2017)
N235	N224I in RAMUCC	(Piotrowska et al., 2017)
R275	R265P in SEPTTR	(FRAC, 2018)
H277Y	H245L in PYRIOR	(Guo et al., 2016)

	H246Y in RHIZCE	(Sun et al., 2017)
	H272R/Y in PENIEX	(Malandrakis et al., 2017)
T278	T253I in VENTIN	(Huf et al., 2017)
	T268A/I in SEPTTR	(FRAC, 2018)

Table 6. SdhC

Position number based on alignment to reference sequence from *Pyrenophora teres* f. sp. *teres* (NCBI gene accession number XM_003302752).

Substitution in archetype	**Homologous position in other species**	**Reference**
S73P	S73P in CORYCA	(Rehfus, 2018)
	A84V in SEPTTR	
	A85V in BOTRCI	
N75S	N86A/K/S in SEPTTR	(Rehfus et al., 2018)
	N87S in RAMUCC	(FRAC, 2018)
G79R	G91R in RAMUCC	(FRAC, 2018)
H134R	H134R in ALTESO	(Mallik et al., 2013)
	H139Y in RHIZCE	(Sun et al., 2017)
	H146L/R in RAMUCC	(FRAC, 2018)
S135R	S135R in ALTEAL	(Sierotzki et al., 2011)
R140	R151M/S/T in SEPTTR	(FRAC, 2018)
H141	H151R in VENTIN	(Huf et al., 2017)
	H153R in RAMUCC	(FRAC, 2018)
F150	I161S in SEPTTR	(Kirikyali et al., 2017)
V155	V166M in SEPTTR	(Rehfus et al., 2018)
G159	G169D/S in UNCINE	(Graf, 2018)
	G171D in RAMUCC	(FRAC, 2018)

Table 7. SdhD

Position number based on alignment to reference sequence from *Pyrenophora teres* f. sp. *teres* (NCBI gene accession number XM_003297196).

Substitution in archetype	**Homologous position in other species**	**Reference**
I66	I50F in SEPTTR	(FRAC, 2018)
D124E/N	D123E in ALTESO	(Mallik et al., 2013)
L130	M114V in SEPTTR	(FRAC, 2018)

H134R	H116D/Y in RHIZCE	(Sun et al., 2017)
	H133Q/R in ALTEAL	(Malandrakis et al., 2018)
H136	H118Y in RHIZCE	(Sun et al., 2017)
G138V	G109V in CORYCA	(FRAC, 2018)
F147	L129I in RHIZCE	(Sun et al., 2017)
E178K	NA	(Rehfus et al., 2016)

Table 8. OS-1

Position number based on alignment to reference sequence from *Botrytis cinerea* (Bos1) (NCBI gene accession number AF435964).

Substitution in archetype	Homologous position in other species	Reference
F127S	NA	(Grabke et al., 2013)
E253D	NA	(Ren et al., 2016)
G262S	NA	(Ren et al., 2016)
G265D	NA	(Ren et al., 2016)
G311R	NA	(Ren et al., 2016)
R319K	NA	(Ren et al., 2016)
V336M	NA	(Ren et al., 2016)
D337N	NA	(Ren et al., 2016)
V346I	NA	(Ren et al., 2016)
A350S	NA	(Ren et al., 2016)
I365N/R/S	I366N in SCLEHO	(Sang et al., 2016)
S426P	NA	(Sang et al., 2018)
V346I	NA	(Ren et al., 2016)
G538R	NA	(Sang et al., 2018)
G545E	NA	(Ren et al., 2016)
N609T	NA	(Ren et al., 2016)
A1259T	NA	(Sang et al., 2018)

Table 9. Oxysterol-binding protein (OSBP)-Related Proteins (ORP)

Position number based on alignment to reference sequence from *Phytophthora infestans* PiORP1 (NCBI gene accession number XP_002902250.1).

Substitution in archetype	Homologous position in other species	Reference
G770A/I/L/P/V	G686V in PHYTNN	(Bittner et al., 2017)

Table 10. Myosin-5

Position number based on alignment to reference sequence from *Fusarium graminearum* (NCBI gene accession number XP_011317208).

Substitution in archetype	Homologous position in other species	Reference
V151	V151A in FUSAOX	(Zheng et al., 2018)
S175	S175L in FUSAME	(Zhang et al., 2018)
K216E/H	K216E/R in FUSAAZ	(Zhang et al., 2017b)
S217L/P/R	S217L/P in FUSAAZ	(Zhang et al., 2017b)
	S219L/P in GIBBFU	(Hou et al., 2018)
S418R	S418T in FUSAOX	(Zheng et al., 2018)
E420G/S	E420G/D in FUSAAZ	(Zhang et al., 2017b)

Table 11. Abbreviations of Species' Names

Abbreviation (EPPO code)	Name of pathogen
ALTEAL	*Alternaria alternata*
ALTESO	*Alternaria solani*
BOTRCI	*Botrytis cinerea*
CANDAL	*Candida albicans*
CERCBE	*Cercospora beticola*
CLADCA	*Venturia effusa*
COLLAC	*Colletotrichum acutatum*
COLLDU	*Colletotrichum truncatum*
CORYCA	*Corynespora cassiicola*
FULVFU	*Cladosporium fulvum*
FUSAAZ	*Fusarium asiaticum*
FUSAME	*Fusarium oxysporum* f. sp. *melonis*
FUSAOX	*Fusarium oxysporum*
FUSAPF	*Fusarium proliferatum*
FUSAVR	*Fusarium verticillioides*
GIBBFU	*Fusarium fujikuroi*
GIBBZE	*Fusarium graminearum*
LEPTNO	*Parastagonospora nodorum*
MONIFC	*Monilinia fructicola*
PENIEX	*Penicillium expansum*

PHYTCP	*Phytophthora capsici*
PHYTMS	*Phytophthora sojae*
PHYTNN	*Phytophthora nicotianae* var. *nicotianae*
PLASVI	*Plasmopara viticola*
PODOXA	*Podosphaera xanthii*
PYRNTE	*Pyrenophora teres* f. sp. *teres*
PYRIOR	*Magnaporthe grisea*
RAMUCC	*Ramularia collo-cygni*
RHIZCE	*Rhizoctonia cerealis*
SCLEHO	*Sclerotinia homoeocarpa*
SCLESC	*Sclerotinia sclerotiorum*
SEPTTR	*Zymoseptoria tritici*
UNCINE	*Erysiphe necator*
USTNVI	*Villosiclava virens*
VENTIN	*Venturia inaequalis*

ACKNOWLEDGEMENTS

Research in the authors' laboratory is funded by the GRDC (CUR00023), GWRDC and Curtin University.

REFERENCES

Adnan M., et al. (2018). Detection and Molecular Characterization of Resistance to the Dicarboximide and Benzamide Fungicides in Botrytis cinerea From Tomato in Hubei Province, China. *Plant Disease* 102, 1299-1306.

Bittner R. J., et al. (2017). Assessing the resistance potential of Phytophthora nicotianae, the causal agent of black shank of tobacco, to oxathiopropalin with laboratory mutants. *Crop Protection* 102, 63-71.

Cai M., et al. (2015). M233I Mutation in the β-Tubulin of Botrytis cinerea Confers Resistance to Zoxamide. *Scientific reports* 5.

Cai M., et al. (2016). C239S Mutation in the β-Tubulin of Phytophthora sojae Confers Resistance to Zoxamide. *Frontiers in microbiology* 7.

Chen S., et al. (2018). Inherent Resistance to 14α-Demethylation Inhibitor Fungicides in Colletotrichum truncatum Is Likely Linked to CYP51A and/or CYP51B Gene Variants. *Phytopathology* PHYTO-02-18-0054-R.

Duan Y., et al. (2018). Molecular and biological characteristics of laboratory metconazole-resistant mutants in Fusarium graminearum. *Pesticide Biochemistry and Physiology* 152, pp.55-61

Duan Y., et al. (2019). Benzimidazole- and QoI-resistance in Corynespora cassiicola populations from greenhouse-cultivated cucumber: An emerging problem in China. *Pestic Biochem Physiol* 153, 95-105.

Fan J., et al. (2014). The Y123H substitution perturbs FvCYP51B function and confers prochloraz resistance in laboratory mutants of Fusarium verticillioides. *Plant Pathology* 63, 952-960.

Fehr M., et al. (2016). Binding of the respiratory chain inhibitor ametoctradin to the mitochondrial bc1 complex. *Pest Management Science* 72, 591-602.

Forcelini B.B., et al. (2016). Resistance in Strawberry Isolates of Colletotrichum acutatum from Florida to Quinone-Outside Inhibitor Fungicides. *Plant Disease* 100, 2050-2056.

FRAC, Succinate Dehydrogenase Inhibitor (SDHI) Working Group 10th Meeting on December 13 (2017). Fungicide Resistance Action Committee (FRAC) (2018).

Grabke A., et al. (2013). Characterization of Iprodione Resistance in Botrytis cinerea from Strawberry and Blackberry. *Phytopathology* 104, 396-402.

Graf S., Characterisation of metrafenone and succinate dehydrogenase inhibitor resistant isolates of the grapevine powdery mildew Erysiphe necator. (2018). PhD Dissertaion, Technische Universität Kaiserslautern, Germany; Permanent Identifyer: urn:nbn:de:hbz:386-kluedo-51233; https://kluedo.ub.uni-kl.de/frontdoor/index/index/docId/5123

Guo M., et al. (2016). Development of a novel strategy for fungal transformation based on a mutant locus conferring carboxin-resistance in Magnaporthe oryzae. *AMB Express* 6, 57.

Hou Y.-P., et al. (2018). Resistance mechanism of Fusarium fujikuroi to phenamacril in the field. *Pest Management Science* 74, 607-616.

Hu J., et al. (2018). Thiophanate-methyl resistance in Sclerotinia homoeocarpa from golf courses in China. *Pesticide Biochemistry and Physiology*.

Huf A., et al., Sensitivity of Venturia inaequalis to Fungicides (2017). In: H. Deising, et al., Eds.), *Modern Fungicides and Antifungal Compounds*, Vol. VIII. Deutsche Phytomedizinische Gesellschaft Braunschweig, pp. 197-198.

Keniya M. V., et al. (2018). Crystal Structures of Full-Length Lanosterol 14alpha-Demethylases of Prominent Fungal Pathogens Candida albicans and Candida glabrata Provide Tools for Antifungal Discovery. *Antimicrob Agents Chemother* 62.

Kirikyali N., et al., Azole and SDHI sensitivity status of Zymoseptoria tritici field populations sampled in France, Germany and the UK during 2015 (2017). In: H. Deising, et al., Eds.), *Modern Fungicides and Antifungal Compounds,* Vol. VIII. Deutsche Phytomedizinische Gesellschaft, Braunschweig, pp. 153–158.

Lehner M., et al. (2015). Fungicide Sensitivity of Sclerotinia sclerotiorum: A Thorough Assessment Using Discriminatory Dose, EC50, High-Resolution Melting analysis, and Description of New Point Mutation Associated with Thiophanate-Methyl Resistance. *Plant Disease* 99, 1537-1543.

Lichtemberg P. S. F., et al. (2017). The Point Mutation G461S in the MfCYP51 Gene is Associated with Tebuconazole Resistance in Monilinia fructicola Populations in Brazil. *Phytopathology* 107, 1507-1514.

Lopez-Ruiz F., et al., Proposal for a unified nomenclature for target site mutations associated with resistance to fungicides; an update (2017). In: H. Deising, et al., Eds.), *Modern Fungicides and Antifungal Compounds. Braunschweig Vol VIII*, pp. 121-126.

Ma D., et al. (2018). Detection and Characterization of QoI-Resistant Phytophthora capsici Causing Pepper Phytophthora Blight in China. *Plant Dis.* 102, 1725-1732.

Mair W., et al. (2016a). Proposal for a unified nomenclature for target-site mutations associated with resistance to fungicides. *Pest Management Science* 72, 1449-1459.

Mair W. J., et al. (2016b). Demethylase Inhibitor Fungicide Resistance in Pyrenophora teres f. sp. teres Associated with Target Site Modification and Inducible Overexpression of Cyp51. *Front Microbiol.* 7, 1279.

Malandrakis A. A., et al. (2017). Characterization of boscalid-resistance conferring mutations in the SdhB subunit of respiratory complex II and impact on fitness and mycotoxin production in Penicillium expansum laboratory strains. *Pesticide Biochemistry and Physiology* 138, 97-103.

Malandrakis A. A., et al. (2018). Biological and molecular characterization of field isolates of Alternaria alternata with single or double resistance to respiratory complex II and III inhibitors. *European Journal of Plant Pathology* 152, 199-211.

Mallik I., et al. (2013). Molecular Characterization and Detection of Mutations Associated with Resistance to Succinate Dehydrogenase-Inhibiting Fungicides in Alternaria solani. *Phytopathology* 104, 40-49.

Mounkoro, P., et al. (2018). Mitochondrial complex III Qi -site inhibitor resistance mutations found in laboratory selected mutants and field isolates. *Pest Manag Sci.* 2019 Aug;75(8):2107-2114. doi: 10.1002/ps.5264. Epub 2018 Dec 24.

Pereira D. A., et al. (2017). Mutations in the CYP51 gene reduce DMI sensitivity in Parastagonospora nodorum populations in Europe and China. *Pest Management Science* 73, 1503-1510.

Piotrowska M. J., et al. (2017). Characterisation of Ramularia collo-cygni laboratory mutants resistant to succinate dehydrogenase inhibitors. *Pest Management Science* 73, 1187-1196.

Qian H., et al. (2018). The Y137H mutation in the cytochrome P450 FgCYP51B protein confers reduced sensitivity to tebuconazole in Fusarium graminearum. *Pest Management Science* 74, 1472-1477.

Rehfus A., Analysis of the emerging situation of resistance to succinate dehydrogenase inhibitors in Pyrenophora teres and Zymoseptoria tritici in Europe (2018). PhD Diss., Institut für Phytomedizin. Universität Hohenheim,Germany.

Rehfus, A., et al. (2016). Emergence of succinate dehydrogenase inhibitor resistance of Pyrenophora teres in Europe. *Pest Management Science* 72, 1977-1988.

Rehfus, A., et al. (2018). Mutations in sdh genes in field isolates of Zymoseptoria tritici and impact on the sensitivity to various succinate dehydrogenase inhibitors. *Plant Pathology* 67, 175-180.

Ren W., et al. (2016). Molecular and Biochemical Characterization of Laboratory and Field Mutants of Botrytis cinerea Resistant to Fludioxonil. *Plant Disease* 100, 1414-1423.

Sang C., et al. (2018). Detection and fitness comparison of target-based highly fludioxonil-resistant isolates of Botrytis cinerea from strawberry and cucumber in China. *Pesticide Biochemistry and Physiology* 147, 110-118.

Sang H., et al. (2016). Molecular Mechanisms Involved in Qualitative and Quantitative Resistance to the Dicarboximide Fungicide Iprodione in Sclerotinia homoeocarpa Field Isolates. *Phytopathology* 107, 198-207.

Sierotzki H., et al., Sensitivity of fungal pathogens to SDHI fungicides (2011). In: H. Dehne, et al., Eds., *Modern fungicides and antifungal compounds, Vol. VI*. Deutsche Phytomedizinische Gesellschaft, Braunschweig, 2011, pp. 179-186.

Standish J. R., et al. (2016). Location of an Intron in the Cytochrome b Gene Indicates Reduced Risk of QoI Fungicide Resistance in Fusicladium effusum. *Plant Disease* 100, 2294-2298.

Sun H. Y., et al. (2017). Homozygous and heterozygous point mutations in succinate dehydrogenase subunits b, c and d of Rhizoctonia cerealis conferring resistance to thifluzamide. *Pest Manag Sci.* 73, 896-903.

Trkulja N. R., et al. (2017). Molecular and experimental evidence of multi-resistance of Cercospora beticola field populations to MBC, DMI and QoI fungicides. *European Journal of Plant Pathology* 149, 895-910.

Vielba-Fernandez A., et al. (2018). Heteroplasmy for the Cytochrome b Gene in Podosphaera xanthii and its Role in Resistance to QoI Fungicides in Spain. *Plant Dis.* 102, 1599-1605.

Wang F., et al. (2015ª). The Y137H mutation of VvCYP51 gene confers the reduced sensitivity to tebuconazole in Villosiclava virens. *Scientific reports* 5.

Wang, Y., et al. (2015b). A new point mutation in the iron-sulfur subunit of succinate dehydrogenase confers resistance to boscalid in Sclerotinia sclerotiorum. *Mol Plant Pathol.* 16, 653-61.

Wang H. C., Zhang C. Q. (2018). Multi-resistance to thiophanate-methyl, diethofencarb, and procymidone among Alternaria alternata populations from tobacco plants, and the management of tobacco brown spot with azoxystrobin. *Phytoparasitica* 46, Issue 5, pp 677–687, https://doi.org/10.1007/s12600-018-0690-6

Watanabe H., et al. (2017). Occurrence of azoxystrobin-resistant isolates in Passalora fulva, the pathogen of tomato leaf mould disease. *Plant Pathology* 66, 1472-1479.

Xu S., et al. (2019). Molecular characterization of carbendazim resistance of Fusarium species complex that causes sugarcane pokkah boeng disease. *BMC Genomics* 20, 115.

Zhang J., et al. (2017a). A Novel Environmental Azole Resistance Mutation in Aspergillus fumigatus and a Possible Role of Sexual Reproduction in Its Emergence. *mBio. 8*, DOI: 10.1128/mBio.00791-17.

Zhang Y., et al. (2017b). Molecular, biological and physiological characterizations of resistance to phenamacril in Fusarium graminearum. *Plant Pathology* 66, 1404-1412.

Zhang Z., et al. (2018). Resistance risk assessment of Fusarium oxysporum f. sp. melonis against phenamacril, a myosin inhibitor. *Pesticide Biochemistry and Physiology* 147, 127-132.

Zheng, Z., et al. (2018). FoMyo5 motor domain substitutions (Val151 to Ala and Ser418 to Thr) cause natural resistance to fungicide phenamacril in Fusarium oxysporum. *Pesticide Biochemistry and Physiology* 147, 119-126.

RESISTANCE MODELLING

Garnault M et al., "Contrasted spatio-temporal dynamics of fungicide resistance and its drivers in the pathogenic fungus *Zymoseptoria tritici* in France", Vol. IX, pp. 201-206. © 2020. Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-16-7

Contrasted spatio-temporal dynamics of fungicide resistance and its drivers in the pathogenic fungus *Zymoseptoria tritici* in France

Garnault M[1,2], Duplaix C[1], Leroux P[1], Couleaud G[3], Carpentier F*[1,2], David O*[2], Walker A-S*[1]

[1]*UMR1290 BIOGER, INRA, AgroParisTech, Université Paris-Saclay, F-78850 Thiverval-Grignon, France*

[2]*MaIAGE, INRA, Université Paris-Saclay, 78350 Jouy-en-Josas, France*

[3]*Arvalis-Institut du Végétal, Boigneville, France* **authors with equal contribution*

Email: anne-sophie.walker@inra.fr

INTRODUCTION

Chemical control is a major tool in the integrated management of many plant diseases. Resistance selection (Delp & Dekker 1985) impedes the efficacy and useful lifetime of fungicides and then crop protection (Brent & Hollomon, 1995). In order to maintain high quality production and, in agreement with the social demand, limit pesticide use, an important challenge for plant protection is therefore to prevent the selection of resistant strains by adopting the most appropriate use strategies.

For *Zymoseptoria tritici*, the pathogen responsible for septoria tritici blotch (STB) on winter wheat, resistance occurred towards all unisite fungicide modes of action authorized in France (i.e. benzimidazoles, QoIs, DMIs and SDHIs). Resistance often results from target modification. Complementary mechanisms with weaker impact are also described, such as target overexpression (for DMIs) or enhanced efflux which leads to the multi-drug resistance phenotype (MDR), i.e. resistance to several modes of action (DMIs, QoIs, SDHIs for *Z. tritici*) (Omrane et *al.* 2017). The high diversity of resistance mechanisms, as well as the strong spatial and temporal heterogeneity of selection pressures, make it difficult to predict the dynamics of resistance in populations.

Our aim was to retrospectively understand the evolution of fungicide resistance in the French populations of *Z. tritici*. We analysed data issued from a longterm monitoring (13 years) that measured the frequencies of resistant strains in populations, over the French territory. First, we quantified the dynamics and heterogeneities observed at the national scale. Second, we highlighted drivers influencing significantly the evolution of resistance frequencies on large scales.

MATERIALS AND METHODS

Resistance frequency data was collected by the "Performance network". Between 2004 and 2017, this network coordinated by Arvalis-Institut du Végétal, conducted 1029 trials in France. In each trial, 30 to 40 upper leaves showing septoria symptoms were randomly collected in the

3-4 unsprayed repetitions and were soaked in sterile water to obtain a spore suspension. This suspension was placed in Petri dishes containing a solid medium amended with discriminating doses of fungicides. The respective doses were designed to determine strains specifically resistant to QoIs (StrR phenotype), or to DMIs (TriLR, TriMR, TriHR phenotype groups, differing according to their resistance factor between azoles and MDR phenotype; Leroux & Walker 2011). The proportion of each resistance phenotypes was estimated after the microscope observation of germ tube elongation after 48 hours of incubation.

For each region between 2004 and 2017, we gathered information about areas sprayed with anti-STB fungicides (data provided by Bayer CropScience's internal panels): 6 active ingredients (AIs) for QoIs and 9 for DMIs were considered. Yield losses induced by STB that were estimated from yield differences measured between sprayed (maximum control) and unsprayed plots cropped with STB-sensitive wheat cultivars (data provided by Arvalis-Institut du Végétal). Proportion of areas under organic wheat, considered as refuges without fungicide selection pressure, were provided by the AgenceBio (agencebio.org).

We estimated the effect of years and regions on resistance frequency using a zero-one-inflated logistic model (MODEL 1). We mapped yearly resistance frequency in France using ponctual observations of the network and a spatial kriging method (MODEL 2), on which we highlighted spatial heterogeneities using a spatial classification algorithm. We also developed a model (DYNAMIC MODEL) to quantify the evolution speed of resistance frequency. In a zero-one-inflated logistic framework, resistance frequency was explained by the initial resistance frequency, the national and regional growth rates and trial conditions (sampling date, wheat cultivar). The exponential growth rates could be interpreted as the relative fitness of the considered resistant phenotype *i.e.* its capacity to survive and to reproduce in the population (Hartl & Clark 1997). A complete description of these three models is given in Garnault et *al*. 2019.

Finally, the EXPLANATORY MODEL was developed to test the effect of (*i*) the selection pressure, (*ii*) the population size and (*iii*) the fraction of refuges, on resistance frequencies evolution at the regional scale. We used as proxy (*i*) the anti-STB fungicide sprayed areas, (*ii*) the yield losses induced by STB and (*iii*) the proportion of surfaces under organic wheat, respectively. This model was similar to the DYNAMIC MODEL, except that regional growth rate parameters were replaced by these three factors. Only the fungicide associated to a resistance was tested to explain the variation of its frequency (QoIs for the StrR phenotype and DMIs for TriR phenotypes). We used a variable selection procedure based on dummy variables to select an optimal set of explanatory variables. The weight of each factor showed its importance for frequencies prediction. It was computed from the sum of squared errors ratio between the model obtained by removing this factor from the full model and the full model.

All models were fitted for each resistance phenotype, in a bayesian framework except for MODEL 2 (estimated using a frequentist approach). Estimations were performed with R using the *rjags*, *spaMM* and *SPODT* packages respectively for the bayesian and frequentist modelling and the spatial partitioning.

RESULTS

Quantification of resistance dynamics

Resistance to QoIs, i.e. StrR phenotype, has been detected in French populations since 2002 and we estimated that its national frequency reached 90% in 2010. In response to the resistance evolution between 2002 and 2010, the use of this mode of action on wheat has decreased (Fig 1).

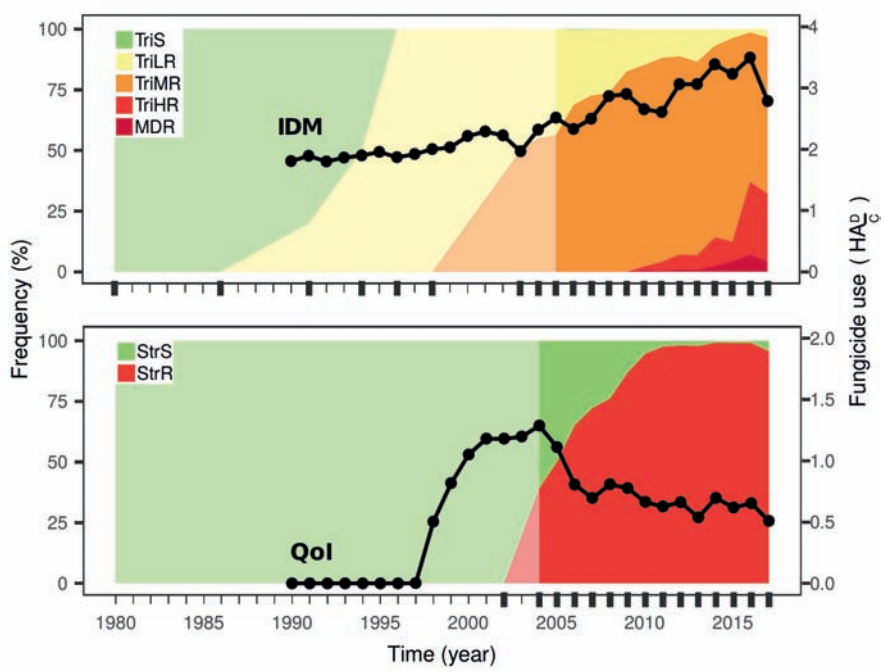


Figure 1. Co-evolution of fungicide uses and frequency of their associated resistance(s) in French populations of *Z. tritici*. Shaded areas: pre-Performance monitoring; Thick x-ticks: years of observation (extracted from Garnault et *al*., 2019).

We estimated that StrR individuals evolved twice as fast as susceptible individuals (Fig. 2). This was the fastest selection among all tested phenotypes. Strong spatial heterogeneities between northern regions (with high frequencies) and southern regions (with no resistance) were detected (Fig. 3A). The front progression speed from North to South was estimated to 145 km/year from 2004 to 2008. Furthermore, the selection of StrR strains was estimated to be significantly slower in northern regions (*e.g.* PIC, CHA) compared to southern regions (*e.g.* MPY, PCH).

DMI fungicides have been authorized in France since the late 1970s and their use is steadily increasing. In response, we observed a sucession of increasingly resistant TriR phenotypes: TriLR ("low" resistance), started to be replaced by TriMR ("moderate" resistance) in the early 2000s, themselves gradually being in a process of becoming replaced by TriHR ("high" resistance) and MDR phenotypes since 2008 (Fig. 1). The growth rates were estimated negative for TriLR, positive before 2011 and negative after for TriMR, positive and the highest among TriR for TriHR (Fig. 2), positive but not significant for MDR phenotypes. This was consistent with the gradual and widespread erosion of the effectiveness of triazoles in European countries, including France. We identified a stable time spatial structure between 2006 and 2013. TriR6 strains were significantly more frequent in the northern regions (Fig. 3B), while the TriR7-TriR8

strains (which remain sensitive to prochloraz) were present with higher frequencies in southwestern regions (Fig. 3C).

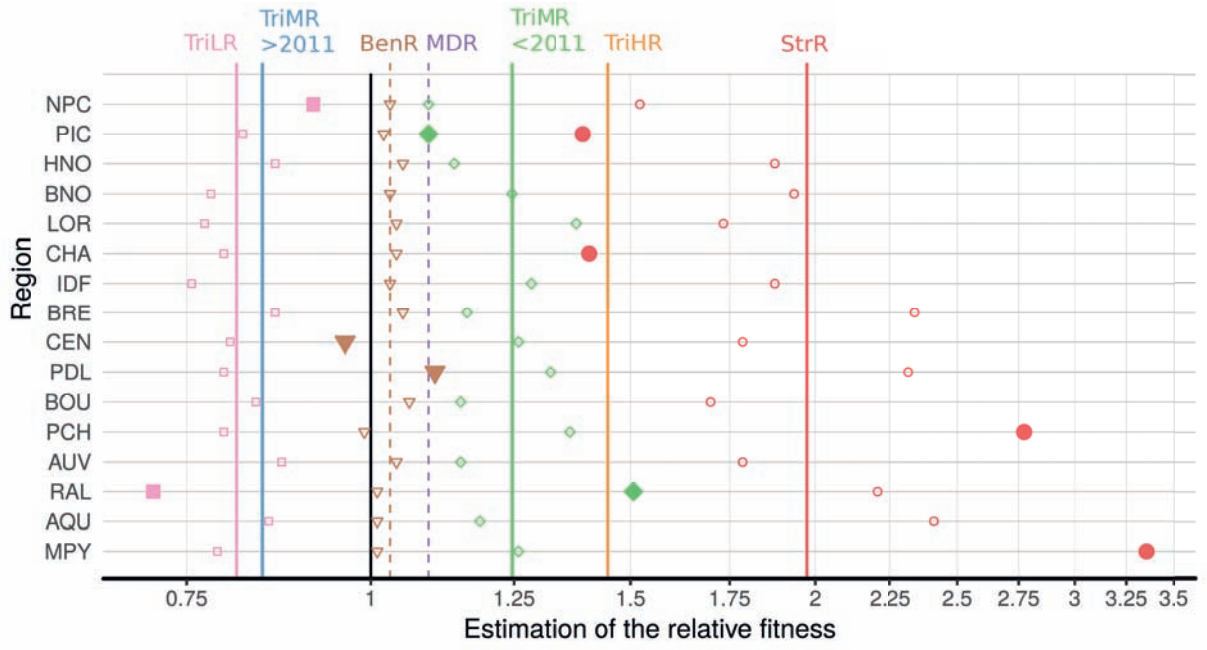


Figure 2. Estimates of the national and regional relative fitness for the main resistant phenotypes in French populations of *Z. tritici* (DYNAMIC MODEL). A relative fitness of 1 (resp. >1 and <1) indicates a steady (resp. increasing and decreasing) frequency. Vertical lines: national growth rate (solid line if significant). Dots: regional growth rates (filled dots if significant). Regions are organized from North to South (extracted from Garnault et *al*., 2019).

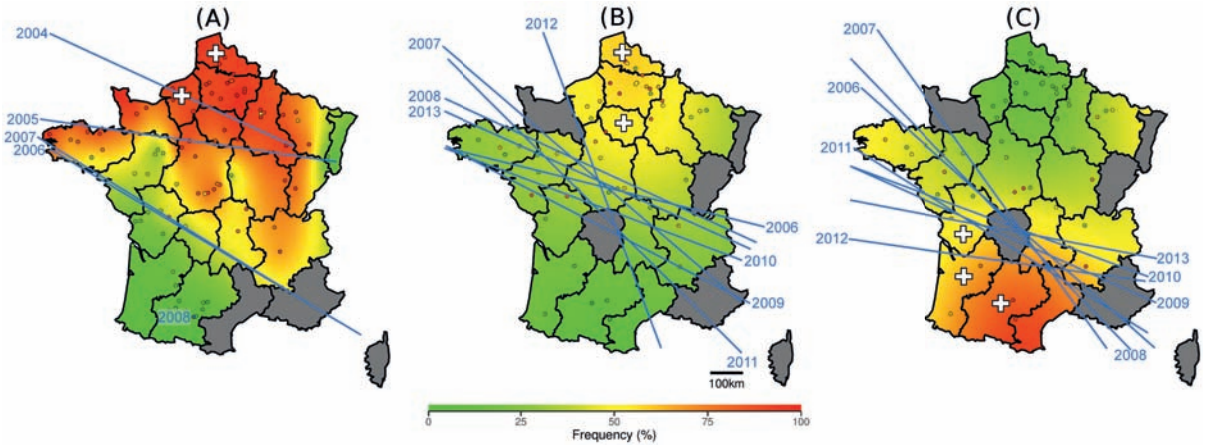


Figure 3. Evolution of spatial partitioning lines for the StrR (A), TriR6 (B) and TriR7-TriR8 (C) phenotypes in France. Dots: Performance trials. Color in dots: observed frequency (in 2006 for StrR; 2013 for TriR6 and TriR7-TriR8). Background color: extrapolated frequencies (MODEL 2). "+": Regions with frequencies significantly higher than the national and interannual average (MODEL 1) (extracted from Garnault et *al*., 2019).

Explanation of spatio-temporal dynamics

Among explanatory factors, the selection pressure exerted by fungicides was the most important to predict resistance frequency (Table 1), according to its relative weight in models (from 0.43

to 0.88 of the predictive quality). The constant growth, representing side factors such as migration, was the second most important parameter, although with much lower weight (from 0.03 to 0.38). Wheat cultivar and sampling date have siginificant effect but very low weight (lower than 0.04 except for one). No effect on yield loss was detected. No effect of any factor was detected for the TriHR phenotype.

Table 1. Estimates (E) and relative weights (W) of explanatory factors tested in the EXPLANATORY MODEL. Yield loss factor is not shown because never significant. The sum of relative weights equals 1, the higher the weight the more important the effect is for frequency prediction. P-value thresholds: *** (P<0.001), ** (P<0.025), * (P<0.05), · (P<0.1).

		StrR		TriMR <2011		TriR6		TriR7-TriR8	
Effect	Levels	E	W	E	W	E	W	E	W
Constant		-3.71*	0.039	-4.12 ·	0.13	4.96***	0.46	1.52 ·	0.383
Fungicides			0.878		0.724		0.491		0.429
	Kresoxym-methyl	4.23***							
	Pyraclostrobin	3.28**							
	Cyproconazole			0		0		2.46**	
	Epoxiconazole			4.94**		0		0	
	Prochloraz			0		3.7***		-3.95***	
	Tebuconazole			0		-11.3***		2.6**	
Organic surfaces		3.26***	0.069	2.56	0.129	0	0	0	0
Wheat cultivar		0.26**	0.005	0.14*	0.007	0.25***	0.022	0.37***	0.151
Sampling date		-0.41***	0.009	-0.17**	0.01	-0.33***	0.027	0.15 ·	0.037

Among the six initial QoI AIs, we estimated a selection effect of kresoxym-methyl and pyraclostrobin on StrR individuals, which are the pioneer and the most used QoIs in France. Prochloraz significantly selected TriR6 strains, whereas it counter-selected TriR7-TriR8 ones. This is consistent with resistance factors observed *in vitro* for these phenotypes. We observed an inversed trend for tebuconazole, *i.e.* selection of TriR7-TriR8 strains along with a counter-selection of TriR6. Epoxiconazole significantly selected TriMR strains, as it could be expected from its heavy use during the 2000s.

DISCUSSION

The large amount of data produced by the Performance network allowed carring out a quantitative, exhaustive and detailed description of the dynamics of resistance in time and space in *Z. tritici* populations. Our frequency-based approach made it possible to process hundreds of populations annually, providing sufficient annual spatial coverage for modeling. We detected original spatial structures, regionalisation and differences of evolution speed between phenotypes and regions, making it possible to describe resistance status in France more quantitatively and accurately than the partial and short-term analyses generally performed.

Using proxy variables, we also explored the effect of (*i*) regional selective pressure in interaction with (*ii*) a population size effect and (*iii*) the fraction of sprayed fields. We showed that the selection pressure *i.e.* fungicide application at large scale, has the greatest explanatory power of the evolution of resistance frequency, providing good prediction of spatio-temporal evolutions for several *Z. tritici* resistances (Garnault et *al.*, in prep.).

These results may help farmers to adapt their spray strategies locally and provide key informations to design regional resistance management. In addition, the methodology we developed here is not specific to *Z. tritici* and can be considered as a generic tool to study resistance evolution in other plant/pathogen systems.

ACKNOWLEDGEMENTS

This work is a part of Maxime Garnault's PhD work at INRA (UMR BIOGER and MaIAGE) funded by a Bayer CIFRE contract. The authors warmly thank the participants of the Performance Network, demonstrating the power of collective action to monitor and manage fungicide resistance. We are grateful to the INRA MIGALE bioinformatics platform (http://migale.jouy.inra.fr) for providing computational resources.

REFERENCES

Delp C; Dekker J (1985). Fungicide resistance: definitions and use of terms. *EPPO Bulletin* 15, 333-335.

Brent KJ; Hollomon DW (1995). Fungicide resistance in crop pathogens: how can it be managed? *Brussels GIFAP*.

Omrane S; Audeon C; Ignace A; Duplaix C; Aouini L; Kema G; Walker AS; Fillinger S (2017). Plasticity of the MFS1 promoter leads to multidrug resistance in the wheat pathoen *Zymoseptoria tritici*. *mSphere* 2, e00393-17.

Leroux P; Walker AS (2011). Multiple mechanisms account for resistance to sterol 14α-demethylation inhibitors in field isolates of *Mycosphaerella graminicola*. *Pest Management Science* 67, 44-59.

Hartl D; Clark A (1997). Principles of population genetics. *Sinauer associates Sunderland* 116, 200-206.

Garnault M; Duplaix C; Leroux P; Couleaud G; Carpentier F; David O; Walker AS (2019). Spatiotemporal dynamics of fungicide resistance in the wheat pathogen *Zymoseptoria tritici* in France. *Pest Management Science*.

Torriani SF; Brunner PC; McDonald BA; Sierotzki H (2009). QoI resistance emerged independently at least 4 times in European populations of *Mycosphaerella graminicola*. *Pest Management Science* 65, 155-162.

DIGITAL PATHOGEN SENSING

Brugger A et al., Extension of hyperspectral imaging to the UV-range for sugar beet-pathogen interactions. In: Deising HB; Fraaije B; Mehl A; Oerke EC; Sierotzki H; Stammler G (Eds), "Modern Fungicides and Antifungal Compounds", Vol. IX, pp. 209-210. © 2020. Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-16-7

Extension of hyperspectral imaging to the UV-range for sugar beet-pathogen interactions

Brugger A, Behmann J, Steiner U, Mahlein A-K

INRES-Plant Diseases and Plant Protection, University Bonn, Nussallee 9, 53115 Bonn, Germany
Corresponding Author: abrugger@uni-bonn.de

INTRODUCTION:

Hyperspectral imaging is focusing on the visible (400 - 700 nm), near-infrared (700 - 1000 nm) and short-wave infrared (1000 - 2500 nm) range of radiation. The ultraviolet range (200 - 380 nm) has not yet been used for digital sensing of plant pathogens and diseases although a variety of plant molecules like flavones, amino acids, anthocyanins or nucleoside feature absorption maxima in this range (Giusti et al. 2014; Talrose et al. 2009). As an example, aromatic phenols are characterized by a maximum absorption at 270 - 274 nm and is produced by a variety of plants as protection against stress (Bhattacharya et al. 2010) while flavonoids who feature two absorption maxima at 240 - 290 and 310 - 370 nm are significant in plant resistance against fungi (Mierziak et al. 2014). In this study, the optical properties of host-pathogen interactions in the UV-range have been studied for the first time. A hyperspectral measuring setup for the UV-range has been established and the influence of abiotic and biotic stress has been investigated.

RESULTS AND DISCUSSION:

To study abiotic stress, barley leaves were placed on phytoagar containing 0 g and 80 g NaCl/l and hyperspectral images were captured from one to five days after incubation (dai). Untreated barley leaves exhibited a constant reflection from 1 to 5 dai which can be linked to consistent amounts of plant molecules absorbing in the UV-range. Barley leaves incubated with 80 g NaCl/l exhibited an increased reflection 4 and 5 dai which is in accordance with the first appearence of chlorosis 4 dai. An increase of reflectance can be linked to a decrease of free amino acids and soluble proteins as a result to a decreased amino acid synthesis as reported for chlorotic apple leaves by Wang et al. (2010). In addition, it is known that high amounts of sodium ions interrupt the protein synthesis and enzyme activity in plants (Carillo et al. 2011).

To investigate biotic stress, sugar beet leaves inoculated with *Cercospora beticola* were measured and spectral signatures revealed that a differentiation between symptom stages and healthy tissue is possible (Fig. 1). Healthy sugar beet tissue is represented by a low reflectance, early and mature symptoms show a similar reflectance while the mid-age symptom is represented by a high reflectance and strong increase from 380 to 500 nm. Since early and

mature symptoms can be clearly separated in the UV-range, but their reflectance is similar in the visible range, an extension of hyperspectral imaging to the UV-range seems to be a promising tool for plant stress detection and phenotyping. However, further time series measurements are needed to evaluate the potential of hyperspectral imaging in the UV-range.

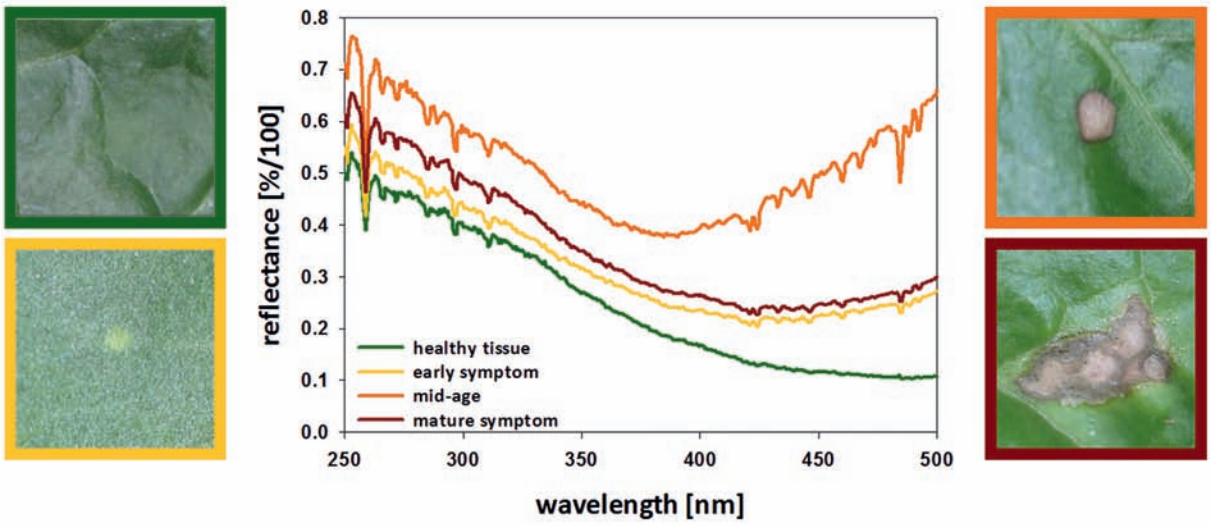


Figure 1 Spectral signatures of sugar beet inoculated with *Cercospora beticola* with n=1000 pixel and RGB images.

REFERENCES

Bhattacharya A; Sood P; Citovsky V (2010). The roles of plant phenolics in defence and communication during *Agrobacterium* and *Rhizobium* infection. *Molecular Plant Pathology* 11, 705–719.

Carillo P; Annunziata M G ; Pontecorvo G; Fuggi A; Woodrow P (2011). Salinity stress and salt tolerance. *Abiotic Stress in Plants-Mechanisms and Adaptations, 1*, 21-38.

Giusti MM; Polit M F; Ayvaz H; Tay D;Manrique I (2014). Characterization and quantitation of anthocyanins and other phenolics in native Andean potatoes. *Journal of Agricultural and Food Chemistry* 62, 4408–4416.

Mierziak J; Kostyn K; Kulma A (2014). Flavonoids as important molecules of plant interactions with the environment. *Molecules* 19, 16240–16265.

Talrose V; Yermakov A; Usov A; Goncharova A; Leskin A; Messineva N; Trusova N; Efimkina M (2009). UV/visible spectra. NIST Chemistry WebBook, NIST Standard Reference Database, p. 20899.

Wang H; Ma F; L C (2010). Metabolism of organic acids, nitrogen and amino acids in chlorotic leaves of ’Honeycrisp’ apple (*Malus Domest. Borkh*) with excessive accumulation of carbohydrates. *Planta* 232, 511–522.

Alisaac E et al, Identification and Discrimination of *Fusarium* Infection at Spikelets of Wheat: Suitability of Different Sensors. In: Deising HB; Fraaije B; Mehl A; Oerke EC; Sierotzki H; Stammler G (Eds), "Modern Fungicides and Antifungal Compounds", Vol. IX, pp. 211-213. © 2020. Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-16-7.

Identification and Discrimination of *Fusarium* Infection at Spikelets of Wheat: Suitability of Different Sensors

Alisaac E[1], Behmann J[1], Dehne H-W[1], Mahlein A-K[2]

[1]*Institute of Crop Science and Resource Conservation (INRES), Plant Diseases and Plant Protection, Rheinische Friedrich-Wilhelms Universität Bonn, Nussallee 9, 53115 Bonn, Germany*

[2]*Institute of Sugar Beet Research (IfZ), Holtenser Landstraße 77, 37079, Göttingen, Germany*

Corresponding Author Email: alisaac@uni-bonn.de

INTRODUCTION

Application of fungicides against plant diseases in cereals needs a precise monitoring of disease incidence and a perfect timing of application to avoid losses in grain yield and quality and to get the highest benefits out of the treatment. This holds true especially for *Fusarium* head blight (FHB) infection and characteristic contamination with mycotoxins. In case of *Fusarium* infection, mycotoxin formation and reduction of grain quality get established before characteristic symptoms become obvious. Recently, various optical sensors have been developed and can be used to identify physiological damage and pathogen infection (Mahlein et al. 2019). In this study, three imaging sensors for hyperspectral imaging (HSI), chlorophyll fluorescence imaging (CFI) and infrared thermography (IRT) have been applied to monitor FHB infection overtime.

MATERIAL AND METHODS

Time-series measurements started 3 days after spray inoculation (dai) with a spore suspension (10^5 conidia/mL) at anthesis of spring wheat Passat (KWS, LOCHOW, Northeim, Germany) (Alisaac et al. 2018).

Spectral vegetation indices (SVIs) were derived from mean spectral signature of the spikelets: normalized differences vegetation index, NDVI = (R800 - R670) / (R800 + R670); photochemical reflection index, PRI = (R531 - R570) / (R531 + R570); pigment specific simple ratio, PSSRa = R800 / R680; PSSRb = R800 / R635; PSSRc = R800 / R470; water index, WI = R900 / R970.

The following indices were derived from chlorophyll fluorescence data of the spikelets: maximal chlorophyll fluorescence (Fm); maximal photochemical efficacy of PSII (Fv/Fm); photochemical efficacy of PSII (Y[II]).

From thermal data of the spikelets, the following parameters were derived: maximum temperature difference (MTD) within spikelets; average temperature difference between air and spikelets (ΔT).

A support vector machine classification (SVM) was run to classify non-inoculated and infected spikelets using the parameters derived from each sensor or a combination of different sensors parameters. Seven data sets were investigated; three from each individual sensor (IRT, CFI and HSI); three from combination of two sensors (IRT-CFI, IRT-HSI and CFI-HSI); one using multi-sensor data (IRT-CFI-HSI).

RESULTS

Classification results showed the possibility to discriminate healthy from infected spikelets as early as 3 days after inoculation (dai) with an accuracy of 78, 56 and 78 % for IRT, CFI and HSI, respectively (Fig. 1). The accuracy increased over the time of the experiment to reach 100 % 5 and 12 dai for IRT and HSI respectively (Fig. 1). Later on, the accuracy decreased 30 dai due to the senescence of non-inoculated spikelets (Fig. 1). Combining data from different sensors could improve the classification accuracy over the time of the experiment for IRT-CFI, IRT-HSI and CFI-HSI respectively (Fig. 2).

CONCLUSIONS

Sensors proved to be an effective tool to detect FHB infection on wheat spikelet and to follow the damage of *Fusarium* on wheat spikes. IRT was most sensitive to identify early response of wheat to FHB infection followed by HSI and CFI. The combination of IRT-HSI led to the superior accuracy over the time of pathogenicity, and this means reliable and applicable systems for FHB monitoring can be established based on IRT-HSI sensors.

REFERENCES

Alisaac E, Behmann J, Kuska MT, Dehne HW, Mahlein AK (2018). Hyperspectral quantification of wheat resistance to *Fusarium* head blight: comparison of two *Fusarium* species. *European Journal of Plant Pathology* 152, 869-884.

Mahlein AK, Alisaac E, Al Masri A, Behmann J, Dehne HW, Oerke EC (2019). Comparison and combination of thermal, fluorescence and hyperspectral imaging for monitoring *Fusarium* head blight of wheat on spikelet scale. *Sensors* 19, 2281.

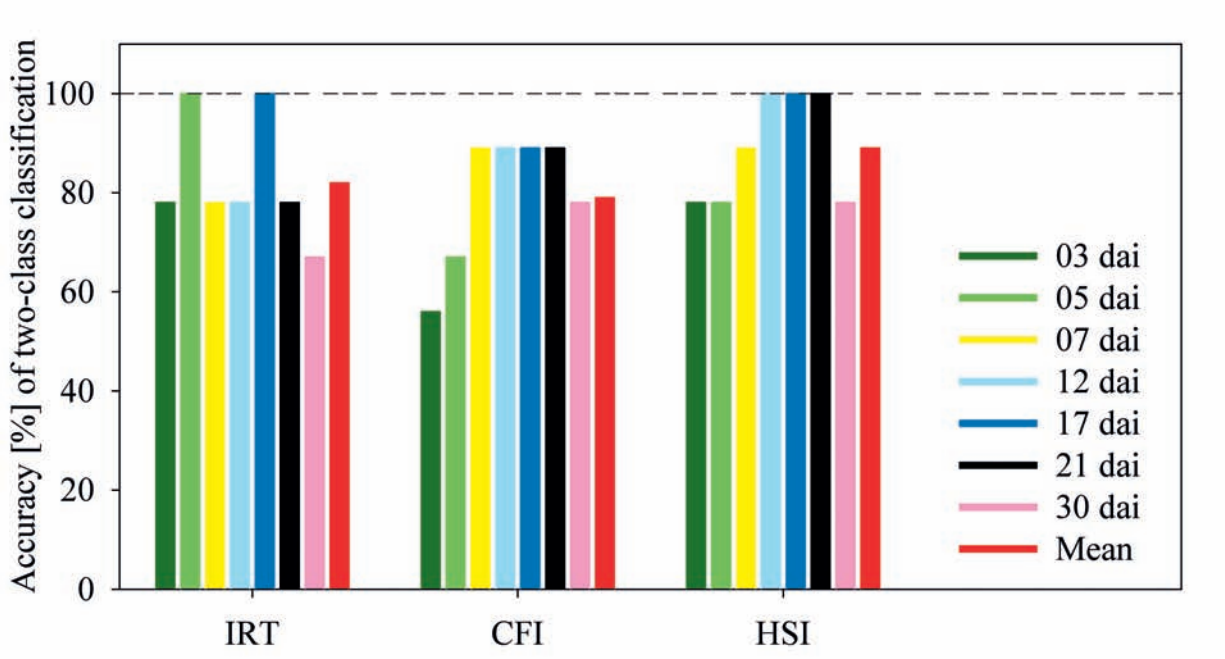


Figure 1 Accuracy of two-class classification (non-inoculated/infected spikelets) using support vector machine (SVM) for each assessment date using the defined parameters derived from each sensor.

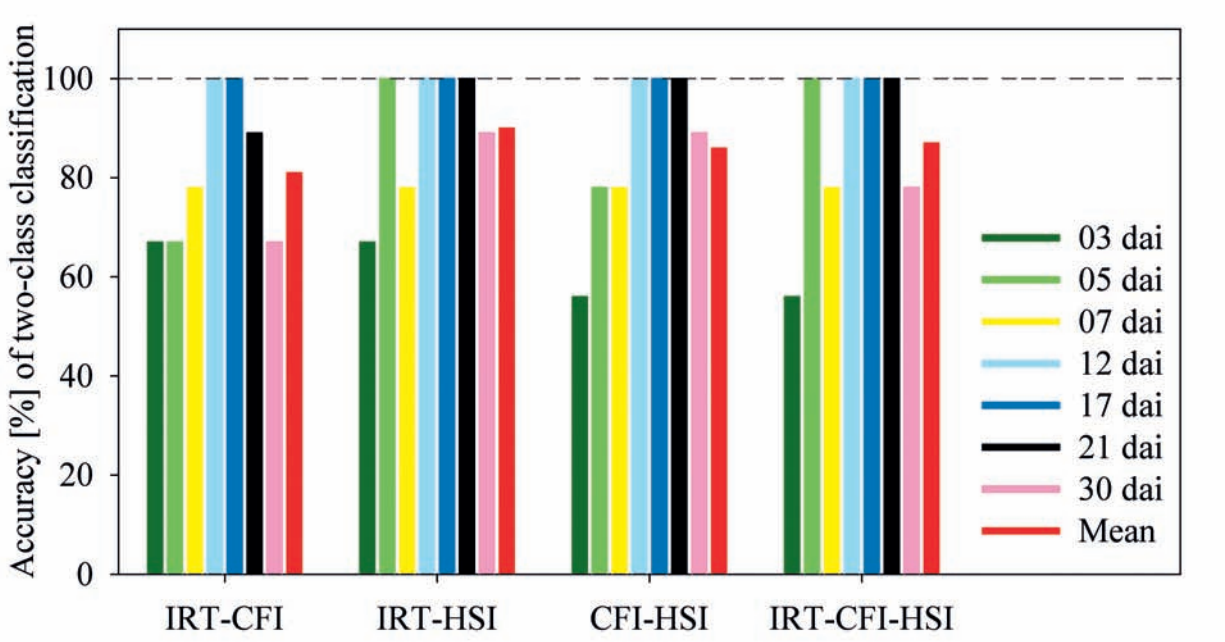


Figure 2 Accuracy of two-class classification (non-inoculated/infected spikelets) using support vector machine (SVM) for each assessment date using different combinations of the defined parameters derived from each sensor.

BIORATIONAL FUNGICIDES / BIOCONTROL

Deising et al., Antagonistic Microorganisms in Plant Protection: Consumers' Friends or Foes? In: Deising HB; Fraaije B; Mehl A; Oerke EC; Sierotzki H; Stammler G (Eds), "Modern Fungicides and Antifungal Compounds", Vol. IX, pp. 217-228. © 2020. Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-16-7

Antagonistic Microorganisms in Plant Protection: Consumers' Friends or Foes?

Deising HB[1], Amorim R[1], De Oliveira Silva A[1], Raschke A[1], Eisermann I[1], Wirsel SGR[1], Csuk R[2], Schmitz LM[3], Arnold N[3]

Martin-Luther-Universität Halle-Wittenberg, [1]Naturwissenschaftliche Fakultät III, Institut für Agrar- und Ernährungswissenschaften, Lehrstuhl für Phytopathologie und Pflanzenschutz, Betty-Heimann-Str. 3, D-06120 Halle (Saale), [2]Naturwissenschaftliche Fakultät II, Institut für Chemie, Lehrstuhl für Organische Chemie, Kurt-Mothes-Str. 2, 06120 Halle (Saale), Germany; [3]Leibniz Institut für Pflanzenbiochemie, Natur- und Wirkstoffchemie, Weinberg 3, 06120 Halle (Saale), Germany.

Email: holger.deising@landw.uni-halle.de

INTRODUCTION

In conventional agriculture the application of plant protection chemistries is action threshold-based, with resistant cultivars and agronomical measures taking the major responsibility in plant protection. Chemistries used to combat diseases and pests overcoming non-chemical measures require stringent analyses of toxicity and eco-toxicity, with tremendous per-substance-risk assessment cost currently estimated at more than $US 70 million (https://agriculture.basf.com/de/Pflanzenschutz/R-D-Strategy.html**)**. In spite of the fact that food quality has improved since synthetic plant protection compounds are used (Rosling et al. 2018), political and societal concerns regarding plant protection chemistries demand banning of these compounds and increasing biological control efforts. A complete shift to organic food production, however, would cause major reduction of yield and yield stability and significant shortfalls in the availability of the vast majority of agricultural products (Goklany 2002; Kirchmann 2019; Knapp & van der Heijden 2018; Seufert et al. 2012). Moreover, the argument that greenhouse gas emissions is reduced in organic farming may apply locally, but not on a global scale, as overseas land use to compensate for shortfalls in domestic supply is predicted to lead to an overall increase in greenhouse gas emission (Smith et al. 2019).

Ignoring the lower yields in organic as compared to conventional farming (Seufert et al. 2012) and well-established benefits provided by approved plant protection chemistries, the political goal envisaged by the German Federal Ministry of Food and Agriculture (BMEL; https://www.bundesprogramm.de/wer-wir-sind/ueber-das-bundesprogramm/, see Federal Programme for Ecological Land Use, BÖLN) is to strengthen organic farming. This, however, would leave farmers without efficient options to control epidemics caused by the occurrence of novel highly virulent pathogen races or species of pathogens. Both, more frequently occuring drought periods and continuous losses of acreages led to reduced wheat yields (Keulemans et al. 2019), and further crop losses cannot be tolerated. As plant protection chemistries are

critically discussed, it is not surprising that alternative strategies for pathogen control are urgently sought. Apart from the development of genetically modified (GM) plants, novel techniques such as RNAi sprays may contribute to plant protection (Koch et al. 2016; Koch et al. 2017). The idea of using antagonistic microorganisms in plant protection exists since long (Wood and Tveit 1955) and becomes increasingly attractive in organic farming systems (Calvo-Garrido et al. 2019; Köhl et al. 2011; Singh 2014). Based on the general assumption that biological compounds are safer than synthetic chemistries, microorganism-based plant protection is thought to be less harmful than the application of pesticides. This notion may be challenged by genetic and biochemical mechanisms activated in confronting microbes and microbial communities.

FUNGAL AND BACTERIAL GENOMES COORDINATE SYNTHESIS OF BIOCHEMISTRIES WITH ANTAGONISTIC POTENTIAL

Prominent examples of microorganisms used as plant protection products belong to the fungal genus *Trichoderma*, and the bacterial genera *Bacillus* and *Pseudomonas* (Lugtenberg 2018, and references therein). Importantly, commercial microbial plant protection products are not only applied to soil, but also as pre- and post-harvest applications onto fruits. Thus, these microorganisms are in direct contact with food. In grapes, commercial biological control products include *Bacillus amyloliquefaciens*, *Bacillus subtilis*, *Aureobasidium pullulans*, *Ulocladium oudemansii*, and *Candida sake* (Calvo-Garrido et al. 2019). The thermostable proteinaceous toxin amylosin produced by *B. amyloliquefaciens* and other *Bacillus* species, including *B. subtilis*, exhibits antimicrobial properties likely due to formation of ion channels in lipid membranes with the consequence of disruption of ion homeostasis, mitochondrial function and energy metabolism not only in microbial, but also in mammalian cells (Apetroaie-Constantin et al. 2009; Rasimus-Sahari et al. 2015). The biocontrol fungus *A. pullulans* commonly found in most phyllospheric habitats, including grapevines, forms antimicrobial compounds inhibiting both Gram-negative and -positive bacteria such as *Pseudomonas fluorescens* and *Staphylococcus aureus*, with the antibacterial property of the fungus attributed to complex chemistries such as 2-propylacrylic acid, 8,9-dihydroxy-2-methyl-4*H*,5*H*-pyrano [3,2-c]-chromen-4-one, 2-methylenesuccinic acid and hexane-1,2,3,5,6-hexol. The antifungal activity of *A. pullulans* results from cyclic depsipeptides called aureobasidins (Bozoudi & Tsaltas 2018). These examples clearly show that microbial antagonism is largely based on antimicrobial chemistries.

The fact that microbial antagonism is chemistry-based may not be surprising, as the access to more than 1000 fungal sequenced and annotated genomes (Grigoriev et al. 2014) indicates an impressively large numbers of secondary metabolism (SM) gene clusters in the vast majority of fungi. For example, *Aspergillus* species, with genome sizes varying between 28 and 40 Mb, typically contain some 50 SM gene clusters per species (Brakhage 2013), and the genomes of the plant pathogenic fungi *Colletotrichum graminicola* and *Colletotrichum higginsianum* contain 42 and 39 SM gene clusters, respectively (O'Connell et al. 2012). Moreover, in the genome of *Fusarium graminearum*, the causal agent of wheat head blight and cob rot in maize, 67

potentially functional SM gene clusters have been identified (Sieber et al. 2014). Surprisingly, even in the budding yeast *Kluyveromyces lactis* four genes form a pulcherriminic acid biosynthesis cluster (Krause et al. 2018). It is of relevance to note that the products of the majority of fungal SM clusters are currently unknown (Brakhage 2013; O'Connell et al. 2012; Sieber et al. 2014), and so are the conditions which activate the transcription factors required for triggering cluster activation. Collectively, these examples strongly suggest that synthesis of secondary metabolites is required for competitive fungal development in the environment. It is not surprising, however, that many secondary metabolites have a significant impact on other organisms, including man.

Aflatoxins, a group of polyketide-derived furanocoumarins, are synthesized by various *Aspergillus* species, including the ubiquitous fungus *A. flavus* and are among the most potent carcinogenic substances known. This group of mycotoxins comprises at least 16 structurally related toxins, four of which, i.e. the aflatoxins B1, B2, G1 and G2, representing the major toxins found in agricultural products, imposing significant health risks (Yu et al. 2004)(Fig. 1A). The ~70 kbp aflatoxin biosynthesis gene cluster in *A. flavus* contains 25 genes, including two adjacent regulatory genes called *aflR* and *aflS* (Smith et al. 2007; Yu et al. 2004)(Fig. 1B). The DNA-binding Zn_2-Cys_6 transcription factor aflR is required for transcriptional activation of most, if not all of the aflatoxin cluster genes, with the aflR-interacting protein aflS acting as a co-activator (Chang 2003).

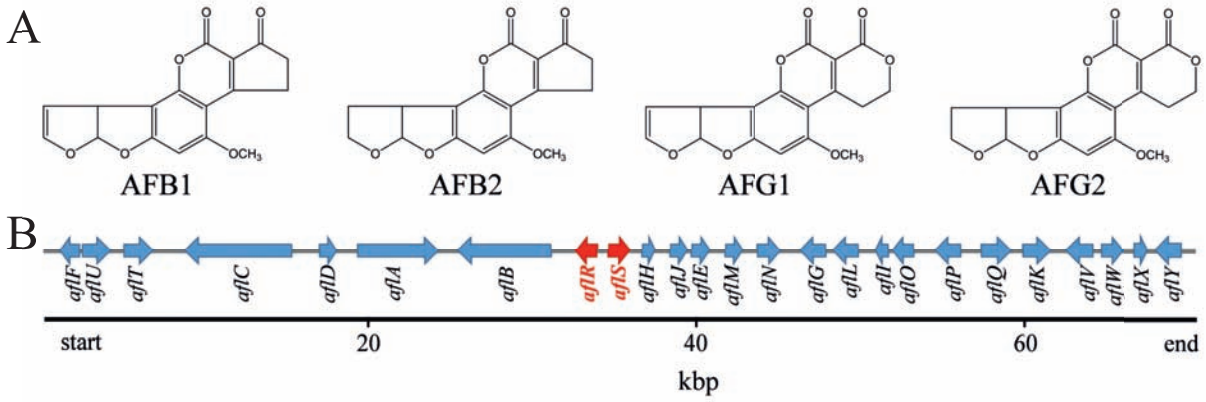


Figure 1 Aflatoxins and aflatoxin gene cluster of *A. flavus*. A. Chemical structure of the four majoraflatoxins associated with food and fodder. B. Organization of the aflatoxin biosynthesis gene cluster. The regulatory genes *aflR* and *aflS* are given in red. Data are from the National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov/nuccore/AY371490); modified after Yu et al. 2004.

Model fungi such as the saprophytes *A. flavus* and *Aspergillus nidulans*, as well as the mammalian pathogen *Aspergillus fumigatus* have been used to study the activation of SM gene clusters in response to environmental stimuli. Collectively, these studies have shown that several factors affect aflatoxin biosynthesis, including carbon and nitrogen availability, temperature,

osmotic potential and pH of the surrounding medium. In addition, the developmental stage of the fungus, oxidative stress and even metabolites of plants that are colonized may affect toxin formation (Yu 2012). Intriguingly, and this is of significant relevance to evaluate the consumer-safety of antagonist-based plant protection, the intimate physical interaction of two or more microorganisms may lead to the formation of as yet unknown compounds. The contact of *A. nidulans* with the soil-inhabiting bacterium *Streptomyces rapamycinicus*, a bacterium identified from a collection of 58 actinomycete species, led to activation of a silent polyketide synthase (PKS) gene cluster required for the production of the archetypal polyketide orsellinic acid, its derivative lecanoric acid, and of two cathepsin K inhibitors (Schroeckh et al. 2009). Chromatin immunoprecipitation experiments revealed that the production of secondary metabolites by *A. nidulans* is accompanied by a global increase in acetylation at lysine 14 of histone H3 (H3K14 acetylation), and acetylation at lysine 9 of histone H3 (H3K9 acetylation) was specifically increased within SM gene clusters (Nützmann et al. 2011), with both epigenetic marks leading to gene activation. Without any doubt, multi-lateral microbial inter-species communication will lead to activation of silent SM gene clusters and formation of a plethora of new compounds (Netzker et al. 2015). This is not only of significant academic interest but allows discovery of novel antibiotics and other molecules of economic interest, e.g. compounds with fungicidal, or herbicidal activity. However, the introduction of non-adapted microorganisms into an ecosystem is likely to result in the formation of novel multi-species communities and trigger an enormous shift in chemistries, with an unknown number of toxic compounds formed.

CONFRONTATIONS MAY CAUSE SM CLUSTER ACTIVATION IN EITHER BIOCONTROL ORGANISMS OR PLANT PATHOGENS

As mentioned above, a number of biocontrol organisms are applied in agriculture, aiming at reduction of microbial disease severity. Examples are the fungus *Aureobasidium pullulans* var. *pullulans*, different *Trichoderma* species, and members of the bacterial genera *Bacillus* or *Pseudomonas* (Lugtenberg 2018).

Interestingly, at first glance *A. pullulans* var. *pullulans* indeed appears to be well-suited as a biocontrol fungus, as its genome harbors only nine SM gene clusters, three of which containing non-ribosomal peptide synthetases, and one each mediating terpene and type I polyketide biosynthesis. In comparison, 32 SM clusters have been found in *A. pullulans* var. *namibiae*, and 37 each in *A. pullulans* var. *melanogenum* and *A. pullulans* var. *subglaciale*, respectively, with the chemical compound(s) produced by these SM clusters being unknown in most cases (Gostinčar et al. 2014). A large and – as compared to the biomass-degrading species *Trichoderma reesei* – significantly expanded repertoire of SM gene clusters has been reported in *Trichoderma* species used in biocontrol. For example, the genomes of the mycoparasitic species *Trichoderma virens* and *Trichoderma atroviride* harbor 18 polyketide synthase (PKS) gene clusters each, and 28 and 16 nonribosomal peptide synthetase (NRPS) gene clusters, respectively. These numbers are approximately twice of those found in *T. reesei* (Mukherjee et al. 2012; Mukherjee et al. 2013). Moreover, several B. subtilis strains show strong antagonistic

effects against fungal plant pathogens such as *Alternaria solani*, *Botrytis cinerea*, *Sclerotinia sclerotiorum* as well as against the oomycete *Phytophthora infestans* (Bóka et al. 2019). Importantly, more than half of the large number of compounds identified in fungi show biological functions and exhibit, for example, antibacterial, antifungal or antitumor activity, clearly illustrating the enormous potential of fungal SM (Boruta 2018; Keller 2019; Peláez 2005; Schueffler & Anke 2014).

Investigating the interaction between the plant pathogenic bacterium *Ralstonia solanacearum*, causing bacterial wilt on several economically important plants such as tomato and potato, and the causal agent of bakanae disease in rice, *Fusarium fujikuroi*, Spraker et al. (2018) discovered that the biosynthesis of the fungal secondary metabolites bikaverin and beauvericin is induced by the bacterial lipopeptide ralsolamycin, with both fungal secondary metabolites antagonising the bacterium. Intriguingly, a bikaverin-producing strain of Botrytis *cinerea* likewise showed activation of bikaverin biosynthesis in response to ralsolamycin, strongly suggesting that responses are conserved in a 'war' employing small molecules produced by taxonomically remote species (Spraker et al. 2018). As a matter of fact, bi-, tri- or multilateral *in vitro* confrontation assays on plates may help identifying microbial antagonists. Indeed, bacterial and fungal antagonists have been isolated from various sources, including soil, aerial plant surfaces, or from plant tissues (Ben Slama et al. 2018; Lahlali & Hijri 2010; Schiewe & Mendgen 1992). Interestingly, Falconi and Mendgen isolated 32 different fungal species from apple leaves, 21 of which were present throughout the season in numbers. Of these, 368 isolates were tested for their antagonistic capacity against the post-harvest apple pathogens *Botrytis cinerea*, *Monilinia fructigena* and *Penicillium expansum*. The exciting finding of this work was that significantly improved control efficiencies were obtained when mixtures of antagonists were applied (Falconi and Mendgen 1994), strongly suggesting that molecular communication in multi-species biocontrol communities may activate formation of novel chemistries with increased antagonistic activities.

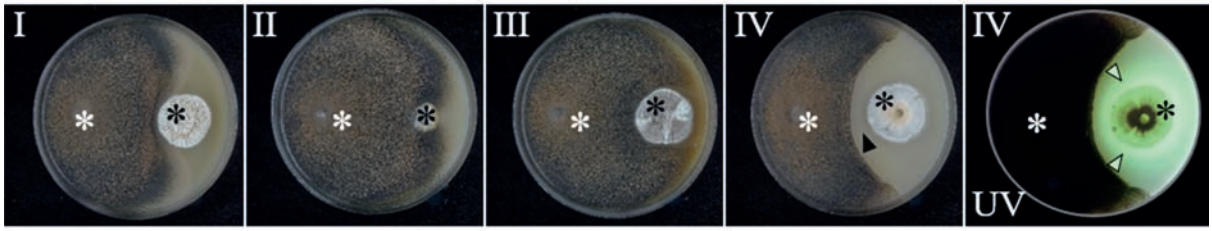


Figure 2 Confrontations between the fungus *C. graminicola* (white asterisk) and four distinct *Streptomyces* species (black asterisk, plates I - IV) on petri dishes. The streptomycetes on plates I – III are not or only marginally inhibitory. The streptomycete on plate IV strongly inhibits growth of the fungus, as indicated by the inhibition zone (plate IV, arrowhead). Exposure of this plate to UV irradiation (λ 312 nm) shows that a greenish fluorescent compound surrounds the colony of the streptomycete, likely indicative of an aromatic antifungal compound secreted by the bacterium.

In bilateral confrontation assays employing the maize pathogen *C. graminicola* and four distinct streptomycete species, we observed strong inhibition zones with only one of the four bacterial species tested (Fig. 2, IV, black arrowhead). Interestingly, exposing the confrontation plate to

UV light showed fluorescing compounds in the inhibition zone (Fig. 2, IV UV, green arrowheads), suggesting the contribution of e.g. aromatic compounds to the inhibitory activity of the inhibitory streptomycete.

INCREASING DIVERSITY OF CHEMISTRIES BY COMPOUND EXCHANGE AND MODIFICATION

Most microbial secondary metabolites play a role in ecological niche reservation, with strobilurin A synthesized by the mushroom *Strobilurus tenacellus* as one of the most prominent examples (Anke et al. 1977). However, though synthesized to intoxicate competing microorganisms, secreted secondary metabolites may be taken up by these and enzymatically converted to putatively more toxic product(s). Exchange and further modification of secondary metabolites by distinct co-cultivated fungal species has been genetically proven, using different *Colletotrichum lagenarium* and *C. graminicola* mutants with defined defects in melanin biosynthesis (Fig. 3) (Deising et al. 2017; Kubo et al. 1983).

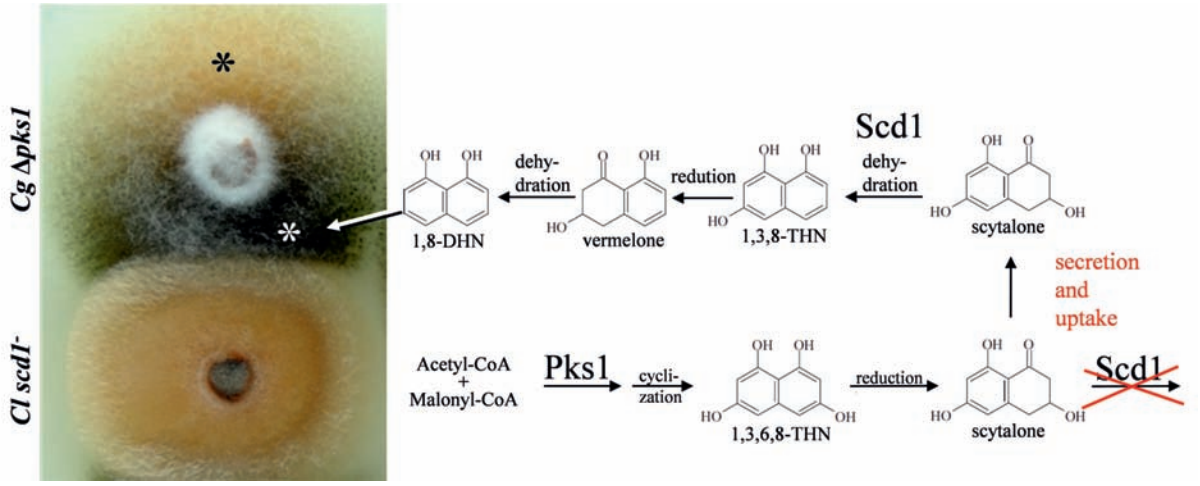


Figure 3 Metabolic cross-species complementation of the melanin-deficient phenotype of Δ*pks1* mutants of *C. graminicola* by co-cultivation with the scytalone dehydratase mutant *scd1*$^{-}$ of *C. lagenarium*. After Deising et al. (2017), with modifications. For details, see text.

The Δ*pks1* mutant of *C. graminicola*, due to a lack of the enzyme polyketide synthase 1 (Pks1), is unable to catalyze the first step in melanin biosynthesis, and thus incapable of forming 1,3,6,8-tetrahydroxynaphthalene (1,3,6,8-THN) and melanin. Therefore, the Δ*pks1* mutant remains salmon-colored (Fig. 3, black asterisk) (Ludwig et al. 2014). In contrast, the *C. lagenarium* scytalone dehydratase (Scd1) mutant *Cl scd1*$^{-}$ is able to initiate the melanin biosynthetic pathway to form scytalone, but unable to further convert this metabolite due to lack of Scd1 (Kubo et al. 1996). As a consequence, scytalone is secreted into the medium. The neighboring Δ*pks1* mutant of *C. graminicola*, however, harbors a functional Scd1 protein, takes up and converts scytalone by successive dehydration and reduction reactions to yield 1,8-

dihydroxynaphthalene (1,8-DHN), which is finally polymerized in the cell wall of the *C. graminicola* Δ*pks1* mutant to form strongly pigmented melanin (Fig. 3, white asterisk). This co-cultivation experiment clearly indicates a cross-species exchange and coordinate cross-species synthesis of secondary metabolites.

Extrapolating this finding to a microbial population in the soil or on aerial plant surfaces, one would expect that complex interactions and chemical multi-component communication would lead to induction of large numbers of SM gene clusters in several members of the consortium. Even in the case of applying biocontrol agents with few SM gene clusters, as reported for *A. pullulans* (see above), the expectation would be that microorganisms and consortia attacked by this biocontrol agent would respond to this biotic stress by massively activating SM gene clusters. It is of importance to note that the compounds formed by microbial consortia may vary considerably, depending on the composition of the consortia and on the secondary metabolites formed by competing partners. Given the large number of compounds formed by microorganisms, given that structural analyses of compounds formed only in areas of confrontation, and derived from that, given the lack of knowledge of toxicity of the chemical compounds synthesized it is simply naive to believe *a priori* that biological control would be a consumer-friendly plant protection strategy (Deising et al. 2017).

WITHOUT GENETIC DATA THE RISK IMPOSED BY BIOCONTROL ORGANISMS CANNOT BE PUT INTO PERSPECTIVE

Challenged by a previous article questioning the safety of biological control measures (Deising et al. 2017), Lugtenberg and others argued that the registration procedure for biological plant protection products is sufficiently stringent to warrant consumer-safety. Furthermore, they argued that the European Commission has spent millions of Euros on risk assessment (Koch et al. 2018; Lugtenberg 2018). These arguments are difficult to follow as microbial consortia forming after application of biocontrol-agents are mostly unknown and probably highly variable. Synthesis of secondary metabolites, as outlined above, depends on several environmental factors and on the interactive metabolic pathway of the consortia, and therefore the nature and toxicity of compounds formed cannot be predicted. The analytical problem indeed is that specific chemicals may be formed in an interaction-specific manner, again emphasizing the important role of the consortia. In this scenario, how can risk-assessment be performed to deliver realistic data?

However, it is not acceptable to ignore that techniques exist allowing performing genome-wide gene expression studies with defined interaction partners, i.e. partners, the genomes of which have been fully sequenced and annotated. Again, several genome initiatives have released some 1000 annotated fungal genome sequences (Grigoriev et al. 2014). These data could be used to design confrontation assays with potential biocontrol strains, and to analyze alterations in SM gene expression. Such an approach would answer the relevant question whether or not SM gene clusters are activated under certain confrontational conditions. Unless these data are available, a skeptical view on spraying biocontrol microorganisms into the field must remain. And unless

it has been shown that SM gene clusters are not activated in either the biocontrol organism(s) used or in the target plant pathogens, we should keep in mind that secondary metabolite cocktails synthesized by microbial consortia may be much more toxic than fungicides that have thoroughly been tested for their consumer-safety prior to legislation. The argument that naturally occurring microorganisms are safe as they are natural is rather naive. The safety of application of biological control agents has to be proven at least on the SM gene cluster expression level. As long as such data are not available, we strongly recommend to base fungal pathogen control primarily on consumer-safe fungicide treatments.

ACKNOWLEDGMENTS

We thank European Social Fund (ESF) for supporting the International Graduate School AGRIPOLY – Determinants of Plant Performance (grant ZS/2016/08/80644 to HBD, project "Chromatin modification in fungal pathogenicity"). Funding of research in the laboratory of H.B.D was funded by the Federal Ministry of Education and Research (BMBF; AZ 031A353A), and the 19th International Reinhardsbrunn Symposium was supported by the German Research Foundation (DFG; DE 403/23-1). HBD, NA and RC acknowledge the support by the ScienceCampus Plant-Based Bioeconomy (Europäischer Fonds für regionale Entwicklung – EFRE, Project ZS/2019/01/96528; WOLF). We also thank Gary Sawers and Marco Fischer, MLU Halle, for providing the Streptomyces species used in this study.

REFERENCES

Anke T; Oberwinkler F; Steglich W; Schramm G (1977). The strobilurins - new antifungal antibiotics from the basidiomycete *Strobilurus tenacellus*. *J. Antibiot.* 30, 806-810.

Apetroaie-Constantin C; Mikkola R; Andersson MA; Teplova V; Suominen I; Johansson T; Salkinoja-Salonen M (2009). *Bacillus subtilis* and *B. mojavensis* strains connected to food poisoning produce the heat stable toxin amylosin. *J. Appl. Microbiol.* 106, 1976-1985.

Ben Slama H; Cherif-Silini H; Chenari Bouket A; Qader M; Silini A; Yahiaoui B; Alenezi FN; Luptakova L; Triki MA; Vallat A; Oszako T; Rateb ME; Belbahri L (2018). Screening for *Fusarium* antagonistic bacteria from contrasting niches designated the endophyte *Bacillus halotolerans* as plant warden against *Fusarium*. *Front. Microbiol.* 9, article 3236.

Bóka B; Manczinger L; Kocsubé S; Shine K; Naiyf S; Alharbi NS; Khaled JM; Münsterkötter M; Vágvölgyi C; Kredics L (2019). Genome analysis of a *Bacillus subtilis* strain reveals genetic mutations determining biocontrol properties. *World J. Microbiol. Biotechnol.* 35, article 52.

Boruta T (2018). Uncovering the repertoire of fungal secondary metabolites: From Fleming's laboratory to the International Space Station. *Bioengineered* 9, 12-16.

Bozoudi D; Tsaltas D (2018). The multiple and versatile roles of *Aureobasidium pullulans* in the vitivinicultural sector. *Fermentation* 4, 85.

Brakhage AA (2013). Regulation of fungal secondary metabolism. *Nat. Rev. Microbiol.* 11, 21-32.

Calvo-Garrido C; Jean Roudet J; Aveline N; Davidou L; Dupin S; Fermaud M (2019). Microbial antagonism toward *Botrytis* bunch rot of grapes in multiple field tests using one *Bacillus ginsengihumi* strain and formulated biological control products. *Front. Plant Sci.* 10, article 105.

Chang P-K (2003). The *Aspergillus parasiticus* protein AFLJ interacts with the aflatoxin pathway-specific regulator AFLR. *Mol. Gen. Genomics* 268, 711-719.

Deising HB; Gase I; Kubo Y (2017). The unpredictable risk imposed by microbial secondary metabolites: How safe is biological control of plant diseases? *J. Plant Dis. Prot.* 124, 413-419

Falconi CJ; Mendgen K (1994). Epiphytic fungi on apple leaves and their value for control of the postharvest pathogens *Botrytis cinerea*, *Monilinia fructigena* and *Penicillium expansum*. *J. Plant Dis. Protect.* 101, 38-47.

Goklany IM (2002). The ins and outs of organic farming. *Science* 298, 1889-1890.

Gostinčar C; Ohm RA; Kogej T; Sonjak S; Turk M; Zajc J; Zalar P; Grube M; Sun H; Han J; Sharma A; Chiniquy J; Ngan CY; Lipzen A; Barry K; Grigoriev IV; Gunde-Cimerman N (2014). Genome sequencing of four *Aureobasidium pullulans* varieties: biotechnological potential, stress tolerance, and description of new species. *BMC Genomics* 15, article 549.

Grigoriev IV; Nikitin R; Haridas S; Kuo A; Ohm R; Otillar R; Riley R; Salamov A; Zhao X; Korzeniewski F; Smirnova T; Nordberg H; Dubchak I; Shabalov I (2014). MycoCosm portal: gearing up for 1000 fungal genomes. *Nucl. Acids Res.* 42, D699-704.

Keller NP (2019). Fungal secondary metabolism: regulation, function and drug discovery. *Nat. Rev. Microbiol.* 17, 167-180.

Keulemans W; Bylemans D; De Coninck B (2019). Farming without plant protection products - Can we grow without using herbicides, fungicides and insecticides? EPRS - European Parliamentary Research Service Scientific Foresight Unit (STOA)PE 634.416.

Kirchmann H (2019). Why organic farming is not the way forward. *Outl. Agric.* 48, 22-27.

Knapp S; van der Heijden MGA (2018). A global meta-analysis of yield stability in organic and conservation agriculture. *Nat. Commun.* 9, article 3632.

Koch A; Biedenkopf D; Furch A; Weber L; Rossbach O; Abdellatef E; Linicus L; Johannsmeier J; Jelonek L; Goesmann A; Cardoza V; McMillan J; Mentzel T; Kogel K-H (2016). An RNAi-based control of *Fusarium graminearum* infections through spraying of long dsRNAs involves a plant passage and is controlled by the fungal silencing machinery. *PLoS Pathog.* 12, e1005901.

Koch A; Biedenkopf D; Furch A; Weber L; Rossbach O; Abdellatef E; Linicus L; Johannsmeier J; Jelonek L; Goesmann A; Cardoza V; McMillan J; Mentzel T; Kogel K-H (2017). An RNAi-based control of *Fusarium graminearum* infections through spraying of long dsRNAs. In: Modern Fungicides and Antifungal Compounds, eds HB Deising; B Fraaije; A Mehl; EC Oerke; H Sierotzki & G Stammler, DPG Verlag, Braunschweig, pp. 63-66.

Koch E; Becker JO; Berg G; Hauschild R; Jehle J; Köhl J; Smalla K (2018). Biocontrol of plant diseases is not an unsafe technology! *J. Plant Dis. Protect.* 125, 121-125.

Köhl J; Postma J; Nicot P; Ruocco M; Blum B (2011). Stepwise screening of microorganisms for commercial use in biological control of plant-pathogenic fungi and bacteria. *Biol. Control* 57, 1-12.

Krause DJ; Kominek J; Opulente DA; Shen XX; Zhou X; Langdon QK; DeVirgilio J; Hulfachor AB; Kurtzman CP; Rokas A; Hittinger CT (2018). Functional and evolutionary characterization of a secondary metabolite gene cluster in budding yeasts. *Proc. Natl. Acad. Sci. USA* 115, 11030-11035.

Kubo Y; Suzuki K; Furusawa I; Yamamoto M (1983). Scytalone as a natural intermediate of melanin biosynthesis in appressoria of *Colletotrichum lagenarium*. *Exp. Mycol.* 7: 208-215.

Kubo Y; Takano Y; Endo N; Yasuda N; Tajima S; Furusawa I (1996). Cloning and structural analysis of the melanin biosynthesis gene *SCD1* encoding scytalone dehydratase in *Colletotrichum lagenarium*. *Appl. Environ. Microbiol.* 62, 4340-4344.

Lahlali R; Hijri M (2010). Screening, identification and evaluation of potential biocontrol fungal endophytes against *Rhizoctonia solani* AG3 on potato plants. *FEMS Microbiol. Lett.* 311, 152-159.

Ludwig N; Löhrer M; Hempel M; Mathea S; Schliebner I; Menzel M; Kiesow A; Schaffrath U; Deising HB; Horbach R (2014). Melanin is not required for turgor generation but enhances cell wall rigidity in appressoria of the corn pathogen *Colletotrichum graminicola*. *Mol. Plant-Microbe Interact.* 27, 315-327.

Lugtenberg B (2018). Putting concerns for caution into perspective: microbial plant protection products are safe to use in agriculture. *J. Plant Dis. Protect.* 125, 127-129.

Mukherjee PK; Horwitz B; Kenerley C (2012). Secondary metabolism in Trichoderma - A genomic perspective. *Microbiology* 158, 35-45.

Mukherjee PK; Horwitz BA; Herrera-Estrella A; Schmoll M; Kenerley CM (2013). Trichoderma research in the genome era. *Annu. Rev. Phytopathol.* 51, 105-129.

Netzker T; Fischer J; Weber J; Mattern DJ; König CC; Valiante V; Schroeckh V; Brakhage AA (2015). Microbial communication leading to the activation of silent fungal secondary metabolite gene clusters. *Front. Microbiol.* 6, article 299.

Nützmann H-W; Reyes-Dominguez Y; Scherlach K; Schroeckh V; Horn F; Gacek A; Schümann J; Hertweck C; Strauss J; Brakhage AA (2011). Bacteria-induced natural product formation in the fungus *Aspergillus nidulans* requires Saga/Ada-mediated histone acetylation. *Proc. Natl. Acad. Sci. USA* 108, 14282-14287.

O'Connell RJ; Thon MR; Hacquard S; Amyotte SG; Kleemann J; Torres MF; Damm U; Buiate EA; Epstein L; Alkan N; Altmuller J; Alvarado-Balderrama L; Bauser CA; Becker C; Birren BW; Chen Z; Choi J; Crouch JA; Duvick JP; Farman MA; Gan P; Heiman D; Henrissat B; Howard RJ; Kabbage M; Koch C; Kracher B; Kubo Y; Law AD; Lebrun MH; Lee YH; Miyara I; Moore N; Neumann U; Nordstrom K; Panaccione DG; Panstruga R; Place M; Proctor RH; Prusky D; Rech G; Reinhardt R; Rollins JA; Rounsley S; Schardl CL; Schwartz DC; Shenoy N; Shirasu K; Sikhakolli UR; Stuber K; Sukno SA; Sweigard JA; Takano Y; Takahara H; Trail F; van der Does HC; Voll LM; Will I; Young S; Zeng Q; Zhang J; Zhou S; Dickman MB; Schulze-Lefert P; Ver Loren van Themaat E; Ma LJ; Vaillancourt LJ (2012). Lifestyle transitions in plant pathogenic Colletotrichum fungi deciphered by genome and transcriptome analyses. *Nat. Genet.* 44, 1060-1065.

Peláez F (2005). Biological activities of fungal metabolites. In: *Handbook of Industrial Mycology*, ed Z An, pp. 49-92. Marcel Dekker, New York.

Rasimus-Sahari S; Teplova VV; Andersson MA; Mikkola R; Kankkunen P; Matikainen S; Gahmberg CG; Andersson LC; Salkinoja-Salonena M (2015). The peptide toxin amylosin of *Bacillus amyloliquefaciens* from moisture-damaged buildings is immunotoxic, induces potassium efflux from mammalian cells, and has antimicrobial activity. *Appl. Environ. Microbiol.* 81, 2939-2949.

Rosling H; Rosling O; Rosling Rönnlund A (2018). Factfulness: Ten Reasons We're Wrong About The World - And Why Things Are Better Than You Think. Hodder and Stoughton Ltd, London, UK.

Schiewe A; Mendgen K (1992). Identification of antagonists for biological control of the post-harvest pathogens *Pezicula malicorticis* and *Nectria galligena* on apples. *J. Phytopathol.* 134, 229-237.

Schroeckh V; Scherlach K; Nützmann HW; Shelest E; Schmidt-Heck W; Schuemann J; Martin K; Hertweck C; Brakhage AA (2009). Intimate bacterial-fungal interaction triggers biosynthesis of archetypal polyketides in *Aspergillus nidulans*. *Proc. Natl. Acad. Sci. USA* 106, 14558-14563.

Schueffler A; Anke T (2014). Fungal natural products in research and development. *Nat. Prod. Rep.* 31, 1425-1448.

Seufert V; Ramankutty N; Foley JA (2012). Comparing the yields of organic and conventional agriculture. *Nature* 485, 229-232.

Sieber CM; Lee W; Wong P; Münsterkötter M; Mewes HW; Schmeitzl C; Varga E; Berthiller F; Adam G; Güldener U (2014). The *Fusarium graminearum* genome reveals more secondary metabolite gene clusters and hints of horizontal gene transfer. *PLoS One* 9, e110311.

Singh HB (2014). Management of plant pathogens with microorganisms. *Proc. Indian Natn. Sci. Acad.* 80, 443-454.

Smith CA; Woloshuk CP; Robertson D; Payne GA (2007). Silencing of the aflatoxin gene cluster in a diploid strain of *Aspergillus flavus* is suppressed by ectopic *aflR* expression. *Genetics* 176, 2077-2086.

Smith LG; Kirk GJD; Jones PJ; Williams AG (2019). The greenhouse gas impacts of converting food production in England and Wales to organic methods. *Nature Commun.* 10, article 4641.

Spraker JE; Wiemann P; Baccile JA; Venkatesh N; Schumacher J; Schroeder FC; Sanchez LM; Keller NP (2018). Conserved responses in a war of small molecules between a plant-pathogenic bacterium and fungi. *mBio* 9, e00820-18.

Wood RKS; Tveit M (1955). Control of plant diseases by use of antagonistic organisms. *Bot. Rev.* 21, 441-492.

Yu J (2012). Current understanding on aflatoxin biosynthesis and future perspective in reducing aflatoxin contamination. *Toxins* 4, 1024-1057.

Yu J; Chang P.-K.; Ehrlich KC; Cary JW; Bhatnagar D; Cleveland TE; Payne GA; Linz JE; Woloshuk CP; Bennett JW (2004). Clustered pathway genes in aflatoxin biosynthesis. *Appl. Environ. Microbiol.* 70, 1253-1262.

Schmitt A et al., Replacement of Contentious Inputs in Organic Farming Systems (RELACS) – a comprehensive Horizon 2020 project. In: Deising HB; Fraaije B; Mehl A; Oerke EC; Sierotzki H; Stammler G (Eds), "Modern Fungicides and Antifungal Compounds", Vol. IX, pp. 229-230. © 2020. Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-16-7

Replacement of Contentious Inputs in Organic Farming Systems (RELACS) – a comprehensive Horizon 2020 project

Schmitt A[1], Pertot I[2a,b], Verrastro V[3], Magid J[4], Moeskops B[5], Möller K[6], Athanasiadou S[7], Experton C[8], Steinshamn H[9], Leiber F[10], Maurer V[10], Bünemann EK[10], Herforth-Rahmé J[10], Tamm L[10]

[1]Julius Kühn-Institut, Institut für Biologischen Pflanzenschutz, Heinrichstr. 243, D-64287 Darmstadt, Germany, [2a]Fondazione Edmund Mach, San Michele all'Adige, Italy, [2b] University of Trento, Trento, Italy, [3]Centro Internazionale di Alti Studi Agronomici Mediterranei, Bari, Italy, [4]University of Copenhagen, Copenhagen, Denmark, [5]International Federation of Organic Agriculture Movements EU Regional Group, Brussels, Belgium, [6]University of Hohenheim, Stuttgart, Germany, [7]Scotland's Rural College, Edinburgh, UK, [8]Institut Technique de l'Agriculture Biologique, Lyon, France, [9]The Norwegian Institute of Bioeconomy Research, Ås, Norway, [10]Research Institute of Organic Agriculture FiBL, Frick, Switzerland

Email: annegret.schmitt@julius-kuehn.de

INTRODUCTION AND OVERVIEW OF THE PROJECT

Organic farmers adhere to high standards in producing quality food while protecting the environment. However, organic farming aims for it´s improvement continuously to keep meeting its ambitious objectives. The project 'Replacement of Contentious Inputs in Organic Farming Systems' (RELACS) is fostering the development and adoption of cost-efficient and environmental safe tools and technologies to:

- Reduce the use of copper and mineral oil in plant protection,
- Identify sustainable sources for plant nutrition, and
- Provide solutions to support livestock health and welfare.

For preparation of the project, the progress achieved over the past decade by research, industry or farmers to replace these inputs was critically reviewed and the technology readiness level (TRL), cost-efficiency and potential for rapid adoption and implementation of alternatives was assessed. Bottlenecks for successful implementation of alternatives were also identified and appropriate actions to facilitate innovation and implementation were devised.

The project was developed by involving actors from research, farming, advisory services and industry from the very start, hence implementing a truly multi-actor approach. RELACS has 29 direct and third-party partners from 13 countries and is coordinated by the Research Institute of Organic Agriculture (FiBL) in Switzerland. RELACS is funded through Horizon 2020, the European Union's research and innovation framework programme under grant agreement No. 773431.

REPLACEMENT OF CONTENTIOUS INPUTS IN PLANT PROTECTION

The need for copper replacement has been recognized already in the 1990ies and international research initiatives were launched by the European Union in 2001 (EU-funded project Blight-MOP). Recently, the European Union launched a specific call (Call SFS-08-2017 Organic inputs – contentious inputs in organic farming) as part of the H2020 research programme in order to reduce the dependency of organic farming on problematic inputs, including copper and mineral oil.

RELACS builds on results of previous research projects and takes far-advanced solutions forward. In the case of copper reduction, plant extracts from *Glycyrrhiza glabra*, *Larix decidua*, and a milk derivative that already proved to be effective (Schmitt et al. 2017), were adopted. In addition, another promising plant extract (SUMB) was included. The crops and diseases addressed in the project are grapevine (infected by *Plasmopara viticola*), apple (*Venturia inaequalis*), and protected cultivation of cucumber (*Pseudoperonspora cubensis*) and tomato (*Phytophthora infestans*).

In the case of reduction of mineral oils, a plant extract from *Clitoria terneata*, orange oil and a vibrational mating disruption technique are applied and refined.

The products and management practices will be evaluated in different pedo-climatic and farming conditions in the EU and other Mediterranean countries. RELACS will develop implementation roadmaps by analysis of the socio-economic conditions required for acceptance and adoption of alternatives and will provide scientific support for relevant EU policies to develop fair, reliable and implementable rules. Rapid dissemination and adoption of techniques along the food value chain will be achieved via established dissemination structures in 12 European countries.

REFERENCE

Schmitt A; Scherf A; Mazzotta S; Kühne S; Pertot I; Köhl J; Markellou A; Andrivon D; Pellé R; Bousseau M; Chauvin JE; Thiéry D; Delière L; Kowalska J; Parveaud CE; Petit A; Giovinazzo R; Brenner J; Kelderer M; Lammerts van Bueren E; Bruns C; Finckh MR; Kleinhenz B; Smith J; Simon-Levert A; Pujos P; Trapman M; Stark J; van Cutsem P; Neerakkal S; Kleeberg H; Peters A; Tamm L (2017). CO-FREE Alternative Test Products for Copper Reduction in Agriculture. In: *Modern Fungicides and Antifungal Compounds*, Vol. VIII, eds H B Deising, B Fraaije; A Mehl; E C Oerke; H Sierotzki; G Stammler, pp. 267-272. Deutsche Phytomedizinische Gesellschaft: Braunschweig.

T.Vlasova, I.Ageeva, The Resistance of Verticillium dahliae to Cotton Phenolic Compounds in the Model System. In: Deising HB; Fraaije B; Mehl A; Oerke EC; Sierotzki H; Stammler G (Eds), "Modern Fungicides and Antifungal Compounds", Vol. IX, pp. 231-232. © 2020 Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-16-7

The Resistance of *Verticillium dahliae* to Cotton Phenolic Compounds in the Model System

Vlasova T[1]; Ageeva I[2]

[1]*Dept. of Plant Physiology, Fac. of Biology, Moscow State Lomonosov-University, Leninskije Gory 1-12, Moscow 119234, Russia, e-mail: tat_vla@list.ru*

[2]*Dept. of Hydrobiology, Fac. of Biology, Moscow State Lomonosov-University, Leninskije Gory 1-12, Moscow 119234, Russia, e-mail: : ageev@phys.chem.msu.ru*

INTRODUCTION

Microscopic fungi of the genus *Verticillium* belong to the most common and harmful phytopathogens. *Verticillium dahliae* Kleb. and *Verticillium albo-atrum* Reink. & Berth are causative agents of Verticillium wilt in many plants, particularly in cotton.

Action of plant antifungal substances reduces the ability of pathogen to penetrate plant tissues and to grow in them. So, the plant phenols and their oxidation products are being discussed as the important factors of the plant defense system, but their role is yet not completely clear. Verticillium wilt is a typical tracheomycosis; the pathogen grows predominantly in the plant vascular system. However, the initial events in the non-vascular tissues are supposed by many authors to be the crucial point of wilt infection establishment. The aim of the present work was to study the initial stage of Verticillium wilt pathogenesis and to reveal the presence of plant phenols and the possible reaction of the fungus to these substances.

MATERIALS AND METHODS

The sterile isolated cotton (*Gossypium hirsutum* L.) roots inoculated with a conidia suspension of a virulent strain of *V. dahliae* were used as a model system. Varieties of cotton were C-4727, highly susceptible to the wilt pathogen, and Tashkent-1, a relatively resistant variety. Light and electron microscopy were used to observe fungal penetration and distribution in roots. The total phenol contents were estimated with Folin's phenol reagent.

RESULTS AND DISCUSSION

The rhizodermal (epidermal) cells of the roots in the areas of inoculum application were almost 100% penetrated by the fungus in both varieties. However, its further propagation through the core parenchyma of the susceptible variety was more intensive.

The total phenol contents in the control and infected roots of the susceptible variety were similar throughout the experiment, while in the infected roots of the more resistant variety the phenol content increased with time compared to the control.

Histochemical tests with $FeCl_3$ revealed the accumulation of phenols in the rhizodermal cells contacting with the fungal mycelium, especially on their outer surface. As the fungus grew deep into the root, the color reaction to phenols in the rhizodermal cells and the core cells became more intense. The reaction was manifested not only in cells containing the fungal hyphae, but also in the cells adjacent to them. Our previous study detected that the activity of terminal oxidases, peroxidase and phenol oxidase, involved in the oxidation of phenols, in both varieties increased at the beginning of the penetration of the pathogen and levelled off gradually.

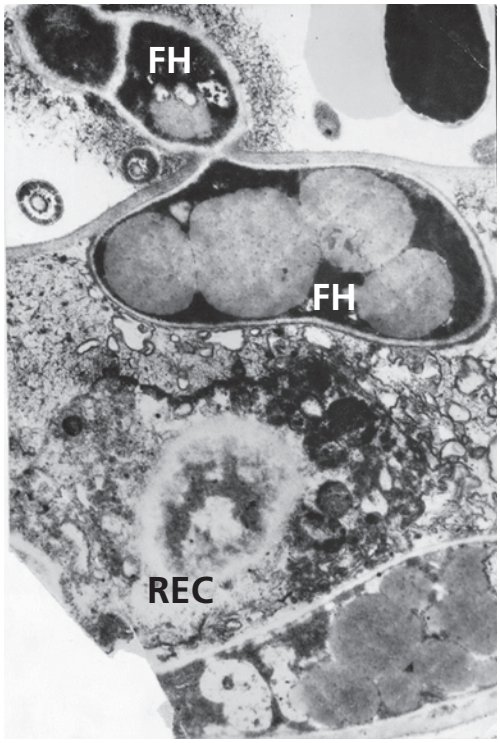


Figure 1

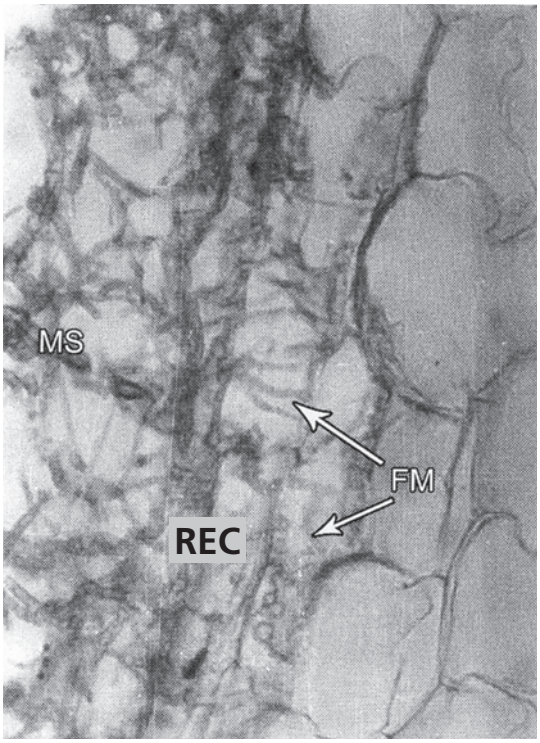


Figure 2

Figure 1 Fungal hypha penetrates root epidermal cell of the resistant cotton variety. Dark substances in root cell and in hypha are in all probability phenols. The large lipid inclusions are considered to be related to the fungal tolerance to plant toxic substances (REC root epidermal cell, FH fungal hypha; electron micrography).

Figure 2 Microsclerotia and hyphae on and in the root epidermis of resistant cotton variety. Histo-chemical reaction to phenols in cell walls of root epidermal cells and upper walls of core cells are darkly colored. Most hyphae are blocked in epi-dermis; however, few are detected in core cells. MS – microclerotia. REC – root epidermal cell. FM – fungal mycelium (light micrography).

The results show, that even intensive accumulation of phenolic compounds in the root cells and substances formed in the process of phenol oxidation do not completely inhibit the growth of *V. dahliae* in the root tissues. It demonstrates certain resistance of the fungus to these compounds.

Hortova B et al., Biological control of apple scab (*Venturia inaequalis*) by biostimulants. In: Deising HB; Fraaije B; Mehl A; Oerke EC; Sierotzki H; Stammler G (Eds), "Modern Fungicides and Antifungal Compounds", Vol. IX, pp. 233-234. © 2020. Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-16-7

Biological control of apple scab (*Venturia inaequalis*) by bio-stimulants

Hortova B, Jaklova P, Kloutvorova J

Research and Breeding Institute of Pomology Holovousy Ltd, Holovousy 129, 50801 Horice, Czechia

Corresponding Author Email: Bronislava.HORTOVA@vsuo.cz

INTRODUCTION

Apple scab, the most important disease in organic and conventional apple orchards, is caused by fungus *Venturia inaequalis* (Cooke) G. Winter (1875) (MacHardy 1996). Apple production is one of the traditional branches of the Czech fruit growing. In the Czech Republic, more than 6 875 thousand hectares of apple-tree plantations are recorded (Buchtova 2018). Management of apple scab in conventional orchards currently depends on applications of fungicides. Plant protection products, for use in organic fruit growing, must comply with the principles for organic production. In this study we tested alternative fungicides, which activate the plant´s natural defense reactions against fungal infections.

MATERIAL AND METHODS

A field trial in the experimental orchard in Holovousy with three bio-stimulants (Chitopron 5 % - active ingredient chitosan hydrochlorid, Imunofol - active ingredients zinc, amino acid and chitosan, and PowerOf-K - active ingredients K_2O, micronutrients, soluble boron, soluble molybdenum) was conducted on variety Golden Delicious. In the trial, we used untreated control and chemical standard (fungicide Captan 80 WG - active ingredient captan). Ten applications were made (April 24; May 01, 07, 14, 22, and 31; June 07, 13, 21, and 27). On July 25th, infection of leaves and fruits was evaluated according to the scale: 0 – no attack, 1 – 1-2 spots per leaf/fruit, 2 – 3-4 spots per leaf/fruit, 3 – 5-10 spots per leaf/fruit, 4 - >10 spots per leaf/fruit. Disease severity (according to Townsend-Heuberger´s formula) and fungicide efficacy (according to Abbott´s formula) were calculated.

RESULTS AND DISCUSSION

Plants of the untreated control had the highest level of scab infection - leaves 42.3 %, fruits 17,3 %). The chemical standard had almost 100% efficiency - percentage scab infection: leaves 0.5 %, fruits 0.0 %). The highest efficacy of bio-stimulants was observed for plots treated with PowerOf-K (percentage scab infection: leaves 7.5 %; fruits 1.0 %). PowerOf-K is a liquid potassium fertilizer with side effects on fungal pathogens. This product has two mechanisms of

antifungal activity: change of osmotic pressure in the fungal cell (dehydration or cracking of the mycelium) and increase of the pH value >10 on the surface of plants, thereby creating zones with unsuitable conditions for actively growing fungi. Kelderer et al. (2008) tested a potassium-based product in a field trial; the percentage of scab infection was: 4.3 and 2.2% on leaves and fruits, respectively. The product with lower efficiency was Chitopron 5% (scab infection: leaves 7.5 %; fruits 2.5 %). The lowest efficiency was in plots treated with Imunofol (scab infection: leaves 9.3 %; fruits 2.3 %). The efficacy of chitosan-based products was tested by Kunze and Hinze (2014), who demonstrated an efficacy of about 75 %. Felipini et al. (2016) did not confirm the positive effect of chitosan against *V. inaequalis*. Meszska et al. (2006) reported that cihitosan activity against apple scab was about 50 %, which was less than that of Captan 80 WG.

The results obtained with bio-stimulants in our study are very variable. Efficacy of the products tested depends on many factors, such as climatic conditions during application, phenological phase of fruit tree, nutritional status of fruit tree, whether the fruit tree is under stress and the doses of the active substances. These products are readily biodegradable, so do not create residues and have a potential to be used in organic fruit production. Our results are for one year only and we will continue to study these substances.

ACKNOWLEDGEMENTS

This research was supported by NPU LO1608.

REFERENCES

Buchtová I (2018). Situační a výhledová zpráva – ovoce: listopad 2018. *Ministerstvo zemědělství České republiky*, p. 89. ISBN 978-80-7434-473-2.

Felipini RB; Boneti JI; Katsurayama Y; Rocha Neto AC; Veleirinho B; Maraschin M; Di Piero RM (2016). Apple scab control and activation of plant defense responses using potassium phosphite and chitosan. *European Journal of Plant Pathology* 145, 929-939.

Kelderer M; Claudio C; Lardschneider E (2008). Formulated and unformulated carbonates to control apple scab (*Venturia inaequalis*) on organic apple.*: Ecofriut - 13th International Conference on Cultivation Technique and Phytopathological Problems in Organic Fruit-Growing*. M. Boos (Ed.), pp 47 -53. Proceedings to the Conference from 18th February to 20th February 2008 at Weinsberg/Germany.

Kunz S; Hinze M (2014). Assessment of biocontrol agents for their efficacy against apple scab. *Ecofruit - 16th International Conference on Cultivation Technique and Phytopathological Problems in Organic Fruit-Growing*. Fördergemeinschaft ökologischer Obstbau e.V. (Ed.), pp 65 -71. Proceedings to the Conference from 17th February to 19th February 2014 at Weinsberg/Germany.

MacHardy WE (1996). Apple Scab, Biology, Epidemiology, and Management. St. Paul, Minn., USA, American Phytopathological Society.

Meszka B; Bielenin A (2006). Non-chemical possibilities for control of apple fungal diseases. *Phytopathol. Pol.* 39, 63–70.

Ageeva L, Vlasova T., Multifactor Effects of Fullerens of Shungite on the Morphology and Heterogeneity of Verticillium dahliae. In: Deising HB; Fraaije B; Mehl A; Oerke EC; Sierotzki H; Stammler G (Eds), "Modern Fungicides and Antifungal Compounds", Vol. IX, pp. 235-236. © 2020 Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-16-7

Multifactor Effects of Fullerenes of Shungite on the Morphology and Heterogeneity of *Verticillium dahliae*

Ageeva I[1], Vlasova T[2]

[1]*Dept. of Hydrobiology, Fac. of Biology, Moscow State Lomonosov-University, Leninskije Gory 1-12, Moscow 119234, Russia, e-mail: ageev@phys.chem.msu.ru*

[2]*Dept. of Plant Physiology, Fac. of Biology, Moscow State Lomonosov-University, Leninskije Gory 1-12, Moscow 119234, Russia,*

Email corresponding author: tat_vla@list.ru

INTRODUCTION

Fullerenes are carbon nanoparticles. It is one of the structural forms of carbon, along with graphite and diamond. Hydrophilic fullerenes exhibit fungicidal and bactericidal activity against microscopic fungi and bacteria that are resistant to many fungicides and antibacterial substances. The mechanism of action of fullerenes as fungicidal and bactericidal substances has not been studied enough and is likely to be multifactorial.

The aim of the research was to study the effect of various concentrations of shungite on the heterogeneity of the pathogenic fungus *Verticillium dahliae* in culture and its morphological characteristics.

MATERIALS AND METHODS

For the experiment, the phytopathogenenic fungus *Verticillium dahliae* Kleb. was cultured in Chapek medium. Shungite in the quantities of 10, 50 and 100 g / l of the medium was added once. Previously, shungite granules were washed with cold water, incubated for 2 days, then washed with distilled water to remove impurities and autoclaved. After this treatment, shungite was added to the culture medium. Fungal culture without the addition of shungite was used as control**.**

The number of cells was counted in a Goryaev chamber under a light microscope. The determination of living and dead cells in the culture was carried out using a luminescent microscope.

RESULTS AND DISCUSSION

In the culture of *V. dahliae* with the addition of shungite in the concentration of 10g/l of the medium, active germination of conidia in deep-seated culture, rapid accumulation of biomass with clearly expressed heteromorphism of mycelial cords were observed. In the stationary culture, the full cycle of development of *V. dahliae* was reproduced.

The addition of 50g/l shungite led to inhibition of the development of the fungal culture, slowing down the rate of conidia germination and the formation of homogeneous mycelium and its differentiation, as well as the lack of oidia formation, i.e., an incomplete development cycle. Heterogeneity of fungal morphology may be explained as a result of toxic action of fullerenes. The cell sizes and contents were different.

The formation of the endospores was also shown in some hyphae. The formation of endospores is accepted by some authors as defense response of fungus to unfavorable environmental conditions.

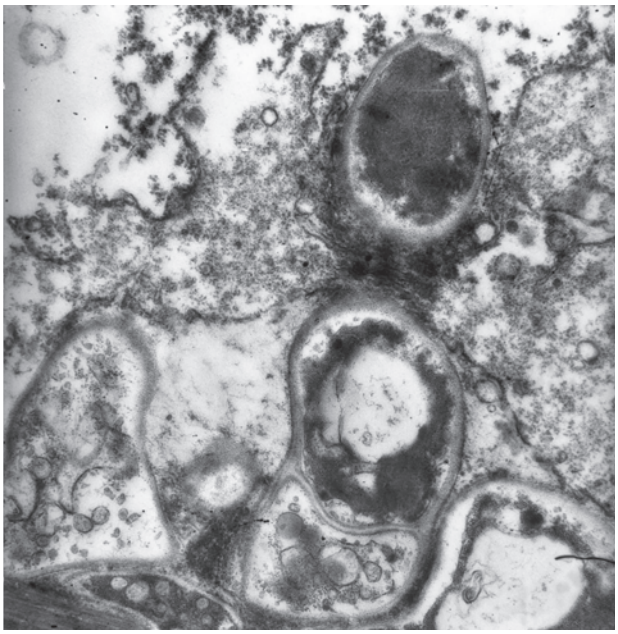


Figure 1

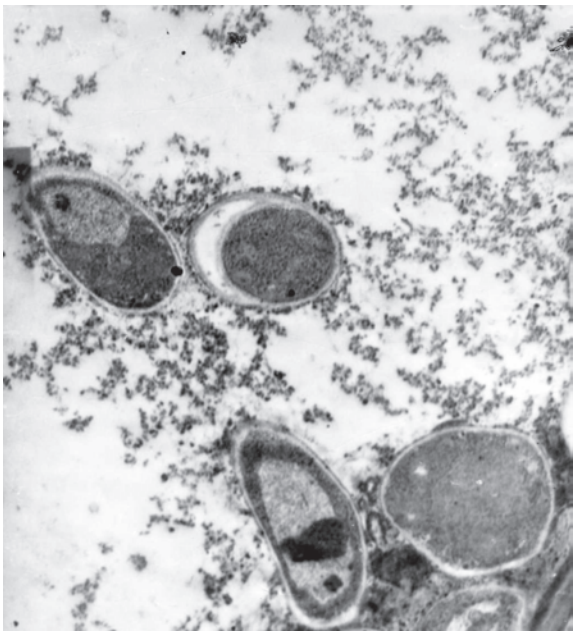


Figure 2

Figure 1 The degradation of some *V. dahliae* hyphae in culture with shungite 100 g/l of the medium.

Figure 2 Heterogeneity of *V. dahliae* cells in culture with shungite 50 g/l of the medium. Endospore formation can be observed in upper right hyphae. Dark osmiophilic inclusions in some fungal vacuoles could be connected with the damage by toxic substances.

When shungite was added in the concentration of 100 g/l, the germination of conidia and the development of the fungus was almost completely blocked; it was accompanied by the formation of resting structures (sclerotia). The results demonstrate that the relation between the concentration of shungite and fullerene, respectively, and changes in fungal development and characteristics is not linear and is rather complicated. It supports the idea that the mode of action of fullerenes may be multifactorial.

NEW TECHNOLOGIES AND APPLICATIONS

Conrath U et al, Bifunctional Fusion Peptides and Microgel-Based Release Systems for Plant Health. In: Deising HB; Fraaije B; Mehl A; Oerke EC; Sierotzki H; Stammler G (Eds), "Modern Fungicides and Antifungal Compounds", Vol. IX, pp. 239-247. © 2020. Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-16-7

Bifunctional Fusion Peptides and Microgel-Based Release Systems for Plant Health

Conrath U[1,4], Schwinges P[1,4], Jakob F[2,3,4], Langenbach C[1,4], Schwaneberg U[2,3,4]

[1]*Department of Plant Physiology, RWTH Aachen University, Aachen 52056, Germany*
[2]*Department of Biotechnology, RWTH Aachen University, Aachen 52056, Germany*
[3]*DWI Leibniz Institute for Interactive Materials, Aachen 52074, Germany*
[4]*Bioeconomy Science Center (BioSC), c/o Research Center Jülich, Jülich 52425, Germany*
Corresponding Author Email: uwe.conrath@bio3.rwth-aachen.de

INTRODUCTION

Pesticides fight insects, microbes, and other possibly harmful organisms (Enserink et al. 2013). Their main use is in agriculture where they help securing crop yield (Oerke 2005). Because many pesticides are washed off by rain (Hunsche et al. 2006) they need to be applied multiple times in a growing season. Sometimes pesticides hit nontarget organisms (Woodcock et al. 2016) and drive the evolution of pesticide-insensitive weeds, insects, and microbes that are hard to control. Thus, although they are safer than ever (Lamberth et al. 2013) some synthetic pesticides can raise ecological and health concerns (Mascarelli 2013; Rauh et al. 2012). Therefore, complementary or alternative pest management products are needed.

Antimicrobial peptides (AMPs) are prevalent in nature. Although they have different modes of action all AMPs destabilize biomembranes (Rangarajan et al. 2013; Tossi et al. 2000). This is probably why they possess antimicrobial activity (Zasloff 2002). AMPs are subjected to residue-free degradation and, therefore, do not pollute the environment. As most pests and pathogens attack plants from the outside, functionalizing the crop surface with AMPs could provide an alternative to the pest and disease management that is based on synthetic chemical compounds. Dermaseptins (DSs), for example, are a family of amphiphilic AMPs of 28-34 amino acid residues (Brand et al. 2002; De Lucca et al. 1998; Leite et al. 2008; Mor 2000; Salay et al. 2011). They originate from the skin of tree frogs (Brand et al. 2002; Leite et al. 2008) and effectively fight bacteria, protozoa, yeasts, and filamentous fungi without harming mammalian cells (Brand et al. 2002; De Lucca et al. 1998; Leite et al. 2008; Mor 2000; Salay et al. 2011). Therefore, DSs are prime candidates for natural pesticides.

The aerial surface of land plants is represented by the cuticle (Bargel et al. 2004). It consists of the crosslinked biopolymer cutin that has intracuticular and epicuticular waxes. The latter are complex mixtures of water-repellent lipophilic compounds (esters of long-chain fatty acids with long-chain aliphatic and cyclic alcohols, phytosterols, pentacyclic triterpenoids, or epicuticular flavonoids) (Bargel et al. 2004). Small peptides that adhere to epicuticular waxes and stick attached AMPs to the wax layer could be used to functionalising the plant surface. They are thus promising for sustainable disease and pest management. In fact, peptide-based adhesion

promoters have been shown to allow for the immobilization of peptides or proteins on multiple surfaces, including hydrophobic ones (Bolivar et al. 2017; Rübsam et al. 2017a; 2017b; Sanghvi et al. 2005).

Soybean (*Glycine max*) provides amino acids, protein, and oil and, thus, belongs to the most important crops on the globe (Singh 2010). Its major disease is caused by *Phakopsora pachyrhizi*, a biotroph fungus that causes Asian soybean rust (SBR) (Langenbach et al. 2016). The disease spreads fast and hits plants hard, defoliating fields within a few days and reducing yield by up to 80% (Pennissi 2010). The best defense would be a soybean variety that resists *P. pachyrhizi*. However, no variety that would resist all genotypes of the fungus is grown today (Langenbach et al. 2016). SBR is rather controlled by synthetic fungicides to which *P. pachyrhizi* increasingly evolves resistance (Godoy et al. 2016; Langenbach et al. 2016). Thus, alternative measures to controlling SBR are urgently needed.

Here we introduce a promising alternative to conventional pest management with synthetic pesticides. We demonstrate that the amphiphilic peptide thanatin (THA), upon spray application, sticks enhanced green fluorescent protein (eGFP) (Zimmer 2002) to the surface of soybean leaves in a rainfast manner. We also report that a bifunctional fusion peptide, DS01-THA, inhibits the germination of *P. pachyrhizi* spores *in vitro* and alleviates SBR symptoms on soybean plants.

RESULTS

Anchor Peptides Stick Protein to Soybean Leaves

To find amphipathic peptides that could serve as adhesion promotors to functionalizing soybean leaves, we based on structural analysis selected the natural amphiphilic peptides tachystatin A2 (TA2) (Osaki et al. 1999), liquid chromatography peak I (LCI) (Gong et al. 2011), THA (Fehlbaum et al. 1996), and lactoferricin B (LFB) (Jones et al. 1995) for testing their binding to soybean leaves (Schwinges et al. 2019). We used genetic engineering to fuse each of these peptides to the eGFP reporter protein to allow for easy detection of the fusion protein in the later assays. We produced the fusion proteins and eGFP (control) in *Escherichia coli*. We then punched out soybean leaf discs and floated them on the adequate *E. coli* cell extract. Floating on buffer served as a control. Ten minutes later, leaf discs were intensively washed in water and assayed for green fluorescence by microscopy. The green fluorescence is indicative of presence of eGFP or the adequate fusion protein. We found that eGFP-LCI and eGFP-THA, but not eGFP-TA2, eGFP-LFB, or eGFP alone bound to the surface of soybean leaf discs in a washing-resistant manner (Schwinges et al. 2019). These findings pointed to LCI and THA as likely suited anchor peptides to functionalizing the soybean leaf surface.

We then purified eGFP-THA, eGFP-LCI, and eGFP from the crude *E. coli* extracts and sprayed aqueous solutions of these proteins onto soybean leaves. After drying off and intensive washing we confirmed the water-resistant binding of eGFP-THA and eGFP-LCI, but not eGFP, to intact soybean leaves (Schwinges et al. 2019).

Long-Term Binding in Semi-Field Condition

To evaluate the persistence of a fusion protein on soybean we sprayed leaflets of the first trifolium of soybean plants with eGFP-THA. Upon drying off, we transferred the plants outdoors and assayed them for presence of eGFP fluorescence at various times over 17 d. By doing so we found that while eGFP fluorescence seemed to decrease with time, eGFP presence was still clearly seen at 17 days after eGFP-THA application (Schwinges et al. 2019). During the test period there was only one moderate rainfall event (0.3 L/m² total precipitation) to which, according to our above findings, eGFP-THA is rather insensitive (Schwinges et al. 2019). Therefore, the observed moderate reduction of eGFP fluorescence over time may be caused by loss of eGFP from the eGFP-THA fusion peptide due to high temperature, sunlight, microbial degradation, or combinations of these possibilities.

Binding to Monocotyledonous Crops

Barley (*Hordeum vulgare*) is used as animal fodder, for brewing distilled beverages (e.g. beer), and as a component of stews, bread and various foods (Zohary et al. 2012). Corn (*Zea mays*), which has become a staple food in many parts of the world, is also used to produce ethanol, animal feed, and other products such as sweet corn and popcorn (Smith 2013). We used eGFP-THA and eGFP-LCI to see whether the amphiphilic peptides THA and LCI, in addition to the eudicot soybean, would anchor eGFP also to the leaf surface of monocotyledonous plants and if they would do so in a water-resistant manner. To do so, we sprayed leaves of barley and corn with the eGFP-THA and eGFP-LCI fusions, as well as with eGFP (control). Upon drying off, sprayed leaves were intensively rinsed with water. We found that the eGFP-THA and eGFP-LCI fusion proteins, but not eGFP alone, sticked to the leaves of barley and corn in a water-resistant manner (Schwinges et al. 2019). Thus, the amphiphilic peptides THA and LCI seem to be useful anchor peptides for functionalizing the surface of monocot and eudicot crops in a rinsing-proof manner.

Peptide-Mediated Anchoring Withstands Rain

Our above results suggested that the THA and LCI-mediated adhesion of eGFP to crop leaves would resist rain. To investigate whether this is true we treated leaves of soybean and barley plants that we had grown in a field with purified eGFP-THA, eGFP-LCI, and eGFP. After treatment, the plants within three days experienced a light, two moderate and a heavy rainfall event (16.2 mm total precipitation). Upon careful inspection we found that almost all (~98%) sprayed eGFP was washed off soybean leaves by the natural rain. However, ~11% of eGFP-LCI and ~40% eGFP-THA remained on the leaves as indicated by remaining green fluorescence. For barley, only ~7% unanchored eGFP, but ~65% eGFP-LCI and ~73% eGFP-THA were still found on the leaf surface after rain (Schwinges et al. 2019). Together, these findings revealed that THA adheres more strongly than LCI to the leaves of soybean and barley plants. Because these findings were made in the field and after natural rain, they disclosed the general suitability of THA and LCI for functionalizing the above-ground crop surface for applied pest and disease management.

Binding to Surface Wax

Next, we investigated whether the amphiphilic peptides THA and LCI would anchor eGFP to leaves by interaction with the hydrophobic surface wax. We sprayed the eGFP-THA and eGFP-LCI fusion peptides on leaves of the surface wax-reduced mutant *cer-j*^{59} of barley (Giese 1976) and on corn leaves of which we had the surface wax removed. Spraying leaves of the barley wild type and corn plants, which both had an intact surface wax layer, served as controls. After drying off, sprayed leaves were thoroughly rinsed with water. We did not include soybean in these assays because wax-deficient soybean mutants are not available and because the aerial soybean surface is highly enriched in trichomes, which precludes noninvasive detachment of surface wax from this species of plant. We found that the eGFP-THA and eGFP-LCI fusion proteins, but not eGFP alone, adhered to leaves of barley and corn in a rinsing-resistant manner. However, both proteins did not adhere to the barley *cer-j*^{59} mutant or to corn leaves with their surface wax removed (Schwinges et al. 2019). This result revealed that the adhesion-promoting peptides THA and LCI bind most likely to epicuticular waxes on plant leaves.

A DS01-THA Fusion Peptide Alleviates SBR

DS01 is an amphiphilic peptide that can protect soybean from *P. pachyrhizi* infection (Brand et al. 2012). To investigate whether DS01, THA, or a bifunctional fusion peptide of DS01 and THA would reduce SBR disease, we sprayed soybean leaves with water (control), THA, DS01, or a bipartite DS01-THA fusion before we inoculated the leaves with urediospores of *P. pachyrhizi*. We found that DS01 and THA alone did not significantly reduce the infection severity of soybean leaves. However, the DS01-THA fusion peptide, in the absence of any surfactant, diminished SBR symptoms by almost 30% (Schwinges et al. 2019). In the field, this would roughly correspond to a 20% yield increase (Dalla Lana et al. 2015). Notably, the protection by DS01-THA was fully retained when treated leaves were extensively rinsed with water before they were inoculated with *P. pachyrhizi* (Schwinges et al. 2019). Moreover, the DS01-THA fusion peptide inhibited the formation of *P. pachyrhizi* appressoria *in vitro*. The antifungal activity of DS01-THA *in vitro* was sensitive to protease digestion which clarifies that the DS01-THA fusion peptide is the likely cause of the observed decrease in SBR symptoms (Schwinges et al. 2019).

DISCUSSION

Some synthetic pesticides cause ecological and health concerns and promote the evolution of pesticide-resistant pests and pathogens. Therefore, alternative approaches for crop management are needed. We showed that THA and LCI, two natural peptides, can be used as adhesion promoters to immobilize proteins or peptides (e.g. eGFP, DS01) to leaves of soybean, barley and corn. The chemical component(s) to which THA and LCI bind in surface wax are still unknown. However, because epicuticular waxes constitute complex mixtures of mainly lipophilic compounds (such as esters of long-chain fatty acids with long-chain aliphatic and cyclic alcohols, phytosterols, pentacyclic triterpenoids, or epicuticular flavonoids) (Bargel et al.

2004; Koch & Ensikat 2008) and because THA and LCI are amphiphilic peptides, a binding through hydrophobic interactions is very likely (Fehlbaum et al. 1996; Gong et al. 2011). The anchor peptide-mediated binding resists washing and rain and seems to not impair the physiology of the plant (Schwinges et al. 2019). On outdoor grown soybean plants, binding of the fusion peptide to a major part also withstands heat, sunlight, and microbial degradation for at least 17 d (Schwinges et al. 2019). Therefore, we conclude that the anchor peptide-mediated binding of the fusion peptide largely withstands major environmental challenges over a reasonable time and, thus, has potential for field application.

The anchor peptide-mediated binding is independent of the type of leaf, as THA and LCI anchored eGFP to primary leaves, and the first and second trifolium of soybean plants (Schwinges et al. 2019). Experiments with the barley *cer-j*^{59} mutant and surface wax-free corn leaves disclosed that the amphiphilic anchor peptides stick to the surface wax layer of leaves, thus presumably allowing noninvasive crop protection.

All land plants possess a cuticle with intracuticular and epicuticular wax or a hydrophobic periderm (Bargel et al. 2004). In addition, amphiphilic anchor peptides seem to stick to the hydrophobic surface of diverse crops, though this seems to be with different strength. Modern methods in protein engineering enabled tailoring of LCI's binding strength to polypropylene and polystyrene (Rübsam et al. 2017b; 2018) which are both hydrophobic. The same techniques can be used to tailoring the binding intensity of anchor peptides to the surface of essentially any land plant. Our approach thus provides a unique opportunity for developing a platform technology to functionalizing the above-ground plant surface for diverse purposes. We expect that bifunctional peptides or proteins consisting of plant-sticking anchor peptides and pesticidal peptides or proteins can be used to fight essentially any pest and disease in a rainfast manner. In fact, a THA-DS01 fusion peptide also inhibits development of the anthracnose-causing pathogen *Colletotrichum graminicola* on corn leaves (Schwinges et al. 2019). Because the here introduced technology utilizes natural AMPs with an unspecific mode of action, there seems to be (i) no threat to human health and the environment, (ii) a reduced chance of developing AMP resistance in the pathogen, and (iii) no need for genetic modification of the targeted crop. In addition, while synthetic pesticides are prone to washing off by rain and dew, bifunctional peptides to a remarkable degree seem to resist these weather events and are thus promising for sustainable next-generation agriculture. In the future, adjustable microgels outfitted with anchor peptides could serve as sticky containers for the controlled release of agrochemicals (Meurer et al. 2017) thus also supporting reduced pesticide use.

CONCLUSIONS

In an interdisciplinary approach of plant pathology and protein engineering we developed a sustainable mean of functionalizing leaves to fight soybean's most severe disease, that is SBR (Figure 1). Using eGFP as a reporter macromolecule, we showed that THA can be used to stick the protein in a rainfast manner to the surface of soybean, barley and corn leaves. The anchoring

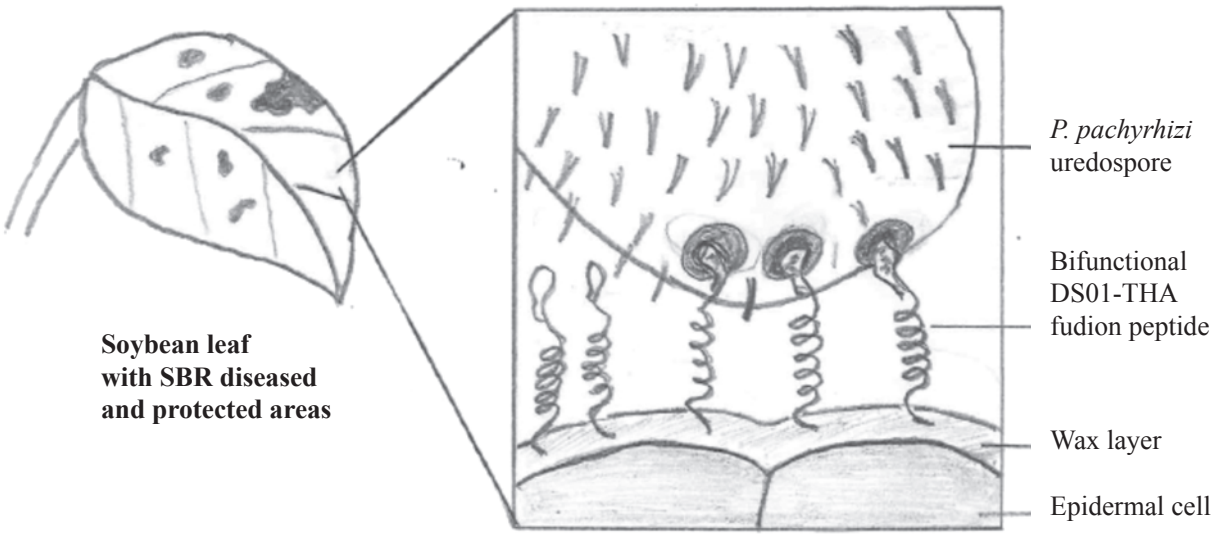


Figure 1 A bifunctional DS01-THA fusion peptide in a rainfast manner sticks to soybean leaves and harms *P. pachyrhizi* thus resulting in SBR reduction.

seems to occur on the surface wax of leaves and withstands high temperature, sunlight, and biotic degradation for at least 17 days. When THA was used to stick the antimicrobial peptide DS01 to soybean leaves, SBR was markedly reduced. We expect that bifunctional peptides or proteins consisting of an adequate adhesion peptide (e.g. THA, LCI) and antimicrobial or pesticidal peptides, proteins, or cells can be used to fight essentially any plant pest and disease in an eco-friendly manner. Adjustable microgels outfitted with anchor peptides could serve as sticky containers for the controlled release of natural or synthetic agrochemicals in next-generation agriculture.

ACKNOWLEDGMENTS

We thank Ulrich Schaffrath for providing the *cer-j*[59] mutant, Holger Deising for the *C. graminicola* strain and Lukas Schreiber for discussion. We appreciate provision of DS01-THA by Numaferm. The scientific activities of the Bioeconomy Science Center are financially supported by the Ministry of Innovation, Science and Research in the NRW Strategy Project BioSC (No. 313/323-400-00213).

CONFLICT OF INTEREST

A patent application (WO 2016/134806) covering the in this article described work has been filed by RWTH Aachen University.

REFERENCES

Bargel H; Barthlott W; Koch K; Schreiber L; Neinhuis C (2004). Plant cuticles: multifunctional interfaces between plant and environment. In: *The Evolution of Plant Physiology*, eds. A R Hemsley & I Poole, pp. 171-194. Elsevier: London.

Bolivar JM; Gascon V; Marquez-Alvarez C; Blanco RM; Nidetzky B (2017). Oriented coimmobilization of oxidase and catalase on tailor-made ordered mesoporous silica. *Langmuir* 33, 5065-5076.

Brand GD; Leite JR; Silva LP; Albuquerque S; Prates MV; Azevedo RB; Carregaro V; Silva JS; Sá VC; Brandão RA; Bloch Jr C (2002). Dermaseptins from *Phyllomedusa oreades* and *Phyllomedusa distincta*. Anti-*Trypanosoma cruzi* activity without cytotoxicity to mammalian cells. *Journal of Biological Chemistry* 277, 49332-49340.

Brand GD; Magalhães MTQ; Tinoco MLP; Aragão FJL; Nicoli J; Kelly SM; Cooper A; Bloch Jr C (2012). Probing protein sequences as sources for encrypted antimicrobial peptides. *PLOS ONE* 7, e45848.

Dalla Lana F; Ziegelmann PK; K, A. de H N Maia A; Godoy CV; Del Ponte EM (2015). Meta-analysis of the relationship between crop yield and soybean rust severity. *Phytopathology* 105, 307-315.

De Lucca AJ; Bland JM; Jacks TJ; Grimm C; Walsh TJ (1998). Fungicidal and binding properties of the natural peptides cecropin B and dermaseptin. *Medical Mycology* 36, 291-298.

Enserink M; Hines, PJ; Vignieri SN; Wigginton NS; Yeston JS (2013). The pesticide paradox. *Science* 341, 729.

Fehlbaum P; Bulet P; Chernysh S; Briand JP; Roussel JP; Letellier L; Hetru C; Hoffmann JA (1996). Structure-activity analysis of thanatin, a 21-residue inducible insect defense peptide with sequence homology to frog skin antimicrobial peptides. *Proceedings of the National Academy of Sciences of the USA* 93, 1221-1225.

Giese B (1976). Roles of the *cer-j* and *cer-p* loci in determining the epicuticular wax composition on barley seedling leaves. *Heredias* 82, 137-148.

Godoy CV; Seixas CDS; Soares RM; Marcelino-Guimarães FC; Meyer MC; Costamilan LM (2016). Asian soybean rust in Brazil: past, present, and future. *Pesquisa Agropecuária Brasileira* 51, 407-421.

Gong W; Wang J; Chen Z; Xia B; Lu G (2011). Solution structure of LCI, a novel antimicrobial peptide from *Bacillus subtilis*. *Biochemistry* 50, 3621-3627.

Hunsche M; Bringe K; Schmitz-Eiberger M; Noga G (2006). Leaf surface characteristics of apple seedlings, bean seedlings and kohlrabi plants and their impact on the retention and rainfastness of mancozeb. *Pest Management Science* 62, 839-847.

Jones EM; Smart A; Bloomberg G; Burgess L; Millar MR (1994). Lactoferricin, a new antimicrobial peptide. *Journal of Applied Bacteriology* 77, 208-214.

Koch K; Ensikat H-J (2008). The hydrophobic coatings of plant surfaces: Epicuticular wax crystals and their morphologies, crystallinity and molecular self-assembly. *Micron* 39, 759-772.

Lamberth C; Jeanmart S; Luksch T; Plant A (2013). Current challenges and trends in the discovery of agrochemicals. *Science* 341, 742-746.

Langenbach C; Campe R; Beyer SF; Müller A; Conrath U (2016). Fighting Asian soybean rust. *Frontiers in Plant Science* 7, 797.

Leite JR; Brand GD; Silva LP; Kückelhaus SA; Bento WR; Araújo AL; Martins GR; Lazzari AM; Bloch Jr C (2008). Dermaseptins from *Phyllomedusa oreades* and *Phyllomedusa distincta*: secondary structure, antimicrobial activity, and mammalian cell toxicity. *Comparative Biochemistry and Physiology - Part A: Molecular & Integrative Physiology* 151, 336-343.

Mascarelli A (2013). Growing up with pesticides. *Science* 341, 740-741.

Meurer RA; Kemper S; Knoop S; Eichert T; Jakob F; Goldbach HE; Schwaneberg U; Pich A (2017). Bifunctional microgel-based fertilizers for controlled foliar delivery of nutrients to plants. *Angewandte Chemie International Edition* 56, 7380-7386.

Mor A (2000). Peptide-based antibiotics: a potential answer to raging antimicrobial resistance. *Drug Development Research* 50, 440-447.

Oerke E-C (2005). Crop losses to pests. *Journal of Agricultural Science* 144, 31-43.

Osaki T; Omotezako M; Nagayama R; Hirata M; Iwanaga S; Kasahara J; Hattori J; Ito I; Sugiyama H; Kawabata S (1999). Horseshoe crab hemocyte-derived antimicrobial polypeptides, tachystatins, with sequence similarity to spider neurotoxins. *Journal of Biological Chemistry* 274, 26172-26178.

Pennissi E (2010). Armed and dangerous. *Science* 327, 804-805.

Rangarajan N; Bakshi S; Weisshaar JC (2013). Localized permeabilization of *E. coli* membranes by the antimicrobial peptide cecropin A. *Biochemistry* 52, 6584-6594.

Rauh VA; Perera FP; Horton MK; Whyatt RM; Bansal R; Hao X; Liu J; Boyd Barr D; Slotkin TA;Peterson BS (2012). Brain anomalies in children exposed prenatally to a common organophosphate pesticide. *Proceedings of the National Academy of Sciences of the USA* 109, 7871-7876.

Rübsam K; Stomps B; Böker A; Jakob F; Schwaneberg U (2017a). Anchor peptides: A green and versatile method for polypropylene functionalization. *Polymer* 116, 124-132.

Rübsam K; Weber L; Jakob F; Schwaneberg U (2017b). Directed evolution of polypropylene and polystyrene binding peptides. *Biotechnology and Bioengneering* 115, 321-330.

Rübsam K; Davari MD; Jakob F; Schwaneberg U (2018). KnowVolution of the polymer binding peptide LCI for improved polypropylene binding. *Polymers*, 10, 423.

Salay LC; Nobre TM; Colhone MC; Zaniquelli ME; Ciancaglini P; Stabeli RG; Leite JR; Zucolotto V (2011). Dermaseptin 01 as antimicrobial peptide with rich biotechnological

potential: study of peptide interaction with membranes containing *Leishmania amazonensis* lipid-rich extract and membrane models. *Journal of Peptide Science* 17, 700-707.

Sanghvi AB; Miller KPH; Belcher AM; Schmidt CE (2005). Biomaterials functionalization using a novel peptide that selectively binds to a conducting polymer. *Nature Materials*, 4, 496-502.

Schwinges P; Pariyar S; Jakob F; Rahimi M; Apitius L; Hunsche M; Schmitt L.; Noga G; Langenbach C; Schwaneberg U; Conrath U (2019). A bifunctional dermaseptin-thanatin dipeptide functionalizes the crop surface for sustainable pest management. *Green Chemistry* 21, 2316-2325.

Singh G (2010). *The Soybean: Botany, Production and Uses*. CAB International: Oxfordshire

Smith AF (2013). *The Oxford Encyclopedia of Food and Drink in America.* 2nd edn. Oxford University Press:Oxford.

Tossi A; Sandri L; Giangaspero A (2000). Amphipathic, alpha-helical antimicrobial peptides. *Biopolymers* 55, 4-30.

Woodcock BA; Isaac NJB; Bullock JM; Roy DB; Garthwaite DG; Crowe A; Pywell RF (2016). Impacts of neonicotinoid use on long-term population changes in wild bees in England. *Nature Communications* 7, 12459.

Zasloff M (2002). Antimicrobial peptides of multicellular organisms. *Nature* 415, 389-395.

Zimmer M (2002). Green fluorescent protein (GFP): applications, structure, and related photophysical behavior. *Chemical Reviews* 102, 759-781.

Zohary D; Hopf M; Weiss E (2012). *Domestication of Plants in the Old World*. 4th edn. Oxford University Press: Oxford.

Koch et al., Short distance spreading of non-coding RNAs: Vesicle trafficking and exosome secretion. In: Deising HB; Fraaije B; Mehl A; Oerke EC; Sierotzki H; Stammler G (Eds), "Modern Fungicides and Antifungal Compounds", Vol. IX, pp. 249-254. © 2020. Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-16-7

Short distance spreading of non-coding RNAs: Vesicle trafficking and exosome secretion

Koch A, Höfle L, Jelonek L, Busche T, Möbus A, Hardt M

Institute for Phytopathology, Centre for BioSystems, Land Use and Nutrition, Justus Liebig University, Giessen, Germany

Email: Aline.Koch@agrar.uni-giessen.de

IMPROVEMENT OF PLANT IMMUNITY USING RNA SILENCING-BASED PLANT PROTECTION STRATEGIES

RNA silencing (also known as RNA interference, RNAi) is a conserved and integral aspect of gene regulation mediated by small RNAs (sRNAs) that direct gene-silencing at the level of transcription but also post-transcriptionally. At the transcriptional level, gene expression is inhibited via RNA-directed DNA methylation (RdDM) while at the post-transcriptional level (PTGS) direct mRNA interference causes inhibition of translation. Originally, RNA silencing is associated with protection against viral infection, control of epigenetic modifications, regulation of genome stability, curbing of transposon movement and regulation of heterochromatin formation (e.g. Koch *et al*. 2017). Besides its natural function, RNA silencing has emerged as a powerful genetic tool for scientific research over the past several years. It has been utilized not only in fundamental research for the assessment of gene function, but also in various fields of applied research, such as agriculture. In plants, RNA silencing strategies have the potential to protect host plants against predation or infection by pathogens and pests mediated by lethal RNA silencing signals generated *in planta* (Host-induced gene silencing, HIGS; e.g. Nowara *et al*. 2010; Koch *et al*. 2013; Abdellatef *et al*. 2015). In addition to the generation of RNA silencing signals *in planta*, plants can be protected from pathogens and pests by exogenously applied RNA biopesticides (Spray-induced gene silencing, SIGS; e.g. Koch *et al*. 2016; Wang *et al*. 2016; Mitter *et al*. 2017). Regardless of how target-specific inhibitory RNAs are applied (i.e. by endogenously or exogenously), the use of target-specific inhibitory RNAs (iRNAs) for plant protection is a potential alternative to conventional pesticides because iRNAs are i) highly specific and easy to produce and ii) can be developed against an unlimited range of pathogens possessing an RNA silencing machinery.

VESICLE-MEDIATED TRANSFER AND UPTAKE OF HIGS-DERIVED RNAS

Despite the striking efficiency of HIGS-based technology holds for agriculture, the mechanisms underlying the transport of RNAs from the plant host to the interacting microbial pathogen are inadequately understood. While in insects and nematodes a transmembrane channel-mediated RNA uptake mechanism based on the *Caenorhabditis elegans* SID-1 (Systemic RNA

interference deficient-1) protein has been described (Huvenne and Smagghe 2010), fungi seem to lack this protein (own observations). Alternatively, it has been hypothesized that small RNAs could be associated with the cargo of extracellular vesicles (EVs) (Cai *et al*. 2018; Rutter & Innes 2017; Rutter & Innes 2018). In line with this hypothesis, the plant-fungal interface, which is comprised of the fungal haustorial plasma membrane (HPM), the fungal cell wall (HCW), the extrahaustorial matrix (EHMx), the extrahaustorial membrane (EHM) and the plant plasma membrane (PPM), is the primary site for plant-fungal recognition (Fig. 1). Uptake of nutrients into the pathogen, delivery of enzymes and toxins into plant cells, secretion of fungal effector proteins and plant antimicrobial molecules (e.g. PR proteins) and biogenesis of cell surface sensors for mutual detection also occur at this interface (Fig. 1). Because most plant pathogens are extracellular, the plant possesses a specialized secretion system, which enables delivery of defense molecules to the site of attack. This secretion pathway mediates the transport of low molecular weight compounds required for immune responses, for example cell surface proteins, which reach their cellular destination via exocytosis from the host cell and is involved in synthesizing the EHM. This latter function indicates that the plant is actively exporting a variety of molecules to the plant-fungal interface, possibly including siRNAs.

Several types of EVs, defined according to their size and origin, have been identified in eukaryotic cells. Exosomes originating from the endosome are only 30-150 nm in diameter and are released through fusion of multivesicular bodies (MVBs) with the plasma membrane (PM). While most of the research on exosomes has been carried out using mammalian cell cultures and animal models such as *Caenorhabditis elegans* and Drosophila, Halperin & Jensen (1967) speculated already more than 50 years ago that a fusion of plant MVBs with the PM may result in the release of small vesicles into the extracellular space. In fact, biogenesis of MVBs and the release of their cargo via exosomes also is inherent to the plant secretory pathway that is activated upon pathogen attack as part of the immune response (An *et al*. 2006a; 2006b; Rutter & Innes 2017). Based on transmission electron microscopy (TEM) studies in barley, MVBs have been identified to proliferate next to cell wall papillae during attack by the powdery mildew fungus, and it was argued that these MVBs release their small vesicles into the paramural space thus leading to the assumption that exosomes exist in plants (An *et al*. 2006a; 2006b). In the latter case, the plant EVs may carry defense compounds to strengthen the plant cell wall at the site of fungal attack. Supporting this notion, proteins, hydrogen peroxide and callose could be identified inside MVBs next to the PM (Xu & Mendgen 1994; An *et al*. 2006b). EVs were also identified in the extrahaustorial matrix of powdery mildew fungus (Micali *et al*. 2011), though it could not be determined in this study whether these vesicles were of plant or fungal origin. Recently, Rutter & Innes (2017) isolated EVs of endosomal origin with a size range of 50-300 nm from the apoplast of *Arabidopsis* leaves, and thus found direct proof that exosomes exist in plants for the first time. These recent findings are in line with our earlier hypothesis that the endosomal vesicle trafficking pathway is the route that transgene-derived siRNAs take to be transferred between individual cells of host and parasite (Koch & Kogel 2014) (Fig. 1).

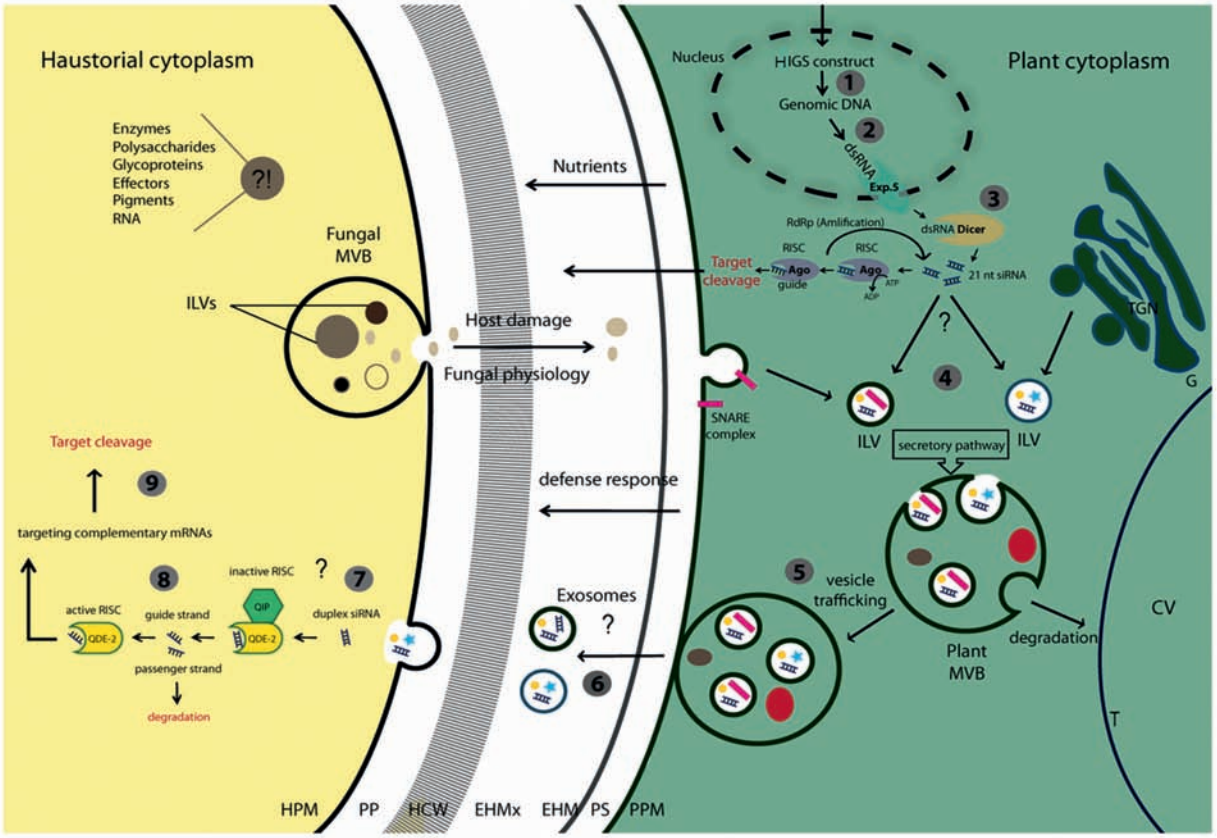


Figure 1 Vesicle-mediated transfer of HIGS-derived RNAs. A potential siRNA translocation pathway is indicated (1-9) (Koch & Kogel 2014).

A potential siRNA translocation pathway involves the integration of a HIGS construct, its transcription into dsRNA and the translocation into the cytoplasm, where it is loaded and processed by DCL enzymes (Fig 1, 1-3). The resulting siRNA duplexes are either delivered to the plant's RNA silencing machinery or are incorporated as duplexes into intraluminal vesicles (ILVs) that originate either from the Golgi body (G) via the trans-Golgi network (TGN) or from endocytosis at the cell membrane, respectively (Fig 1, 4). The ILVs, consisting of several cargos, are internalized by MVBs that enter the secretory pathway (Fig 1, 5). MVBs fuse to the PPM followed by subsequent release of ILVs (now called exosomes) (Fig 1, 6). Exosomes cross the cellular interface, entering the fungal cell and release their cargo, possibly including plants siRNAs (process unknown) (Fig 1, 7). The siRNAs may subsequently enter the fungal RNAi machinery resulting in target gene silencing where they are wrenched by the AGO protein QDE-2 (quelling deficient-2), while the passenger strand is removed by the exonuclease QIP (Fig 1, 8). The guide strand remains in the RNA-induced silencing complex (RISC) which is activated and targets complementary mRNAs, resulting in degradation and gene silencing, respectively (Fig 1, 9). Translocation of sRNAs via exosomes might require membrane associated receptors for attachment at the fungal cell layers and for further entry into the fungal RNA silencing machinery. However, alternative routes for siRNA delivery also may exist, including (i) converted bidirectional trafficking of RNA species across the plant-fungal cellular interface via several exocytic/endocytic receptors; (ii) uptake during fungal nutrient acquisition via specific

transporters; or (iii) passive crossing via trans-cell wall diffusion or through various transmembrane channels or pores. The latter could be a reasonable possibility if the siRNA size is sufficiently small.

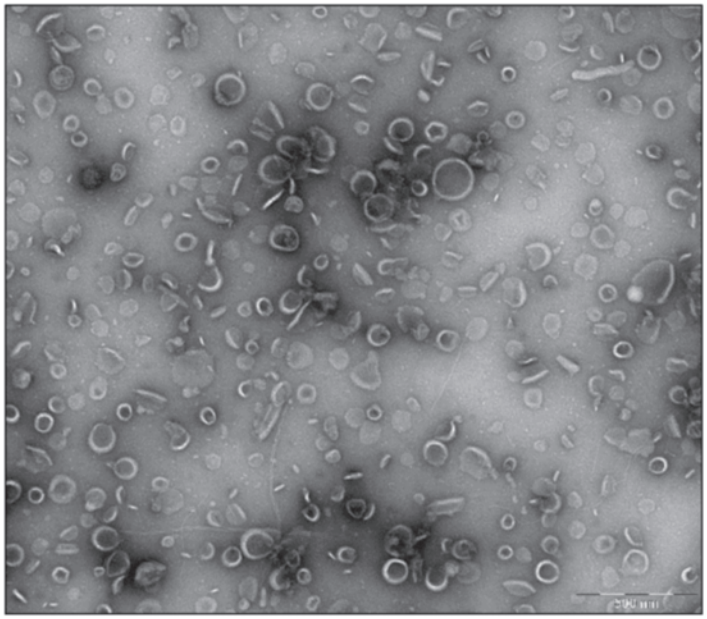


Figure 2 Isolation of EVs from the apoplast of CYP3RNA-expressing *Arabidopsis* leaves. Vesicles were analysed by negative staining and TEM (unpublished).

To test the hypothesis that sRNA transfer during HIGS requires the secretion of EVs, we adjusted/ adapted recently developed protocols (Rutter & Innes 2017) for the isolation of EVs and their cargo from *Arabidopsis* leaves. Vesicles isolated by these methods were around 100 nm in diameter (Fig. 2), which is in good agreement with the size range reported for exosomes from mammalian cells (30-150 nm, Raposo & Stoorvogel 2013) as well as plants (50-300 nm, Rutter & Innes 2017). The physical appearance of vesicles in the TEM analysis was comparable to typical exosome preparations from cell culture supernatants (Li *et al*. 2017), especially as they were surrounded by a characteristic lipid bilayer which has an average thickness of around 5 nm in diameter (Fig. 2). Next, we assessed the RNA cargo of EVs isolated from the apoplastic fluid of transgenic *Arabidopsis* plants expressing a 791 nt dsRNA. Therefore, RNA was isolated and subjected to RNA sequencing. Mapping of siRNA reads to the dsRNA precursor identified dsRNA-precursor-specific siRNAs, while no specific reads were detected in the EVs from the controls (unpublished). In conclusion, our findings further corroborate that EVs of transgenic dsRNA producing HIGS plants contain siRNAs derived from the transgene. However, a better understanding the mechanistic basis (i.e. processing, translocation and uptake) of RNA silencing-based plant protection strategies such as HIGS and SIGS is critical for the successful implementation of these technologies for future field application. While we found strong indication for vesicle-mediated transport during HIGS-mediated plant protection, questions that remain open are:

- How are siRNAs (or dsRNAs) packed into vesicles?
- What is the nature of the transported RNA?
- How can these vesicles cross the plant-fungus interface?
- How are they released into fungal cells?

Thus, the overall scientific goal of our research aims to clarify central mechanisms concerning the uptake and translocation of non-coding RNAs, necessary for enhancing the efficacy and specificity of RNA silencing-based plant protection technologies.

REFERENCES

Abdellatef E; Will T; Koch A; Imani J; Vilcinskas; Kogel KH (2015). Silencing the expression of the salivary sheath protein causes transgenerational feeding suppression in the aphid Sitobion avenae. *Plant Biotechnology Journal* 13, 849-57.

An Q; Ehlers K; Kogel K-H; van Bel AJE; Huckelhoven R (2006a). Multivesicular compartments proliferate in susceptible and resistant MLA12-barley leaves in response to infection by the biotrophic powdery mildew fungus. *New Phytologist* 172, 563–576.

An Q; Huckelhoven R; Kogel K-H; van Bel AJE (2006b). Multivesicular bodies participate in a cell wall-associated defence response in barley leaves attacked by the pathogenic powdery mildew fungus. *Cell Microbiology* 8, 1009–1019.

Cai Q; He B; Kogel KH; Jin H (2018). Cross-kingdom RNA trafficking and environmental RNAi — nature's blueprint for modern crop protection strategies. *Current Opinion Microbiology*, 46, 58–64.

Halperin W; Jensen WA (1967). Ultrastructural changes during growth and embryogenesis in carrot cell cultures. *Journal of Ultrastructure Research* 18, 428–443.

Huvenne H; Smagghe G (2010). Mechanisms of dsRNA uptake in insects and potential of RNAi for pest control: A review. *Journal Insect Physiology* 56, 227-235.

Koch A; Biedenkopf D; Furch A; Weber L; Rossbach O; Abdellatef E; Linicus L; Johannsmeier J; Jelonek L; Goesmann A; Cardoza V; McMillan J; Mentzel T; Kogel K-H (2016). An RNAi-based control of Fusarium graminearum infections through spraying of long dsRNAs involves a plant passage and is controlled by the fungal silencing machinery. *Plos Pathogens*, 12, e1005901

Koch A; Kang HG; Steinbrenner J; Dempsey DA; Klessig DF; Kogel KH (2017). MORC Proteins: Novel Players in Plant and Animal Health. *Frontiers in Plant Science*. doi.org/10.3389/fpls.2017.01720

Koch A; Kogel KH (2014). New wind in the sails: improving the agronomic value of crop plants through RNAi-mediated gene silencing. *Plant Biotechnology Journal* 12, 821-831.

Koch A; Kumar N; Weber L; Keller H; Imani J; Kogel KH (2013). Host-induced gene silencing of cytochrome P450 lanosterol C14α-demethylase-encoding genes confers strong resistance to Fusarium spec. *Proc Natl Acad Sci USA* 110, 19324-19329.

Li P; Kaslan M; Lee SH; Yao J; Gao Z (2017). Progress in Exosome Isolation Techniques. *Theranostics* 7, 789–804.

Micali CO; Neumann U; Grunewald D; Panstruga R; O'Connell R (2011). Biogenesis of a specialized plant-fungal interface during host cell internalization of *Golovinomyces orontii* haustoria. *Cell Microbiology*, 13, 210–226.

Mitter N;Worrall EA; Robinson KE; Li P; Jain RG; Taochy C; Fletcher SJ; Carroll BJ; Lu GQ; Xu ZP (2017). Clay nanosheets for topical delivery of RNAi for sustained protection against plant viruses. *Nature Plants* 3, 16207.

Nowara D; Gay A; Lacomme C; Shaw J; Ridout C; Douchkov D; Hensel G; Kumlehn J; Schweizer P (2010). HIGS: host-induced gene silencing in the obligate biotrophic fungal pathogen *Blumeria graminis*. *The Plant Cell* 22, 3130–3141.

Raposo G; Stoorvogel W (2013). Extracellular vesicles: exosomes, microvesicles, and friends. *Journal Cell Biololgy* 200, 373–383.

Rutter BD; Innes RW (2017). Extracellular Vesicles Isolated from the Leaf Apoplast Carry Stress-Response Proteins. *Plant Physiology* 173, 728-741.

Rutter BD; Innes RW (2018). Extracellular vesicles as key mediators of plant-microbe interactions. *Current Opinion Plant Biology* 44, 16–22.

Wang M; Weiberg A; Lin F-M; Thomma B, Huang H-D; Jin H (2016). Bidirectional cross-kingdom RNAi and fungal uptake of external RNAs confer plant protection. *Nature Plants* 2, 16151.

Xu H; Mendgen K (1994). Endocytosis of 1,3-β-glucans by broad bean cells at the penetration site of the cowpea rust fungus (haploid stage). *Planta* 195, 282-290.

Cook NM et al., Novel Strategies for Monitoring Fungicide Resistance in Cereal Pathogens, In: Deising HB; Fraaije B; Mehl A; Oerke EC; Sierotzki H; Stammler G (Eds), "Modern Fungicides and Antifungal Compounds", Vol. IX, pp. 255-256. © 2020. Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-16-7

Novel Strategies for Monitoring Fungicide Resistance in Cereal Pathogens

Cook NM, Radhakrishnan GV, Frey R, Sattler U, Oliver RP, Torriani SFF, Saunders DGO

John Innes Centre, Norwich Research Park, Colney Lane, Norwich, Norfolk, UK
Corresponding Author Email: nicola.cook@jic.ac.uk

Monitoring and quantifying the presence of mutations that confer fungicide resistance within fungal populations is crucial to implement informed resistance management strategies and thereby prolong the efficacy of commercially available fungicides. Many researchers, both in academia and the agrichemical industry, perform rigorous fungicide sensitivity testing of field isolates from different economically important fungi. To detect resistance-associated mutations, genotyping methods such as pyrosequencing, qPCR and loop-mediated isothermal amplification are widely used (Gobeil-Richard et al. 2016; Manjunatha et al. 2018). However, these current techniques have their limitations as prior knowledge of fungicide resistant mutations is required to design assays that can detect both the presence and frequency of a mutation within a given pathogen's population. Separate assays have to be optimised for each pathogen and the individual mutations to monitor which can be very time consuming and labour intensive. These targeted methods are also unable to identify novel fungicide resistant mutations. Recent innovations in genomics-based molecular techniques hold the promise of facilitating the development of near real-time diagnostic methods for known and novel fungicide resistant mutations. This knowledge would enable more effective and customised chemical control programmes to be developed, maximising effectiveness while limiting chemical input.

We have developed a platform that incorporates the latest sequencing technology to detect the presence of fungicide resistant mutations in a high-throughput manner. Using this method, multiple mutations from different fungicide target genes can be monitored simultaneously from leaf samples containing different fungal species. The method has initially been set up to monitor six fungal pathogens deemed to be economically important (Dean et al. 2012): *Blumeria graminis* f. sp. *tritici*, *Puccinia triticina*, *Puccinia striiformis* f. sp. *tritici* and *Zymoseptoria tritici* which infect wheat, as well as *Pyrenophora teres* f. sp. *teres* and *Ramularia collo-cygni* which infect barley. Within these pathogens, fungicide resistance mutations can be detected within the *Cyp51, SdhB, SdhC* and *SdhD* genes from each pathogen. Both the *Cyp51a* and *Cyp51b* genes were included for *P. teres* f. sp. *teres*. This method is currently capable of detecting the presence and relative abundance of the six targeted pathogens with a limit of detection of 1 in 100 genotypes within a DNA sample.

This new genotyping platform comprises of five stages: DNA Extraction, PCR, Library Construction, Sequencing and Data Analysis (Figure 1). To facilitate high-throughput sample processing, all stages were developed to be compatible with a 96 well plate format. The DNA extraction method uses a standard tissue disruption and column-based protocol, which enables the extraction of DNA from 192 samples in 4 hours. The PCR step then uses primers that are unique for each pathogen to amplify the entire length of the five or six genes. The primers were

combined into three primer pools based on their relative efficiency and PCR parameters were optimised to unify conditions for all reactions. The concentrations of primers within the pools were also adjusted to ensure equal amplification across all 31 genes. The library construction and sequencing steps have been optimised for use on both Illumina and Oxford Nanopore Technologies sequencing platforms of which the latter has the advantage of being both mobile and real-time. The data analysis pipeline is streamlined and requires minimal user input to detect the given pathogens within an infected field sample as well as any mutations that may lead to fungicide resistance.

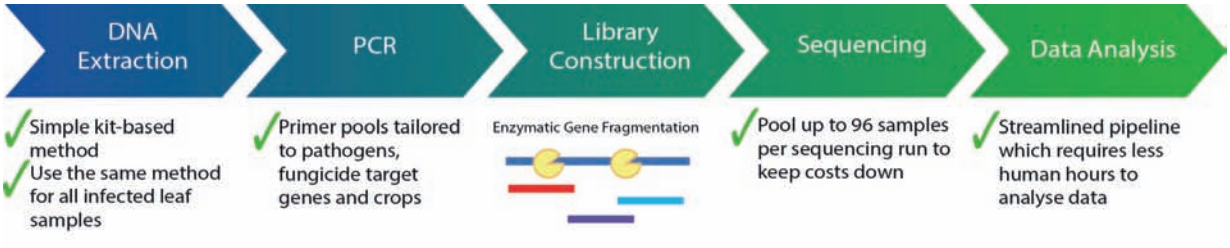


Figure 1 Pipeline detailing the steps of the high-throughput monitoring method.

We have developed a high-throughput genotyping method that is capable of monitoring the presence of fungicide resistance mutations within several key fungicide target genes for multiple pathogens simultaneously using the same standardised technique. The method is capable of being fully automated making it particularly attractive in an industrial setting. While this method is capable of detecting the presence and frequency of potential fungicide resistance mutations, the full length genotype of the target genes cannot be elucidated using this method. This prevents the identification of the number of mutations within the Cyp51 gene for each genotype which determines the levels of fungicide resistance exhibited by each genotype within the sample. To rectify this, the full length of the fungicide target genes could be sequenced on third generation sequencing platforms such as the MinION (Oxford Nanopore Technologies, Oxford, UK). As a result, the genotyping platform in its current state is more suitable to monitor the fungicide target genes which large levels of resistance is conferred through one mutation such as the *SDH* genes. The high-throughput nature of the method also makes it well suited to large-scale screening of samples from field trial sites to study the evolution of fungicide resistance with a view to better inform the fungicide management strategies used in agriculture.

REFERENCES

Dean R; Van Kan JAL; Pretorius ZA; Hammond-Kosack KE; Di Pietro A; Spanu PD; Rudd JJ; Dickman M; Kahmann R; Ellis J & Foster GD (2012). The Top 10 fungal pathogens in molecular plant pathology. *Molecular Plant Pathology*, 13, 414-430.

Gobeil-Richard M; Tremblay DM; Beaulieu C; Van Der Heyden H & Carisse O (2016). A pyrosequencing-based method to quantify genetic substitutions associated with resistance to succinate dehydrogenase inhibitor fungicides in *Botrytis* spp. populations. *Pest Management Science*, 72, 566-573.

Manjunatha C; Sharma S; Kulshreshtha D; Gupta S; Singh K; Bhardwaj SC & Aggarwal R (2018). Rapid detection of *Puccinia triticina* causing leaf rust of wheat by PCR and loop mediated isothermal amplification. *PLOS ONE*, 13, e0196409.

NEW FUNGICIDES

Strobel et al., Mefentrifluconazole (Revysol®) - The First Isopropanol-Azole. In: Deising HB; Fraaije B; Mehl A; Oerke EC; Sierotzki H; Stammler G (Eds), "Modern Fungicides and Antifungal Compounds", Vol. IX, pp. 259-264. © 2020. Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-16-7

Mefentrifluconazole (REVYSOL®) – The First Isopropanol-Azole

Strobel D[1], Bryson R[1], Semar M[1], Stammler G[1], Kienle M[1], Smith J[2]

[1]BASF SE, Agricultural Solutions, Speyerer Strasse 2, D-67177 Limburgerhof

[2]ADAS Rosemaund, Preston Wynne, HR1 3PG Hereford, United Kingdom

Corresponding Author Email: dieter.strobel@basf.com

ABSTRACT

Revysol® is a novel triazole fungicide with high activity against a broad range of foliar diseases and a favorable regulatory profile. Unlike conventional azoles, the unique chemical structure results in a high level of selectivity and excellent efficacy even against existing triazole-adapted isolates of *Zymoseptoria tritici*. With Revysol, the triazole 'head' sits on the 'neck' of a slim isopropanol linker. This chemical constellation gives a high degree of structural flexibility that is unique among the DMIs. When Revysol docks on to its target site, the flexible linker allows it to form a hook, which fits into the enzyme's binding pocket, resulting in strong inhibition of enzyme activity, even with existing target site mutations.

In planta, Revysol is rapidly taken up into the leaf and is then slowly but consistently translocated apically via the xylem, resulting in a high level of curative activity. The limited translocation leads to the formation of an inner-leaf reservoir which allows a well-balanced, long lasting systemic activity. As a result, Revysol also shows a high residual activity, because the leaf deposits are protected inside the leaf. Revysol will be developed in different formulations, including with Xemium® and F 500® as partners for use in row and specialty crops around the globe. Market introduction in major countries is targeted for 2019/2020.

INTRODUCTION

Azole fungicides have been in practical use for more than 40 years. Despite problems with resistance and the challenges of the new hazard-based regulatory system in Europe, their history is a remarkable story of innovation and success (Lucas 2016).

Revysol (common name: mefentrifluconazole) is a novel triazole fungicide with high activity against a broad range of foliar diseases and a favorable regulatory profile. Unlike conventional azoles, the unique chemical structure results in a high level of selectivity and excellent efficacy even against triazole-adapted isolates of *Zymoseptoria tritici*, the causal agent of Septoria leaf blotch. The number of effective active ingredients is increasingly limited, especially in Europe, due to resistance developments and consequences of the regulation EC 1107/2009 (Bryson et al. 2016). In this paper we demonstrate that Revysol can play an essential role for integrated disease management.

MODE OF ACTION

Based on the fungicide mode of action classification of the Fungicide Resistance Action Committee (FRAC), Revysol is a fungicide belonging to the group of the sterol biosynthesis inhibitors (SBI, mode of action class G). Within the SBIs, it belongs to the sub group of demethylation inhibitor (DMI, G1) and the chemical group of triazoles. The primary mode of action of Revysol is – as with all other DMIs - the blocking of ergosterol biosynthesis through inhibition of cytochrome P450 sterol 14α-demethylase (CYP51). The depletion of ergosterol and accumulation of non-functional 14α-methyl sterols results in inhibition of growth and cell membrane disruption.

Studies on cross resistance with other DMIs

Various mutations in the target gene have evolved for major plant pathogens, namely *Z. tritici*, with variable effects on different DMIs. Target gene mutations might be combined and accumulate and can result in higher levels of resistance (Cools & Fraaije 2013). The number and complexity of *CYP51* haplotypes in "shifted strains" is now at a high level mainly in several European countries, and the latest update is available by Huf et al. 2019. In addition, target site overexpression and/or enhanced efflux can also be found simultaneously (Stammler & Semar 2011, Cools & Fraaije 2013). Isolates belonging to different *CYP51*-haplotypes have been shown to vary in their sensitivity to different DMIs (Stammler & Semar 2011). For Revysol, high intrinsic activity and a low correlation of sensitivity is observed for important DMIs used for *Z. tritici* control such as prothioconazole (Figure 1).

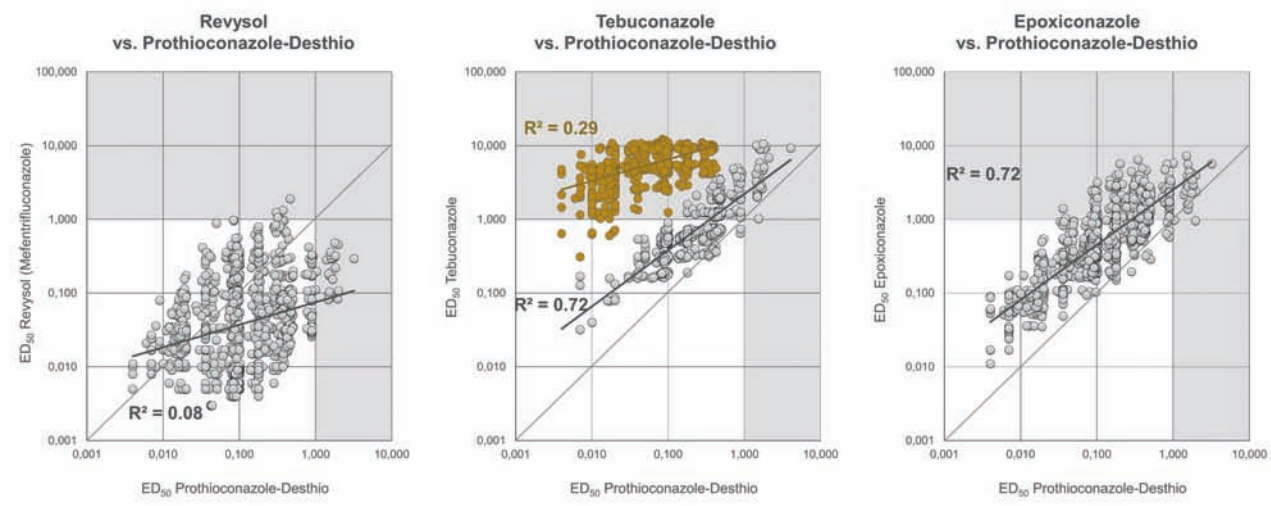


Figure 1 Sensitivity of *Z. tritici* isolates towards Revysol, tebuconazole and epoxiconazole in comparison to prothioconazole-desthio. Microtiter tests by EpiLogic, Germany (1.347 single pycnidial isolates from field samples out of European countries 2014-2018; test of a range of different DMI concentrations and subsequent ED_{50} calculation; mgl^{-1}). Tebuconazole forms two subgroups due to the presence (lower group) or absence (upper group) of the V136A mutation in the *CYP51*. Prothioconazole-desthio was used instead of prothioconazole due to its' recognised role in disease control (Parker et al. 2013).

High structural flexibility due to the isopropanol linker

The high structural flexibility of the Revysol molecule explains the observed limited cross-resistance of Revysol compared with the leading azoles used against *Z. tritici*: Mutations in the *CYP51* gene cause alterations of the binding site, often the binding pocket becomes wider which affects the binding of conventional DMIs. In contrast, the Revysol molecule is more flexible in its structure compared with other DMIs and is therefore able to bind even if the binding pocket shape is altered. This flexibility comes from the fact that the triazole 'head' sits on the 'neck' of a slim isopropanol linker. This chemical constellation ensures a high degree of structural flexibility that is unique among the DMIs (Figure 2). This slim linker requires less energy to adjust compared to conventional DMIs. When Revysol approaches the active site of the target enzyme C14-demethylase (CYP51), the flexible linker allows it to easily form a "hook", which fits perfectly into the enzyme's binding pocket, resulting in strong inhibition of enzyme activity even where target site mutations have evolved.

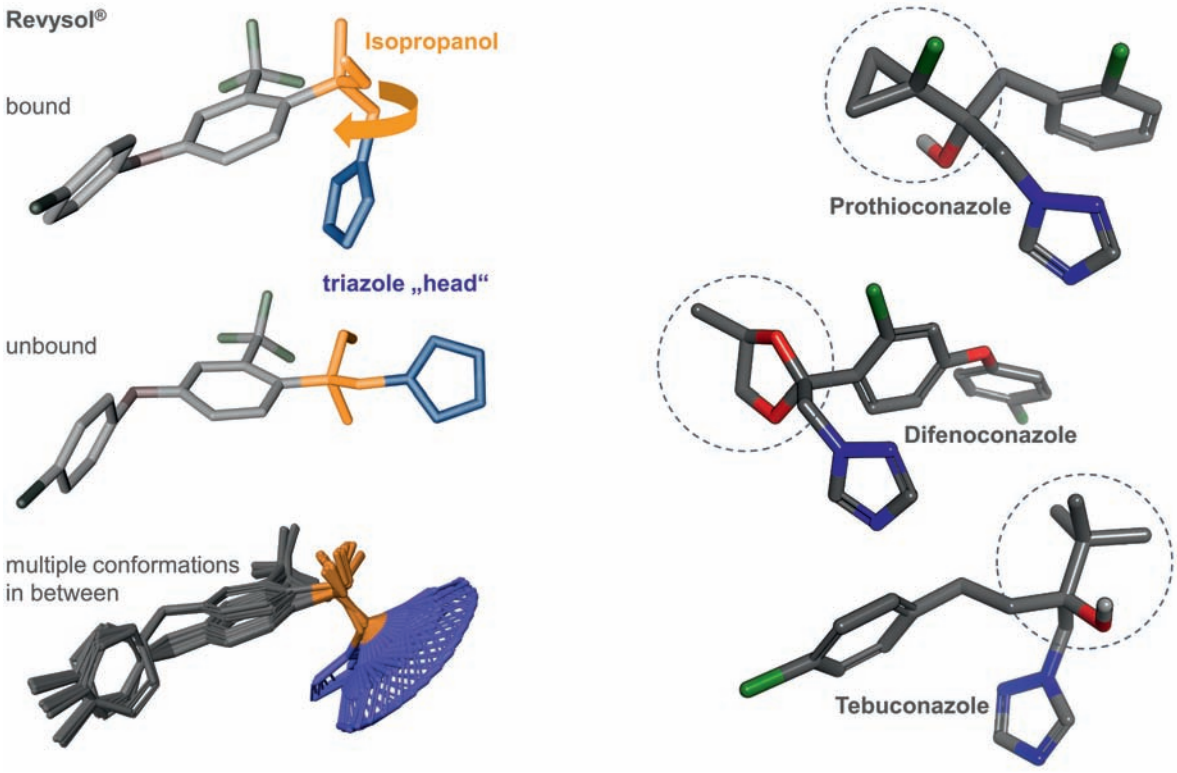


Figure 2 Chemical structure and possible conformations of Revysol. The isopropanol-unit (orange) is a thinner linker compared to other azoles allowing the molecule to easily form flexible conformations (simple quantum-chemistry energy calculations).

Efficacy against DMI-adapted strains in glasshouse and field

Further tests were made to show the activity of Revysol on DMI-adapted isolates. Efficacy of DMI compounds against a range of adapted *Z. tritici* isolates (Table 1) was studied under controlled conditions in the glasshouse (1-day preventative application, 1/3 of the registered field rate). Whereas conventional DMIs gave poor efficacy against some of the adapted strains, Revysol was able to show full control (Figure 3).

Table 1 Sensitivity of *Z. tritici* isolates to DMI fungicides, determined in microtiter tests as described by Stammler & Semar 2011

Code	Origin	Detected Mutations	ED_{50} Revysol [mgl^{-1}]	ED_{50} prothioconazole [mgl^{-1}]	ED_{50} tebuconazole [mgl^{-1}]	ED_{50} epoxiconazole [mgl^{-1}]
-	Wildtype	-	< 0,003	0,070	0,025	0,005
Strain A	Ireland	V136A + S524 T	0,019	1,930	0,198	0,214
Strain B	Ireland	V136A + I381V + S524 T	0,018	6,270	0,544	0,653
Strain C	Ireland	V136A + I381V + S524 T	0,055	11,210	0,657	1,740
Strain D*	France	I381V*	0,515	2,110	6,450	1,730
Strain E	Ukraine	A379G + I381V	0,067	0,402	6,840	0,188
Strain F	France	V136C + DEL	0,064	1,390	3,450	0,430
Strain G	Ireland	A379G + I381V + S524T + DEL	1,910	14,740	10,000	1,770

*phenotype with increased efflux

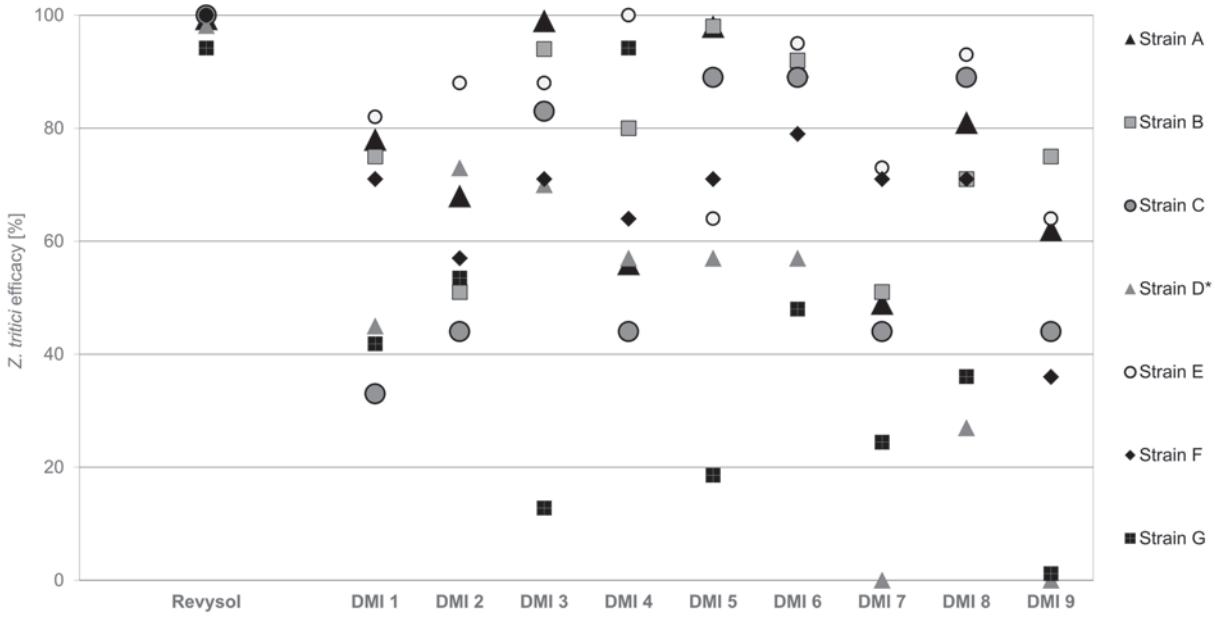


Figure 3 Efficacy of various DMI compounds against highly adapted strains of *Z. tritici* in the glasshouse (inoculation one day after application; 33% of the registered dose rate). *Strain D = Phenotype with increased fungicide efflux.

In a field study with *Z. tritici*, Revysol was compared to a conventional DMI for the control of highly DMI-shifted strains in comparison to natural infection. Whereas Revysol was able to maintain the level of efficacy irrespective of the DMI-adaptation level, the conventional DMI showed inferior efficacy with the natural population and failed to control the highly azole-shifted strains (Table 2).

UPTAKE AND CURATIVE ACTIVITY

In order to be effective, DMI fungicides need to reach the target CYP51 inside the fungal cell, as at the time of application the fungus may already be developing inside the plant. From its chemical properties, Revysol shows an optimum octanol-water coefficient (Log P_{OW} = 3.4),

Table 2 Efficacy of a single treatment of DMI compounds against *Z. tritici* populations, field trial ADAS Rosemaund, UK 2018. Application of test products 5 days after inoculation; assessment 29 days after application (flag leaf).

Z. tritici population	mean RF**	% severity *Z. tritici*	% efficacy Revysol 150 gha^{-1}	% efficacy DMI 1 200 gha^{-1}
natural infection	119	39	66	43
highly azole-shifted*	211	57	63	7

*Inoculation at BBCH 39: Mix of *CYP51* variant [L50S, V136C, S188N, I381V, Y461H, S524T] and [L50S, D134G, V136A, I381V, Y461H, S524T]; Spore density 1 x 10^7.

**Resistance Factor. Average level of adaptation to DMI 1 in comparison to the wildtype population based on sensitivity (ED_{50}, microtiter tests)

but a very low solubility (0.81 mgl^{-1} in water at 20°C). Novel approaches to the formulation of Revysol were therefore necessary to ensure a high level of plant uptake.

The level of uptake was evaluated by applying the optimized Revysol formulation onto wheat leaves: Deposits on the surface and the wax layer were removed by cellulose acetate treatment ("stripping"), and both the surface/wax deposit and the remaining leaves were analysed by UPLC-MS-MS to quantify the amount of active ingredient. Assessments were repeated at different points of time after treatment. The level of leaf uptake was expressed as a percentage (amount in leaf tissue in relation to the applied amount) and compared to the findings with commercial azole formulations. The results show a very quick and high level of uptake for Revysol (Table 3), which can explain the high level of curative activity observed in field trials (Smith 2019).

Table 3 Leaf uptake quantification over time following droplet application on wheat leaves with a spray concentration in 200 lha^{-1} water.

Active ingredient	Formulation	Dose rate [lha^{-1}]	leaf uptake [%] 1h	2h	5h	24h	48h
Revysol	100 gl^{-1} EC	1.5	27.3	33.5	47.2	64.9	63.3
DMI 1	250 gl^{-1} EC	0.8	4.3	5.8	15.2	29.1	44.3
DMI 2	125 gl^{-1} SC	1.0	1.7	3.5	6.0	15.8	25.6

TRANSLOCATION AND LONG-LASTING PERFORMANCE

Despite the observed quick and high uptake levels, there is only moderate systemic translocation of Revysol in an apical direction. After 7 days, only 10% of the total Revysol amount is translocated in a wheat leaf experiment, whereas for DMI 1 and DMI 2 38% and 16% translocation was measured, respectively. This phenomenon can be explained by the low solubility of Revysol: Only traces of the active ingredient are able to follow the water flow in the Xylem, but leaf-systemic activity is still high. As a result, the majority of the active

ingredient stays within the application zone as an “inner-leaf reservoir”, resulting in a consistent and very durable translocation process and thus protection.

This long-lasting performance was studied in field experiments. Following a single application on to the fully emerged flag leaf (BBCH 39), Revysol gave a significantly longer level of control compared to a conventional DMI (Smith 2019).

CONCLUSION

Despite the severe challenges of bringing a new active ingredient to the market, BASF has developed an effective new azole fungicide. Revysol, although a DMI, is the first isopropanol azole carrying the triazole ‘head’ on the ‘neck’ of a slim isopropanol linker. This novel constellation increases the binding efficiency of Revysol, ensuring a high level of control even of highly shifted isolates with complex *CYP51* haplotypes. With the continual threat and emergence of pathogen resistances towards commercial fungicides, Revysol is likely to play a pivotal role in disease control in many crops in the future.

REFERENCES

Bryson RJ; Brix HD and Bruns J (2016). How will EU pesticide legislation impact on the Crop Protection Innovatoin pipeline? *Proceedings of the Crop Protection in Northern Britain conference*, *Dundee*, 9- 14.

Cools H; Fraaije BA (2013). Update on mechanisms of azole resistance in *Mycosphaerella graminicola* and implications for future control. *Pest Management Science* 69, 150-155

Huf A; Rehfus A; Lorenz KH; Bryson RJ; Voegele RT and Stammler G (2019). Proposal for a new nomenclature for CYP51 haplotypes in *Zymoseptoria tritici* and analysis of their distribution in Europe. *Plant Pathology* DOI: 10.1111/ppa.12891

Lucas JA (2016). Azole fungicides: Still live after all these years. *Outlooks on Pest Management – August 2016*, 150-151.

Parker J; Warrilow A; Cools H; Fraaije B; Lucas J; Rigdova K; Griffiths W; Kelly D and Kelly S (2013). Prothioconazole and prothioconazole-desthio activities against Candida albicans sterol 14-α-demethylase. *Applied and Environmental Microbiology* 79, 1639-45.

Smith J (2019). Efficacy of a novel triazole fungicide mefentrifluconazole against adapted strains of *Zymoseptoria tritici* in wheat. In preparation.

Stammler G; Semar M (2011). Sensitivity of *M. graminicola* to DMI fungicides across Europe and impact on field performance. *Bulletin OEPP/EPPO Bulletin* 41, 149–155.

Matsuzaki Y et *al.*, Biological profile of metyltetraprole - a new QoI-fungicide: discovery of the tetrazolinone chemical class and *in vitro/in vivo* analysis of activity against QoI-resistant fungal strains. In: Deising HB; Fraaije B; Mehl A; Oerke EC; Sierotzki H; Stammler G (Eds), "Modern Fungicides and Antifungal Compounds", Vol. IX, pp. 265-270. © 2020. Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-16-7

Biological profile of metyltetraprole, a new QoI-fungicide: discovery of the tetrazolinone chemical class and *in vitro*/*in vivo* analysis of activity against QoI-resistant fungal strains

Matsuzaki Y, Iwahashi F

Sumitomo Chemical Co., Ltd. Health and Crop science Research Laboratory, 2-1 Takatsukasa 4-chome, Takarazuka, Hyogo, 665-a8555 Japan

E-mail: matsuzakiy2@sc.sumitomo-chem.co.jp

INTRODUCTION

After the first emergence of resistant fungal strains and the consequent declining efficacy of QoIs in the early 2,000s, industrial agrochemical research has mainly focused on the improvement and diversification of succinate dehydrogenase inhibitors (SDHIs). However, populations of some destructive pathogen species have acquired resistance to SDHIs in recent years (Torriani et *al*. 2017). Thus, another solution for disease management is demanded by crop growers. However, improvements in QoIs that overcome the current spread of resistant populations have not yet been achieved, especially those conferred by target site mutations corresponding to the G143A amino acid substitution in cytochrome *b*. Under such situations, we hypothesized that modification of the pharmacophore and the substituent on the central linking ring could recover the interaction between the G143A mutant cytochrome *bc*1 complex and QoIs (Figure 1), which is speculated to be under severe steric hindrance (Leadbeater et *al*. 2012, Sierotzki et *al*. 2015 and Matsuzaki et *al*. 2019). Here, we report the biological profile of metyltetraprole, a new tool for the management of current fungal populations with resistance to existing fungicides.

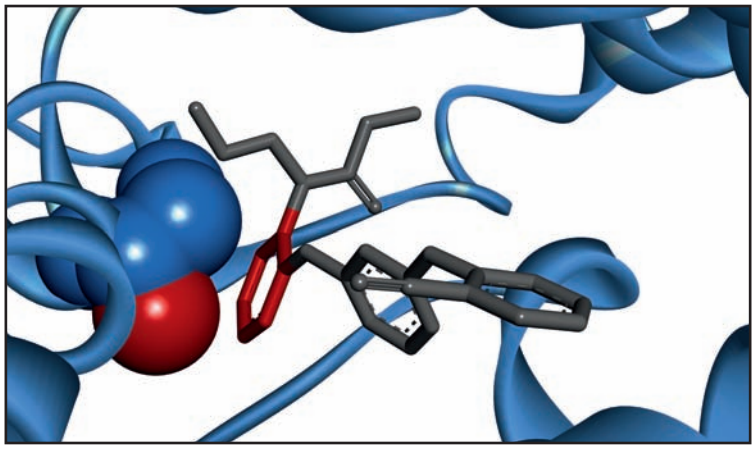


Figure 1 Steric hindrance between the methyl group of the alanine residue at position 143 of cytochrome *b* and the central linking ring of a QoI (azoxystrobin). Modified from PDB:3L71.

DISCOVERY OF TETRAZOLINONE-QOI

Following intensive screening of the sample library of Sumitomo Chemical, we identified a sample with a tetrazolinone moiety, which exhibited highly similar activity between the wild type and the G143A mutant of *Zymoseptoria tritici*. We made several attempts to increase the activity of tetrazolinone compound by modifying the partial structure and discovered metyltetraprole (Table 1 and 2). The electron transport chains of the G143A mutant of *Z. tritici,* as well as the F129L mutant of *Pyrenophora teres,* were strongly inhibited by metyltetraprole (Suemoto et *al.* 2019), although the binding site of metyltetraprole was confirmed to be the Qo site of the cytochrome *bc*1 complex, consistent with existing QoIs (Matsuzaki et *al*. 2019). Interestingly, the resistance factor (RF) of metyltetraprole was lower than that of azoxystrobin and pyraclostrobin in the F129L mutant of *P. teres*, in which the mechanism of lower sensitivity to QoIs is distinct from that of the G143A mutant. In silico analysis suggested that the smaller compact structure of the pharmacophore part of metyltetraprole, namely the tetrazolinone-moiety, might be relevant to the lower structural interference in the F129L mutant Qo site, although further elucidation would be required for confirmation (Arakawa et *al*. 2018).

Table 1 Chemical structures and physicochemical property of metyltetraprole.

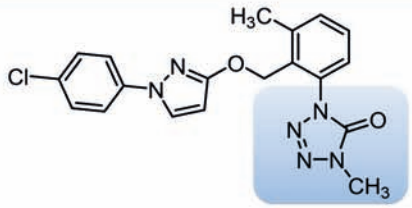
Tradename of active ingredient	Pavecto®
ISO Common name	Metyltetraprole
Chemical name （IUPAC）	1-(2-{[1-(4-chlorophenyl)-1*H*-pyrazol-3-yl]oxymethyl}-3-methylphenyl)-1,4-dihydro-4-methyl-5*H*-tetrazol-5-one
Chemical structure	
Chemical class	Tetrazolinone (proposal)
LogPow	4.16 (pH 7.4, 20 ° C)
Solubility (water)	0.12 mg/L (pH 7-8, 20 ° C)
Vapor Pressure	3.6×10^{-9} Pa (20 ° C)

Table 2 Antifungal activity against *Zymoseptoria tritici* (microtiter tests)

Compound	EC_{50} mg L^{-1} Wild type	EC_{50} mg L^{-1} G143A mutant	Resistance factors*
Metyltetraprole	0.002	0.002	1
Azoxystrobin	0.02	> 1	>200
Pyraclostrobin	0.001	0.2	200

*Resistance factor (RF): [EC_{50} of resistant isolate]/[EC_{50} of wild type isolate]

PHARMACOKINETICS OF METYLTETRAPROLE

The uptake and movement of metyltetraprole were measured in wheat. After spraying water-diluted metyltetraprole (60 g L^{-1} EC) at 120 g ha^{-1}, the amount of metyltetraprole on the surface of the leaf and inside the leaf tissue (epicuticular wax layer plus the more inner part) was measured by HPLC. The amount of metyltetraprole collected after briefly washing the leaves with acetonitrile (which does not dissolve the epicuticular wax layer of the plants), and the amount retained inside the leaves were compared. The experiments confirmed the uptake of metyltetraprole by wheat leaves (Table 3) and results were consistent with those of previous studies showing that metyltetraprole exhibits post-infection activity (Suemoto et *al* 2019). We also visualized the systemic movement of metyltetraprole using radioactive metyltetraprole ([^{14}C] metyltetraprole, pyrazolyl-3-^{14}C, 1 × 10^6 dpm). Droplets of [^{14}C] metyltetraprole as a water dilution of the EC formulation were placed on the leaves and the radioactivity was measured by autoradiography. Only a weak signal was detected downstream of the xylem 3 days after treatment (Figure 2). Therefore, the movement of metyltetraprole along with xylem flow after uptake was estimated to be slow and limited. The majority of metyltetraprole was retained at the position where the droplet was received rather than moving quickly. This pharmacokinetic feature of metyltetraprole is expected to contribute to its long-lasting efficacy.

Table 3 Distribution of metyltetraprole in/on wheat leaves

Compound and formulation	Part of wheat leaf	ng active ingredient per 1 cm^2 leaf area (mean* ± SD)			
		0 DAT**	3 DAT	20 DAT	35 DAT
Metyltetraprole 60 g L^{-1} EC	Surface	253 ± 69	178 ± 37	141 ± 14	69 ± 15
	Inner	73 ± 23	108 ± 20	35 ± 9	29 ± 4
Fluxapyroxad 62.5 g L^{-1} EC (ref.)	Surface	231 ± 56	164 ± 75	116 ± 24	102 ± 10
	Inner	66 ± 13	74 ± 7	50 ± 12	44 ± 2

*All tests were performed in triplicate

** DAT: days after treatment

SPECTRUM AND IMPACT OF G143A/F129L MUTATION AMONG DIFFERENT PATHOGEN SPECIES

In addition to antifungal tests performed using microtiter and agar plates (Suemoto et *al*. 2019), seedling bioassays were performed in greenhouses to profile the spectrum of metyltetraprole activity. Although metyltetraprole showed activity against a broad range of fungal pathogens (Ascomycota and Basidiomycota) and against Oomycetes. Particularly, pathogen species belonging to Dothideomycetes within the Ascomycota, including a number of leaf spot-type pathogens such as *Zymoseptoria*, *Pyrenophora*, *Ramularia*, *Cercospora*, and *Stagonospora*, were highly sensitive to metyltetraprole (EC_{50}<0.01 mg L^{-1}). We also confirmed the RFs in G143A and F129L mutants among different pathogen species and confirmed that metyltetraprole had a consistently lower RF compared with azoxystrobin and pyraclostrobin. The mean RFs of metyltetraprole in the G143A mutant strains were always <2 among the

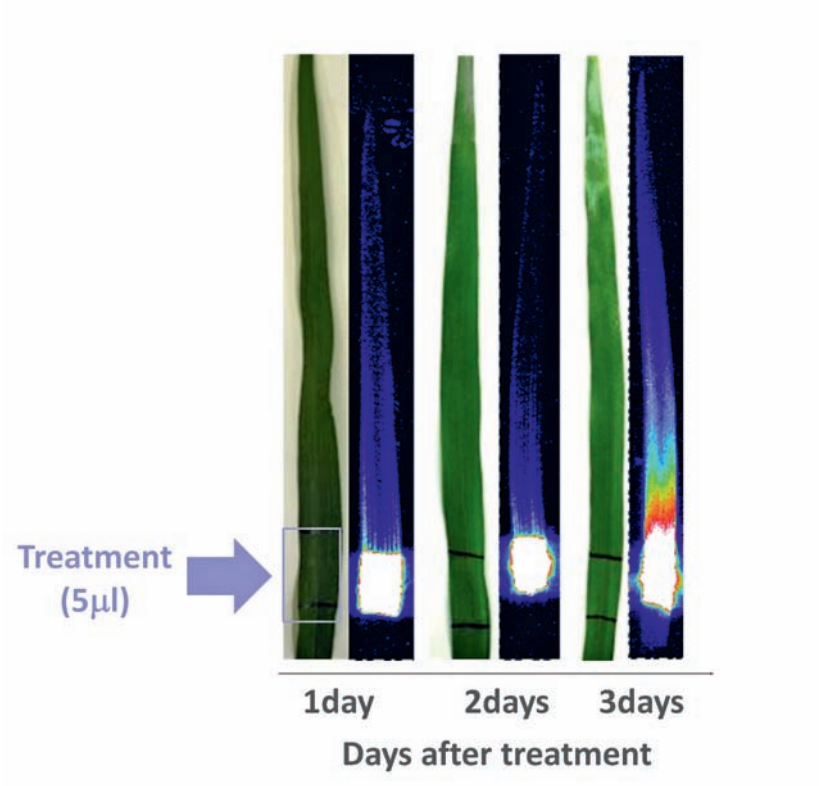


Figure 2 Translocation of metyltetraprole over time in wheat flag leaf (variety: Apogee) visualized by [^{14}C] metyltetraprole; 5 µL droplet of [^{14}C] metyltetraprole as a water dilution of the EC formulation was placed on the leaves and radioactivity was measured by autoradiography.

different species whereas those of pyraclostrobin and azoxystrobin were consistently >100. The corresponding mean RF in the F129L strains for metyltetraprole was 2.1 while those of pyraclostrobin and azoxystrobin were 15.8 and 90.6, respectively. Therefore, metyltetraprole is expected to be a new tool for control of QoI-resistant mutants of the above-mentioned species.

FIELD TRIAL EXPERIMENTS

The results of field experiments across west Europe, in accordance with guidelines of the European and Mediterranean Plant Protection Organization (http://pp1.eppo.int/), confirmed the efficacy and spectrum of metyltetraprole as a fungicide for cereals. The performance of metyltetraprole was stable in the presence of G143A QoI-resistant strains of *Z. tritici* (Septoria leaf blotch of wheat, Table 4) and was also effective against F129L QoI-resistant strains of *P. teres* (net blotch of barley, Table 5). We also confirmed the reliable control of *Pyrenophora tritici-repentis* (tan spot of wheat) and *Ramularia collo-cygni* (ramularia leaf spot of barley) in our trials (data not shown). These results are important since both QoI and SDHI resistance are emerging problems in these species. Therefore, the inclusion of metyltetraprole in spray programs will complement currently used fungicides.

Table 4. Results *Zymoseptoria tritici* field tests on wheat

Location	% G143A *(N of isolates G143A/total)*	% Severity Untreated plot	% Efficacy Metyltetraprole (120 g ha^{-1})	% Efficacy Pyraclostrobin (220 g ha^{-1})
France (1)	80 *(4/5)*	85.5	93.2	36.0
France (2)	100 *(5/5)*	86.4	96.2	14.8
Belgium	100 *(5/5)*	27.8	93.2	1.8
UK (1)	100 *(5/5)*	72.4	90.3	46.3
UK (2)	100 *(5/5)*	36.9	94.0	54.7
Ireland (1)	100 *(5/5)*	44.2	97.9	52.7
Mean	-	58.9	94.1	34.4

Table 5. Results *Pyrenophora teres* field tests on barley

Location	% F129L *(N of isolates F129L/total)*	% Severity Untreated plot	% Efficacy Metyltetraprole (120 g ha^{-1})	% Efficacy Pyraclostrobin (220 g ha^{-1})
France (1)	N.D.	19.9	88.4	51.3
France (2)	12.5 *(2/16)*	22.5	88.9	84.4
France (3)	55.6 *(5/9)*	14.8	94.6	3.4
France (4)	16.7 *(2/12)*	18.6	94.1	97.3
Mean	-	19.0	91.5	59.1

SUMMARY AND CONCLUSIONS

Metyltetraprole is an innovative QoI fungicide with a unique tetrazolinone-pharmacophore instead of methoxyacrylate or its analogous structure. It is less affected by currently known QoI-resistant mutations, especially for G143A, which induces strong resistance to existing QoIs. The intrinsic activity of metyltetraprole is particularly high against Ascomycota. As a foliar fungicide, metyltetraprole can penetrate leaves and is retained well at the position that received the droplets. Metyltetraprole offers a new solution for disease management to crop growers who have difficulty controlling crop pathogens resistant to various current fungicides. However, a resistance management strategy for metyltetraprole should also be carefully established to avoid the development of newly emerging resistant mutants to tetrazolinone-type QoIs.

REFERENCES

Arakawa A; Kasai Y; Yamazaki K; Iwahashi F (2018). Features of interactions responsible for antifungal activity against resistant type cytochrome bc1: A data-driven analysis based on the binding free energy at the atomic level. PLoS One 13, e0207673.

Leadbeater A (2012). Resistance risk to QoI fungicides and anti-resistance strategies. In: *Fungicide Resistance in Crop Protection*, eds T S Thind, pp. 141-154. CABI: Wallingford.

Matsuzaki Y; Yoshimoto Y; Arimori S; Kiguchi S; Harada T; Iwahashi F (2019). *Pest Management Science*, submitted.

Suemoto H; Matsuzaki Y; Iwahashi F (2019). Metyltetraprole, a novel putative complex III inhibitor, targets known QoI - resistant strains of Zymoseptoria tritici and Pyrenophora teres. *Pest Management Science* 75, 1181-1189.

Sierotzki H (2015). Respiration inhibitors: Complex III. Fungicide Resistance in Plant Pathogens. In: *Fungicide resistance in plant pathogens*, eds H Ihii and D H Hollomon, pp. 119–143. Springer: Tokyo.

Torriani SFF; Frey R; Buitrago C; Wullschleger J; Waldner M; Kuehn R; Scalliet G; Siertzki H (2017). Succinate-Dehydrogenase Inhibitor (SDHI) Resistance Evolution in Plant Pathogens. In: *Modern Fungicides and Antifungal Compounds*, eds Deising HB et al., Vol. VIII, pp. 89-94. Deutsche Phytomedizinische Gesellschaft: Braunschweig.

Craig IR et al., Molecular Insight into the Binding of Metyltetraprole: A New QoI Fungicide. Contribution to the Reinhardsbrunn Symposium Proceedings Volume. In: Deising HB; Fraaije B; Mehl A; Oerke EC; Sierotzki H; Stammler G (Eds), "Modern Fungicides and Antifungal Compounds", Vol. IX, pp. 271-276. © 2020. Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-16-7

Molecular Insight into the Binding of Metyltetraprole: A New QoI Fungicide

Craig IR, Stammler G, Bryson R, Rheinheimer J, Klappach K

BASF SE, Carl-Bosch-Strasse 38, D-67056 Ludwigshafen, Germany
Corresponding Author Email: ian.craig@basf.com

ABSTRACT

Metyltetraprole is a new, structurally-unique QoI fungicide based on tetrazolinone chemistry. By combining computational and experimental investigations molecular-level insight is obtained into the binding geometry of metyltetraprole at the Qo site. Importantly, in contrast to existing QoIs, metyltetraprole retains activity against G143A-mutated phytopathogens.

BACKGROUND

Metyltetraprole (Pavecto®) is a new, structurally-unique Qo inhibitor (QoI) fungicide. Like marketed QoIs it exerts its biological effect by binding to the Qo site of the cytochrome bc1 complex, thus inhibiting the fungal respiratory chain (Suemoto 2019). Over recent decades the QoIs have been one of the most important classes of agrochemical fungicides because they have provided effective control of a broad range of phytopathogens (AgriService 2016). However, various fungal diseases have developed resistance to the marketed QoIs, causing their field efficacy to decline. Although several resistance mechanisms have been observed, the G143A mutation in the Qo site has caused the largest loss of activity amongst existing QoIs.

A key point in the history of the QoI fungicides was the structural elucidation of Qo-binding natural products such as strobilurin A and oudemansin A in the mid 1970s. This work showed that these molecules share a common enoletherester structural component (see Figure 1). Due to a hydrogen-bonding interaction made by the carbonyl of this functional group with a backbone amide-NH, this part of the QoI molecular structure is here named the “backbone binding unit” (BBU). From these starting points, roughly twenty years of research and development led to kresoxim methyl and azoxystrobin entering the market as the first QoI fungicides in the mid 1990s (AgriService 2016). The lead optimization efforts had resulted in significant changes to the structures of the rest of the molecules, but the BBUs of these first QoIs ended-up largely or entirely unchanged from the initial natural products. The low structural variation of the backbone binding unit continued as further QoIs were launched in the early 2000s, with fluoxastrobin (market entry 2004) perhaps showing the greatest dissimilarity.

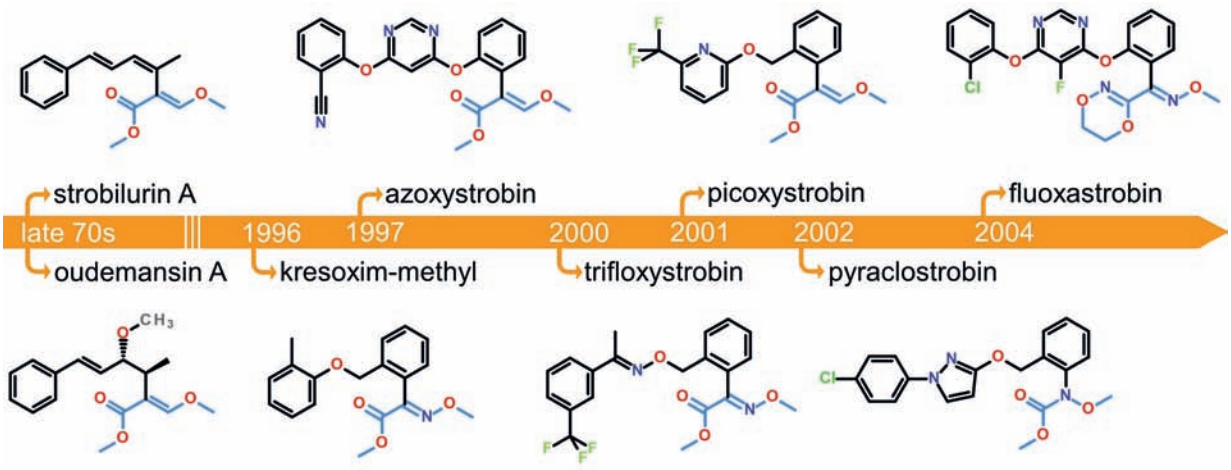


Figure 1. Timeline showing molecular structures and market entry dates for selected QoIs (AgriService 2016). The backbone binding units (BBUs) are highlighted in blue.

CHEMICAL & BIOLOGICAL PROPERTIES OF METYLTETRAPROLE

The molecular structure of metyltetraprole possesses two unique features not found in marketed QoIs. Firstly, metyltetraprole is based on a tetrazolinone backbone binding unit. This makes it the first QoI fungicide to possess an aromatic ring BBU and means that metyltetraprole represents a significant development beyond the trend observed along the timeline above. Secondly, the molecule features a novel methyl substituent on the central phenyl ring (Figure 2). Aside from these two aspects, the hydroxypyrazole side-chain is identical to that of pyraclostrobin.

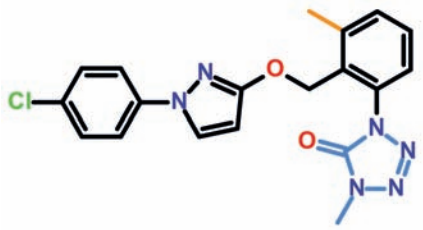


Figure 2. Molecular structure of metyltetraprole. The tetrazolinone backbone binding unit is highlighted blue and the novel methyl substituent in orange.

In additional to these unique structural features, metyltetraprole also exhibits a unique biological property. In particular, in contrast to marketed QoIs the molecule retains strong activity against G143A-mutated strains of plant pathogens (Suemoto 2019). Additional evidence of this is shown in Table 1 which presents half-maximal inhibition concentrations (IC50s) derived from an enzyme assay using mitochondrial membrane preparations. This assay measures loss of enzymatic activity of cytochrome bc1 complex, for example due to QoI inhibition. The assay was performed with both wild-type (WT) and G143A-mutated cytochrome bc1 complex from the wheat pathogen *Zymoseptoria tritici* using experimental procedures analogous to those described previously (Dreinert 2018). Whereas the three QoIs in Table 1 inhibit the wild-type complex to a similar degree, there are significant differences in inhibition of the G143A-mutated

complex. The strobilurins azoxystrobin and pyraclostrobin are hundreds of times weaker against the mutated Qo site. Metyltetraprole, in contrast, shows a similar level of inhibition of WT and G143A-mutated complex.

QoI	*Z. tritici* WT IC50 [µM]	*Z. tritici* G143A IC50 [µM]	Resistance Factor G143A
metyltetraprole	0.06	0.01	~1
pyraclostrobin	0.01	2.4	240
azoxystrobin	0.2	> 100	> 500

Table 1. Half-maximum inhibition concentrations (IC50s) derived from enzyme assays for three QoIs against wild-type (WT) and G143A-mutated cytochrome bc1 complex from *Z. tritici*. The IC50 of azoxystrobin against the G143A complex was too weak to be properly determined and therefore set to the highest measured concentration.

BINDING GEOMETRY

The key to understanding the relationship between novel aspects of a structure of a molecule and any unusual enzyme inhibition properties is often knowledge of the enzyme-ligand binding geometry ("binding mode"). Computational homology modelling was therefore used to derive a model protein structure of the *Z. tritici* cytochrome-bc1:tetrazolinone complex, based on a crystallographically-determined tetrazolinone-bound yeast structure (unpublished; Hunte lab, University of Freiburg, Germany). The first step in this process is an alignment of the protein sequences of yeast and *Z. tritici* cytochrome bc1 complexes. For cytochrome b, this revealed that 60% of the sequence positions have the same amino-acid in yeast and *Z. tritici*. In the Qo site, the sequence identity rises to 85%. The high sequence identity between the yeast template and *Z. tritici* target indicates high potential for a reliable homology model. In addition, the alignments also reveal specific amino-acid positions differing between the two organisms. In the Qo site these are I125 in yeast to M125 in *Z. tritici* and I122 to V (yeast numbering). Starting from the yeast template structure the homology modelling software implements the identified amino-acid substitutions. A force-field-based energy minimization procedure is then applied to mitigate against any steric clashes introduced before the final *Z. tritici* model is produced. In this work homology models for both WT and G143A-mutated *Z. tritici* cytochrome bc1 complex were produced with MOE (MOE 2019).

The experimentally-derived binding-mode of metyltetraprole in the homology model of cytochrome bc1 complex from wild-type *Z. tritici* is shown in Figure 3. Comparison of the bound conformation of metyltetraprole with the binding-site surface indicates good spatial fit and therefore attractive van der Waals interactions. A number of directional interactions are also observed. There is a strong hydrogen bond between the tetrazolinone carbonyl oxygen and the backbone NH of Glu273 and a weaker one between the tetrazolinone N3 a backbone CH bond. Multiple CH-π interactions are also formed with the aromatic rings of the hydroxypyrazole side-

chain. Taken together, the binding geometry and molecular interactions predicted to form between metyltetraprole and the WT *Z. tritici* Qo-site rationalize the strong inhibition measured in the experiments described above.

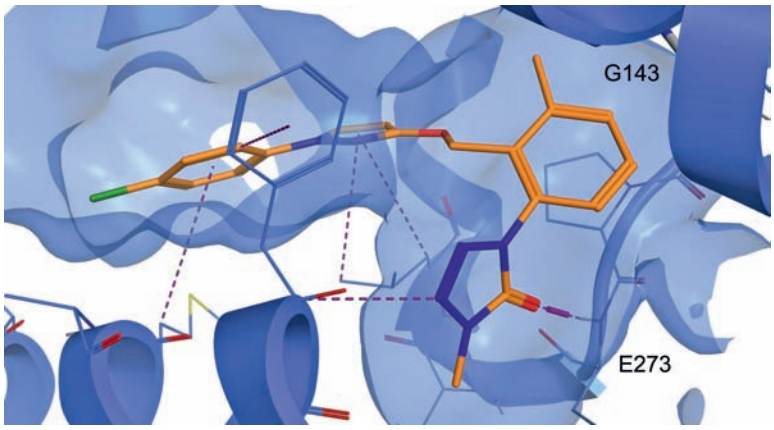


Figure 3. Experimentally-derived binding mode of metyltetraprole at the Qo site of a homology model of cytochrome bc1 complex from wild-type *Z. tritici*. Hydrogen bonds and CH-π interactions are represented with purple dashed lines. The binding-site molecular surface is rendered blue. Figure produced with MOE (MOE 2019).

MOLECULAR HYPOTHESIS FOR ACTIVITY AGAINST G143A

The experimentally-derived binding modes of metyltetraprole at the wild-type and G143A-mutated Qo sites of *Z. tritici* form the basis of a molecular hypothesis rationalizing the activity of the molecule against G143A-mutated pathogens. In addition, binding modes for marketed QoIs are required to differentiate metyltetraprole from existing fungicides, which show significant loss of activity against G143A mutants. They were derived for pyraclostrobin and azoxystrobin at the WT and G143A *Z. tritici* Qo sites using in-house and publicly-available protein structures respectively.

As can be seen by comparison of panel (a) and (b) in Figure 4, the G143A mutation introduces an additional methyl group pointing into the Qo site, thus narrowing it. When the WT binding mode of pyraclostrobin is confronted with this alteration (panel (b)), some atoms of the BBU and central phenyl ring approach the additional carbon at position 143 at short distances significantly below the sum of the atom's van der Waals radii. Although not shown in Fig. 4, the same is true for azoxystrobin. These "steric clashes" are repulsive and weaken the binding of the marketed QoIs. Due to the smaller size of the tetrazolinone ring, metyltetraprole suffers only one minor steric clash (panel (d)). It is therefore proposed that the stark differences in resistance factors reported in Table 1 are primarly due to differences in the size and shape of each QoI's backbone binding unit, since these lead to differences in the number and strength of steric clashes with the G143A mutation. Previous work used different, computationally-predicted binding modes to reach a similar view (Arakawa 2018).

If this qualitative hypothesis for metyltetraprole's activity against G143A-mutated cytochrome bc1 is correct, accurate binding-energy calculations for the respective BBU-phenyl fragments

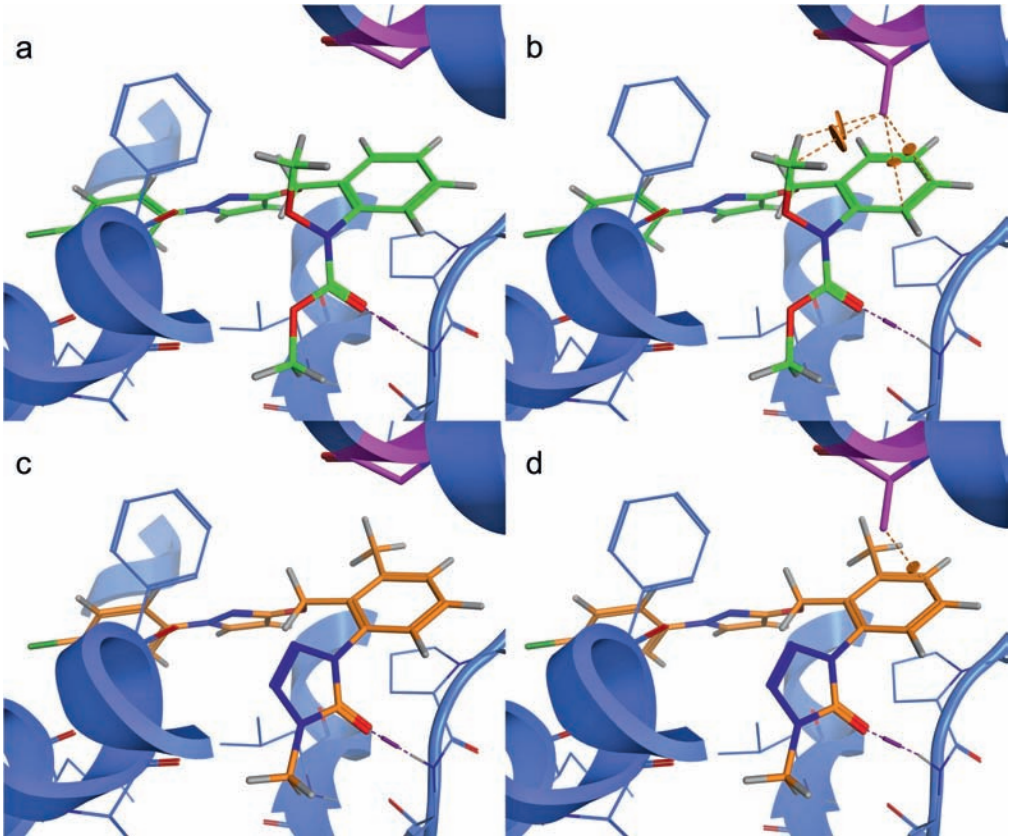


Figure 4. Binding modes at the Qo sites of homology models of *Z. tritici* cytochrome bc1: (a) pyraclostrobin at WT site, (b) pyraclostrobin at G143A site, (c) metyltetraprole at WT site, and (d) metyltetraprole at G143A site. Position 143 is highlighted pink. Steric clashes (see text) are indicated by orange dashed lines and discs.

binding to the key amino-acids involved (G143/A143, P272, and E273) should recapitulate the trend in Table 1. To test this, density functional theory (DFT) calculations were used to evaluate the energy of: (i) the free BBU-phenyl fragments (in their bound conformations), (ii) the empty three-residue binding-site models defined above, and (iii) the complexes formed from (i) and (ii). For each QoI / binding-site combination, subtracting the energies of the first two components from that of the latter gives the binding-energy. The DFT calculations used the ωB97X functional, the 6-31G**++ basis set, and the counterpoise correction for basis-set superposition error. They were performed with Jaguar (Bochevarov 2013).

The differences in the DFT-calculated WT and G143A binding energies are shown in Table 2. The value for metyltetraprole is more negative than those for the two marketed QoIs, consistent with lower loss of binding energy due to the mutation and with the measured resistance factors. Since these minimal binding models neglect parts of the binding site (and solvation), the absolute values of the energy differences should not be overinterpreted. However, the difference of ~2.7 kcal/mol between the binding-energy differences of metyltetraprole and the existing QoIs is thermodynamically consistent with the order-of-magnitude of the difference in the measured resistance factors (hundreds). Overall, the DFT calculations support the above hypothesis for metyltetraprole's retained inhibition of G143A phytopathogens.

QoI	DFT binding energy difference G143A-WT [kcal/mol]	Measured resistance factor G143A (from Table 1)
metyltetraprole	-1.08	~1
pyraclostrobin	+1.79	240
azoxystrobin	+1.36	> 500

Table 2. DFT binding energy differences for minimal models of QoI binding.

SUMMARY

Combining protein crystallography with molecular modelling and visualization has led to the hypothesis that the size and shape of the unique tetrazolinone backbone binding unit results in fewer and weaker steric clashes with the G143A-mutated Qo site and the retention of inhibition of G143A-mutated fungal strains.

REFERENCES

AgriService (2016). *AgriReference Products*, 269-276. Phillips McDougall: Midlothian, UK.

Arakawa A; Kasai Y; Yamazaki K; Iwahashi F (2018). Features of interactions responsible for antifungal activity against resistant type cytochrome bc1. *PLoS ONE* 13, e0207673.

Bochevarov AD; Harder E; Hughes TF; Greenwod JR; Braden DA; Phillip DM; Rinaldo D; Halls MD; Zhang J; Friesner RA. Jaguar. *Internation Journal of Quantum Chemistry* 113 (18), 2110-2142.

Dreinert A; Wolf A; Mentzel T; Meunier B; Fehr M (2018). The cytochrome bc1 complex inhibitor Ametoctradin has an unusual binding mode. *Biochimica et Biophysica Acta (BBA) – Bioenergetics* 1859 (8), 567-576.

MOE (2019). Chemical Computing Group ULC: Montreal, Canada.

Suemoto H; Matsuzaki Y; Iwahashi F (2019). Metyltetraprole, a novel putative complex III inhibitor, targets known QoI-resistant strains of Zymoseptoria tritici and Pyrenophora teres. *Pest Management Science* 75, 1181-1189.

Yoshimoto Y, Arimori S, Discovery of Pavecto® - a new Q_OI Fungicide. In: Deising HB; Fraaije B; Mehl A; Oerke EC; Sierotzki H; Stammler G (Eds), "Modern Fungicides and Antifungal Compounds", Vol. IX, pp. 277-278. © 2020. Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-16-7

Discovery of Pavecto® - a new Q_OI Fungicide

Yoshimoto Y, Arimori S

Sumitomo Chemical Co., Ltd. Health and Crop science Research Laboratory, 2-1 Takatsukasa 4-chome, Takarazuka, Hyogo, 665-8555 Japan
Corresponding Author Email: yosimotoy@sc.sumitomo-chem.co.jp

INTRODUCTION

Pavecto® (ISO name: metyltetraprole, Figure 1) is a novel QoI fungicide discovered by the Sumitomo Chemical Company. It has a unique chemical structure including a tetrazolinone moiety and methyl group on the central linking ring and exhibits potent activity against both QoI resistant strains and wild types.

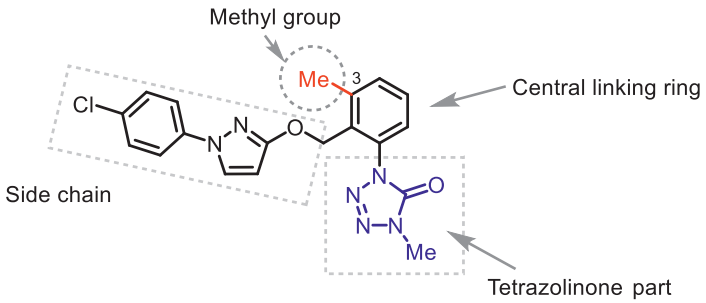


Figure 1 Structure of metyltetraprole

SEED GENERATION

First, the microplate assay of *Zymoseptoria tritici* (QoI-resistant (G143A) and -sensitive strains) was performed. After screening approximately 200 QoI samples, a group of samples with a tetrazolinone moiety as the pharmacophore (e.g. compound 1, Figure 2) were identified, which showed similar fungicidal activity against both strains. The half-maximal effective concentration (EC_{50}) values of compound **1** were 0.38 mg/L and 0.18 mg/L against QoI-resistant and -sensitive strains, respectively.

DISCOVERY OF LEAD COMPOUND AND OPTIMIZATION TO METYLTETRAPROLE

Attempts to increase the fungicidal activity of compound **1** were made by substituting its side chain with different types of groups. As a result, compound **2** with a *p*-chlorophenyl pyrazole was identified as the lead compound (Figure 2). Although compound **2** exhibited enhanced antifungal activity (EC_{50} = 0.02 mg/L) against *Z. tritici*, its biological activity was not comparable to that of the existing fungicides. Therefore, to further enhance the activity of **2**, a

part of the central linking ring was modified. The effects of various substituents were determined via these modifications. The introduction of various substituents on the third position of the benzene ring significantly increased the antifungal activity compared to that of the unsubstituted compound **2**. Tetrazolinone derivatives with substituents on the third position showed extremely potent fungicidal activity against *Z. tritici* (resistant and sensitive strain). Based on the results of various tests, metyltetraprole was finally selected as the most promising compound (EC_{50} = 0.002 mg/L).

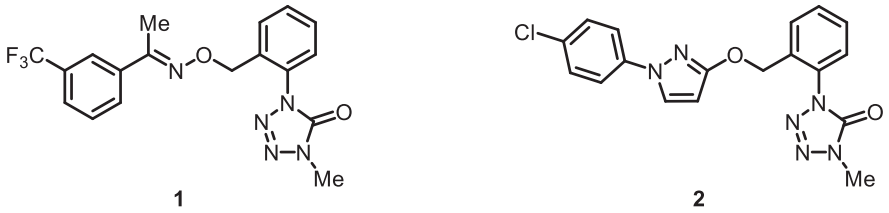


Figure 2 Structures of **1** and **2**

The potent efficacy of metyltetraprole was confirmed in the antifungal microtiter test as well as *in planta* tests including the field trials (Suemoto et *al*. 2019).

CONCLUSIONS

Metyltetraprole is a novel QoI fungicide discovered by the Sumitomo Chemical Company. It differs from the existing QoI fungicides because it is effective against pathogens that have developed resistance toward strobilurin fungicides currently available on the market. Metyltetraprole has a unique chemical structure including a tetrazolinone moiety and methyl group on the central linking ring. The tetrazolinone group contributes to the increased efficacy against QoI-resistant fungal pathogens compared to the conventional QoIs. The methyl group at the third position of the benzene ring plays an important role in enhancing the fungicidal activity against various plant pathogens.

Metyltetraprole effectively controls major plant diseases and improves the crop produce and profitability. It will be submitted for registration in many countries, and we look forward to making metyltetraprole formulated products available to more farmers around the world.

REFERENCES

Arakawa A; Kasai Y; Yamazaki K; Iwahashi F (2018). Features of interactions responsible for antifungal activity against resistant type cytochrome *bc*1: A data-driven analysis based on the binding free energy at the atomic level. *PLoS ONE* 13(11), e0207673.

Suemoto H; Matsuzaki Y; Iwahashi F (2019). Metyltetraprole, a novel putative complex III inhibitor targets known QoI-resistant strains of *Zymoseptoria tritici* and *Pyrenophpra teres*. *Pest Management Science* 75(4), 1181-1189.

Young D et al., Fenpicoxamid Provides a New Target Site for Control of Septoria Leaf Blotch in Cereals. In: Deising HB; Fraaije B; Mehl A; Oerke EC; Sierotzki H; Stammler G (Eds), "Modern Fungicides and Antifungal Compounds", Vol. IX, pp. 279-284. © 2020. Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-16-7

Fenpicoxamid Provides a New Target Site for Control of Septoria Leaf Blotch in Cereals

Young D, Kemmitt G, Leader A

Corteva Agriscience, 9330 Zionsville Road, Indianapolis, IN 46268, USA

Email: david.young@corteva.com

ABSTRACT

Fenpicoxamid (Inatreq™ active) belongs to a new chemical class of fungicide (picolinamide) for control of Septoria (*Zymoseptoria tritici*) and other diseases. Fenpicoxamid is derived from the antifungal natural product UK-2A, which is produced by fermentation of an actinomycete (*Streptomyces sp. 517-02*). It undergoes a minor synthetic modification post-fermentation for stabilization, then in plants or fungi is metabolized to release UK-2A. Fenpicoxamid will be the first product in the cereals market to act at the Qi site of the cytochrome bc_1 complex in mitochondria. Because it has a novel mode of action in cereals, there is no target site-based cross-resistance between fenpicoxamid and current fungicides. Baseline sensitivity testing of European *Z. tritici* isolates has been conducted to characterize sensitivity of the existing population to fenpicoxamid. Continued field monitoring will enable early detection of any potentially less sensitive *Z. tritici* strains that may develop after product introduction. Widespread resistance to strobilurin fungicides, an erosion in efficacy of azole fungicides, and detection of isolates with resistance to the more recently introduced SDHI fungicides has created an urgent need for a compound such as fenpicoxamid with a new mode of action to control *Z. tritici*. However, implementation of a sound resistance management strategy will be critical to maintain its long-term effectiveness.

BIOLOGICAL PROPERTIES AND MODE OF ACTION

Control of Septoria leaf blotch currently relies heavily on azole and SDHI fungicides, as well as the multi-site fungicide chlorothalonil. The efficacy of azoles continues to decrease, SDHI efficacy is threatened by populations with reduced sensitivity, and chlorothalonil is to be withdrawn from the European market due to regulatory concerns. Consequently, there is an urgent need for a fungicide like fenpicoxamid with a new mode of action that controls resistant *Zymoseptoria tritici* populations.

Fenpicoxamid is a derivative of the natural product UK-2A, and was discovered at Dow AgroSciences LLC through a collaboration with Meiji Seika Pharma Co. Ltd. In the manufacture of fenpicoxamid, UK-2A is produced by large scale fermentation of the producing organism, *Streptomyces* sp. 517-02, and is converted to fenpicoxamid through a single chemical modification of the picolinamide head group (Figure 1).

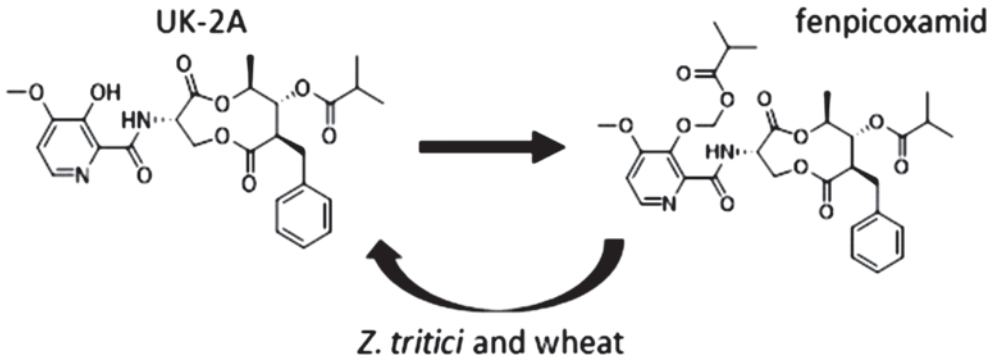


Figure 1. Fenpicoxamid is produced from UK-2A and reverts to UK-2A in *Z. tritici* and wheat.

UK-2A exhibits high *in vitro* fungitoxicity, however its efficacy in the greenhouse and field is limited by oxidation and UV instability on the plant surface (Owen et al. 2017). In fenpicoxamid, derivatization of the hydroxyl of UK-2A with an isobutyryloxymethyl ether group greatly improved both stability on the plant surface and disease control. Against *Z. tritici*, efficacy of fenpicoxamid in greenhouse protectant and curative tests exceeded that of UK-2A by 12-fold and 63-fold, respectively.

In field trials across Europe over 2 years (2014 and 2015), fenpicoxamid at 100 g/ha delivered disease control and yield benefits (Figure 2) which were superior to prothioconazole at 198 g/ha, and equivalent to a leading mixture product of bixafen and prothioconazole at 281 g/ha.

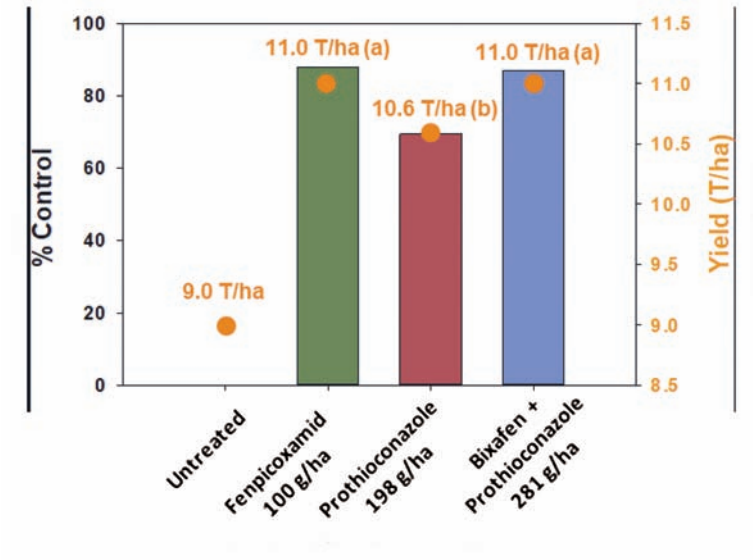


Figure 2. Control of Septoria leaf blotch and grain yield across 22 trials in 6 European countries using 2 applications in each trial.

In addition to protectant activity, fenpicoxamid provides translaminar control and protects new growth through local systemicity. Slow penetration of fenpicoxamid into leaves over time,

which is enhanced by the formulation, results in excellent residual activity. After uptake into leaves, fenpicoxamid is converted to UK-2A which is mobile and relatively stable in tissue.

UK-2A inhibits respiration by binding to the Qi site of cytochrome bc_1 (complex III) in the mitochondrial electron transport chain. As such, its site of action is different from other products used for Septoria control, including the QoI fungicides (strobilurins), SDHIs and azoles. Structurally, UK-2A is related to the classic Qi site inhibitor antimycin A, and is a highly potent inhibitor of complex III (IC50 = 6.71 nM in a cytochrome *c* reductase assay), comparable to both antimycin (7.14 nM) and the highly potent QoI inhibitor pyraclostrobin (3.48 nM). Ligand docking studies using the crystal structure *Saccharomyces cerevisiae* cytochrome bc_1 in combination with Qi site mutant sensitivity data have revealed the likely docking pose for UK-2A, and its target site interactions (Young et al. 2018). Mutants with reduced sensitivity to UK-2A and fenpicoxamid contained single amino acid substitutions N31K, G37C and L198F in cyt *b*, which were located at the Qi site and conferred cross-resistance to antimycin A. The exchange N31K likely disrupts a key salt bridge interaction between Asp-229 and the pyridyl N of UK-2A, while the G37C and L198F substitutions probably displace the bislactone ring system from its optimal binding conformation though steric interference.

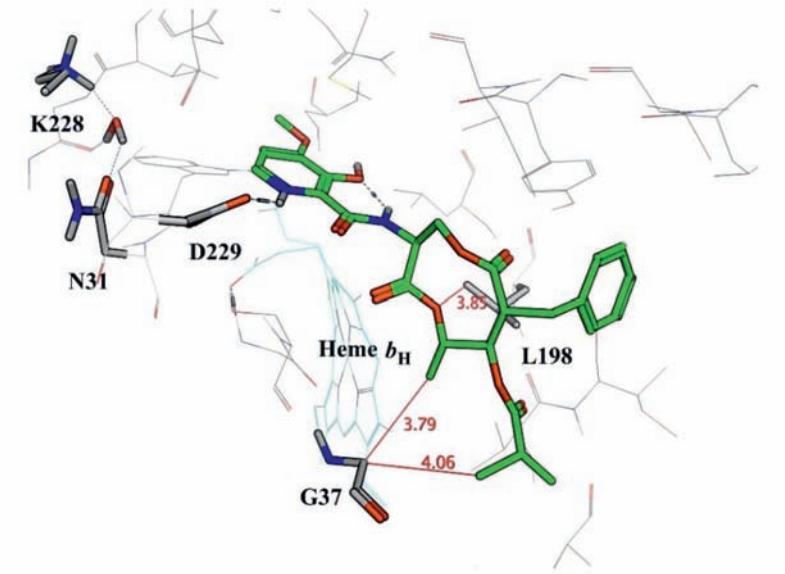


Figure 3. Binding of UK-2A at the Qi site of *S. cerevisiae* cytochrome *b*.

Mode of action classification by FRAC places fenpicoxamid in the QiI fungicide group 21, along with cyazofamid and amisulbrom, which are used exclusively to control Oomycete diseases. Fenpicoxamid represents a new chemical class, “picolinamide”, in this group and is the first QiI fungicide with activity against Ascomycete and Basidiomycete fungi.

Since fenpicoxamid provides a new mode of action in the cereal market, cross-resistance to current products would not be expected and this has been confirmed in testing against resistant isolates. Efficacy of fenpicoxamid is not affected by the G143A mutation at the Qo site in

cytochrome *b*, which confers resistance to strobilurin fungicides, and controls azole-resistant strains containing CYP51 mutations associated with resistance to azole fungicides (Table 1).

Table 1. Control of fungicide-resistant *Z. tritici* isolates by fenpicoxamid in low volume greenhouse tests (adapted from Owen et al. 2017).

Fungicide	MoA	Wild Type[a]	UK-4[b]	UK-7	UK-10	UK-12	UK-13
Fenpicoxamid	QiI	1.8[c]	1.8	1.3	1	1.2	6.2
Fluxapyroxad	SDHI	6.7	6.8	6.9	4.1	5.8	27
Epoxiconazole	DMI	4.2	98.3	>100	>100	>100	>100
Pyraclostrobin	QoI	3.8	>100	>100	>100	>100	>100

[a]Wild type strain ATCC26518.

[b] All "UK" strains contain the G143A exchange in cytochrome *b*, which confers strobilurin resistance, as well as various CYP51 mutations associated with azole resistance. UK-10 and UK-12 overexpress *CYP51*. UK-12 and UK-13 overexpress the efflux pump gene *MgMFS1*.

[c] Data are EC_{80} values (g/ha).

Efficacy of fenpicoxamid against *Z. tritici* strains with reduced sensitivity to SDHIs has been evaluated in laboratory-generated mutants (Table 2). Compared to the wild type parent strain IPO323, strains with mutations in succinate dehydrogenase show a range of reduced sensitivity to the SDHI bixafen, but no significant change in sensitivity to fenpicoxamid.

Table 2. Sensitivity of SDHI-resistant lab mutants of *Z. tritici* with amino acid substitutions in succinate dehydrogenase subunits B, C and D to bixafen and fenpicoxamid.

				EC_{50} (mg/L) ± SD	
Strain	SdhB	SdhC	SdhD	Bixafen	Fenpicoxamid
IPO323[a]	wt	wt	wt	0.0694 ± 0.0108	0.0104 ± 0.0046
M36	H267Y	wt	wt	1.06 ± 0.13	0.00934 ± 0.00542
M38	wt	wt	D129T	2.67 ± 0.19	0.0240 ± 0.0104
M62	I269V	wt	wt	0.365 ± 0.137	0.0157 ± 0.0127
M112	wt	wt	D129E	0.347 ± 0.135	0.0145 ± 0.0071
M142	wt	N86K	wt	>10	0.0105 ± 0.00464
M148	C137R	wt	wt	0.995 ± 0.091	0.0159 ± 0.0094
M171	S221P	wt	wt	0.666 ± 0.176	0.0205 ± 0.0096

[a]Parent strain for all mutants.

BASELINE SENSITIVITY AND RESISTANCE MANAGEMENT

Baseline sensitivity of the European *Z. tritici* population to fenpicoxamid has been analyzed by *in vitro* testing of almost 4,000 isolates from 15 countries between 2011 and 2018 (Figure 4). The mean EC_{50} value across isolates was 0.040 mg/L and values for >90% of the population were within a relatively narrow 10-fold concentration range of 0.01 – 0.1 mg/L. Ongoing monitoring of field populations after product launch will be carried out to detect any future shift in sensitivity.

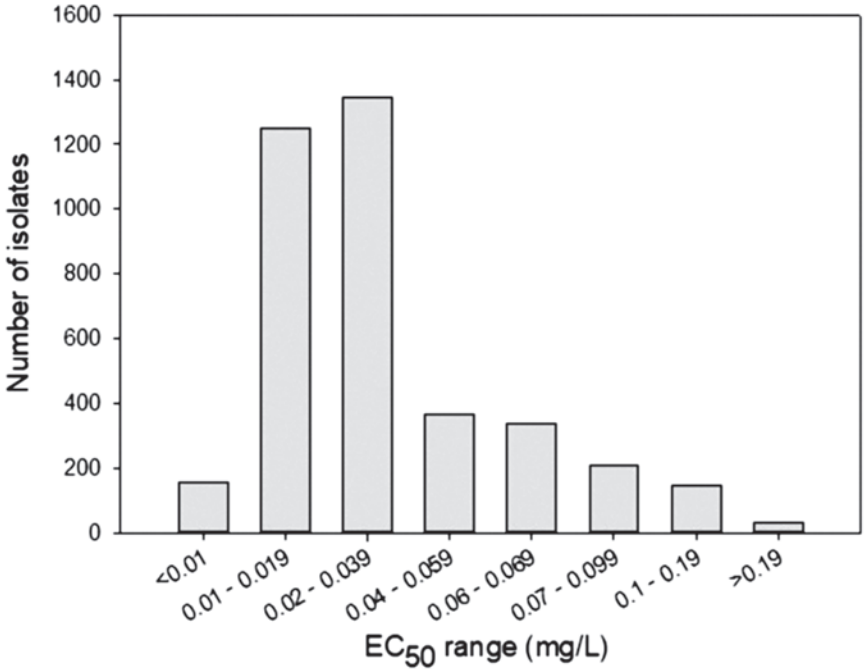


Figure 4. Baseline sensitivity of European *Z. tritici* field isolates to fenpicoxamid.

Complex III inhibitors have a long history of use as fungicides and provide useful context in assessing the resistance risk for fenpicoxamid and strategies for resistance management. Strobilurins were introduced as the first complex III inhibitors in 1996, and several factors contributed to intense selection pressure. At that time, there was a belief that resistance risk was low because cytochrome *b* is encoded by a mitochondrial gene and this encouraged poor resistance management practices, including multiple applications per season and use in solo applications rather than mixtures. This situation was aggravated by the introduction of multiple competing products in a short space of time. Three mutations have been identified in the field, and the G143A mutation has been especially problematic since it confers very high resistance in many different pathogens with generally little impact on fitness. In the case of *Z. tritici*, the G143A mutation was detected 4 years after introduction of strobilurins with a dramatic loss in efficacy after 7 years (Sierotzki 2015). Similarly, in *Plasmopara viticola* the G143A mutation was detected after 2 years with loss in efficacy after 4 years.

The first QiI fungicides, cyazofamid and amisulbrom, were introduced in 2001 and 2003, respectively, and are used only to control Oomycete diseases. The most recent complex III inhibitor, ametoctradin, is also used to control Oomycetes and binds to both the Qo and Qi sites. For these compounds, there have been few reports of resistance, although different Qi site mutations conferring resistance to cyazofamid and ametoctradin in *P. viticola* were recently reported in France (Note Technique Commune Resistances 2019). The reason why these more recent complex III inhibitors did not experience the dramatic loss in efficacy observed for strobilurins is unclear, but poor resistance management strategy for strobilurins was probably an important factor. While target site based resistance is known for all complex III inhibitors, the impact on efficacy has been variable and may be mitigated by good resistance management strategy.

For fenpicoxamid, studies in yeast (Young et al. 2017) and more recently in *Z. tritici* (Fouche et al. 2020) have shown that resistant mutants can be isolated under laboratory conditions. No evidence for resistance has been found in field populations. However, since the resistance risk is assumed to be medium to high, implementation of a sound resistance management strategy is essential. To preserve the long term effectiveness of fenpicoxamid, recommendations will include only one application per season and use in mixture with another effective mode of action.

ACKNOWLEDGEMENTS

We are grateful to B. Fraaije for providing *Z. tritici* strains and SDHI cross-resistance data, and to many colleagues at Corteva for their scientific contributions and technical support.

REFERENCES

Owen WJ; Yao C; Myung K; Kemmitt G; Leader A; Meyer K; Bowling AJ; Slanec T; Kramer VJ (2017). Biological characterization of fenpicoxamid, a new fungicide with utility in cereals and other crops. *Pest Management Science* 73, 2005-2016.

Young DH; Wang NX; Meyer ST; Avila-Adame C (2018). Characterization of the mechanism of action of the fungicide fenpicoxamid and its metabolite UK-2A. *Pest Management Science* 74, 489-498.

Sierotzki H (2015). Respiration inhibitors: complex III. In: *Fungicide Resistance in Plant Pathogens*, eds H Ishii & DW Hollomon, pp. 119-143. R A Kay & W J Wood, pp. 103-119. Springer: Tokyo.

Note Technique Commune Resistances 2019 Maladies de la Vigne: Mildiou, Oidium, Pourriture Grise, Black-Rot. Available at https://www.r4p-inra.fr.

Fouche G; Debieu D; Young D; Meunier B; Walker AS; Fillinger S (2020). Assessing the risk of resistance selection towards quinone inside inhibitor fungicides (QiIs) in *Zymoseptoria tritici*. In: this volume.

REGULATORY ASPECTS

Matyjaszczyk E, Registered doses of active substance in fungicides. Seed versus foliar treatment. In: Deising HB; Fraaije B; Mehl A; Oerke EC; Sierotzki H; Stammler G (Eds), "Modern Fungicides and Antifungal Compounds", Vol. IX, pp. 287-294. © 2020. Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-16-7

Registered doses of active substance in fungicides. Seed versus foliar treatment.

Matyjaszczyk E

Institute of Plant Protection – National Research Institute ul. Władysława Węgorka 20, 60-318 Poznań, Poland

Corresponding Author Email: e.matyjaszczyk@iorpib.poznan.pl

ABSTRACT

A study into doses of seed and foliar fungicides containing identical active substances registered to control the same pest in the same crop was carried out. The search was performed in nine European Union member states: Austria, Czech Republic, Belgium, Finland, Germany, Italy, Poland, Slovakia and Spain. The results show that the use of seed treatment is usually connected with a significantly lower release of active substance per hectare when compared with foliar treatments. For fungicide use, the difference is often 8-fold or higher.

These results apply to fungicides only and should not lead to the general conclusion that seed treatment is a more environmentally friendly method of application of plant protection products in comparison to foliar application. In case of insecticides much higher dose per treated area is usually recommended while using seed treatment, as shown by another study of the same author.

INTRODUCTION

Reduction of dependency on chemical control is one of the aims of implementation of obligatory integrated pest management (IPM) in the European Union. The professional user should keep the use of pesticides and other forms of intervention to levels that are necessary (Directive 128/2009). Several measures can be used to reach this goal. The emphasis on preventive measures of pest control, as well as use of plant protection products of biological origin are among others encouraged in IPM (Feldmann 2017, Matyjaszczyk 2018). Reduction of pesticide use is also possible by using appropriate application techniques and adjuvants (Kucharski et al. 2015). There are also other means to monitor and reduce pesticide use (Łącka 2018, Szpyrka et al. 2017, Baranowska et al. 2019, Mazurkiewicz et al. 2017).

For protection against pests during the early growth stages of the crop different choices may be possible: seed dressing and/or foliar treatments. Several sources stresses that plant protection product applications in the form of seed dressings allow for the reduction of the amount of an active substance per hectare in comparison with foliar treatment (Taylor and Harman 1990, Stevens 2002). Matyjaszczyk (2017) has shown that this statement is only partially true. In fact,

for insecticides, the consumption of an active substance for seed treatment is, as a rule, much higher than for foliar application. However, the use of fungicide seed treatment is usually connected with a significantly lower release of active substance per hectare when compared to foliar treatments.

It is difficult to find data on how much plant protection products farmers really use. However, the registration data are quite clear and show how much plant protection products farmers can use.

The aim of this paper is to answer the question how significant the difference in recommended dose of active substance per hectare between seed and foliar treatment in the protection against diseases is.

METHODS

Research into the registers of plant protection products in European Union member states was carried out. The member states analyzed were Austria, Czech Republic, Belgium, Finland, Germany, Italy, Poland, Slovakia and Spain. The search was performed in 2016 (Austria, Belgium, Finland, Germany, Italy, Poland, Spain) and 2017 (Czech Republic, Slovakia).

The objective of the research was to find matches between seed dressings and products for foliar application registered in the same member state, containing the same active substance or combination of identical active substances and registered to control the same pests in the same crops. The doses of active substance recommended per hectare were compared. To calculate the amount of an active substance per hectare, it was estimated that the foliar products as well as seed dressings were applied according to the maximum recommended dosage. The maximum recommended sowing rate was followed in the case of seed dressings.

Following the label recommendation, seed dressings and foliar treatments are applied at different growth stages of the crop: seed dressing on sowing material, it means before sowing, while foliar treatments are applied after seedling emergence. When comparing amount of active substance used for seed versus foliar treatment this difference is unavoidable.

RESULTS

All EU Member States have a common list of approved active substances which is published as part of Regulation 540/2011. However, plant protection products containing these active substances are registered separately in every single EU Member State due to different climatic, crop and pest conditions in particular regions. Therefore, the availability of plant protection products differs among the member states (Matyjaszczyk 2011, Matyjaszczyk & Sobczak 2017). It may appear that the registered doses differ among the member states as well.

The attached Table 1 presents the comparison of matches of foliar plant protection products in comparison to seed dressings containing the same active substances and registered to protect the same species of arable plants against the same diseases.

In the majority of the analyzed cases of fungicides, the registered use of active substance per hectare is lower when using seed dressing. The difference is usually significant and is rather measured in multiples than in fractions. Only in two member states: Czech Republic and Italy a case was found (in both cases protection of wheat using fluxapyroxad) where the registered use of active substance per hectare was similar for both application methods. For fluquinconazole and prochloraz, fungicides registered in Austria and Poland for the protection of seedborne wheat disease, the recommended dose of one active substance was lower, while the second was much higher. The ratio differed between Austria and Poland, due to slightly different doses recommended for use in both member states. However the recommended dose of fluquinconazole was higher in seed dressing (20% higher in Austria, while 50% higher in Poland when compared to the foliar spray product), but at the same time the recommended dose of prochloraz was very significantly lower (14 times lower in Austria and 12.4 times lower in Poland) when compared to foliar spray product.

The highest difference in registered use of active substance per hectare in a product containing one active substance was noted in Czech Republic for fungicides containing tebuconazole; it amounted to a 34.4 times higher dose in the foliar spray product. In case of products containing two active substances, the maximum difference in registered use of one of them was 64 times more in foliar fungicide and it was again for tebuconazole in Czech Republic. The second active substance of this product was prothioconazole and it was registered in a dose that was 4.2 times higher than in the foliar spray product.

It is very difficult to make any comparisons between recommended use of different active substances. Therefore to conclude what exactly is the difference in the registered use of product if the recommended use of one active substance is 4.2 times more in the foliar product and the second 64 times more is very challenging. However it is still possible to conclude that this difference is very significant and a much higher amount of active substance is recommended for use in foliar applications.

CONCLUSIONS

After analyzing the data presented in Table 1, it is possible to observe that the difference in registered use of active substance of fungicides between seed and foliar treatments. A 20-fold higher rate in foliar products is not uncommon, while 8-fold higher doses are common. The difference of 8-fold lower registered use in the case of seed treatment translates into the fact, that even if only once per seven years seed treatments achieve positive results, the release of active substance into the environment will still be lower than in the case of emergency foliar treatment after the confirmed disease's symptoms (assuming that 1 foliar treatment will be sufficient to effectively control the developing disease).

It therefore may be concluded that in case of fungicides, the difference in recommended dosage of an active substance per hectare between seed and foliar treatment is most often sufficiently significant to compensate for the occasional, unnecessary use of seed dressings. Therefore

considering the rules of integrated pest management (Directive 2009, Matyjaszczyk 2019) fungicidal seed treatment should be recommended in integrated pest management rather than foliar application if such an alternative exists.

However, that should not lead to the general conclusion that seed treatment is more environmental friendly method of application of plant protection products in comparison to foliar application. In case of insecticides, a much higher dose per treated area is recommended for using seed treatment in comparison with foliar spray applications, as shown by Matyjaszczyk (2017).

REFERENCES

Baranowska A; Mystkowska I; Zarzecka K; Radwańska K (2019). Impact of growth biostimulators and herbicide on the content of ascorbic acid in edible potato tubers (*Solanum Tuberosum L.*) *Applied Ecology and Environmental Research*, 17, 2105-2118. DOI: 10.15666/aeer/1702_21052118

Commission Regulation (EU) No 540/2011 of 25 May 2011 implementing Regulation (EC) No 1107/2009 of the European Parliament and of the Council as regards the list of approved active substances. *Official Journal of the European Union* L 153/1 (2011)

Directive 2009/128/EC of the European Parliament and of the Council of 21 October 2009 establishing a framework for Community action to achieve the sustainable use of pesticides *Official Journal of the European Union* L 309/71

Feldmann F (2017). Efficacy and risks of „biorationals“ in organic and integrated pest management–acceptable? In: *Book of abstracts of the 8th international symposium plant protection and plant health in Europe*, Braunschweig, 13–14 December 2017. http://plant-protection.net/fileadmin/Tagungen/PPPHE/2017/Documents/PPPHE2017_Report.pdf.

Kucharski M; Kalitowska O; Wujek B (2015). New adjuvants in chemical plant protection *Przem. Chem.* 94: 397-399

Łącka I (2018). Innovativeness of Polish chemical industry and its prospects. *Przem Chem* 97, 209-2013 DOI:10.15199/62.2018.2.5

Matyjaszczyk E (2011). Selected aspects of plant protection in Poland, five years on from EU accession. *Outlook on Agriculture* 40, 119-123

Matyjaszczyk E (2017). Comparison between seed and foliar treatment as a tool in integrated pest management. *J. Agric. Food Chem*. 65, 6081–6086 DOI:10.1021/acs.jafc.7b01095

Matyjaszczyk E (2018). „Biorationals” in integrated pest management strategies. *Journal of Plant Diseases and Protection* 125, 523-527 https://doi.org/10.1007/s41348-018-0180-6

Matyjaszczyk E (2019). Problems of Implementing Compulsory Integrated Pest Management. *Pest Management Science* https://doi.org/10.1002/ps.5357

Matyjaszczyk E; Sobczak J (2017). Common EU registration rules and their effects on the availability of diverse plant protection products: A case study from oilseed rape and potato in 5 Member States. *Crop Protection* 100, 73–76 DOI 10.1016/j.cropro.2017.06.006

Mazurkiewicz A; Tumialis D; Pezowicz E; Skrzecz I; Błażejczyk, G (2017). Sensitivity of *Pieris brassicae*, *P. napi* and *P. rapae* (Lepidoptera: Pieridae) larvae to native strains of *Steinernema feltiae* (Filipjev, 1934) *Journal of Plant Diseases and Protection*, 124, 521-524. DOI: 10.1007/s41348-017-0118-4

Stevens MM (2002). Seed Treatments. In: *Encyclopedia of Pest Management*; Pimentel, D, Ed.; Marcel Dekker, Inc.: New York, 2002;pp 754−756.

Szpyrka E; Matyaszek A; Słowik-Borowiec M (2017). Dissipation of chlorantraniliprole, chlorpyrifos-methyl and indoxacarb-insecticides used to control codling moth (*Cydia Pomonella L.*) and leafrollers (Tortricidae) in apples for poduction of baby food . *Environmental Science and Pollution Research*, May; 24(13), 12128-12135 https://doi.org/10.1007/s11356-017-8821-z

Taylor AG; Harman GE (1990). Concepts and technologies of selected seed treatments. *Annu. Rev. Phytopathol.* 28, 321−339.

Table 1 Comparison of doses of foliar fungicides and seed dressings containing the same active substances and registered to control the same diseases in the same crops registered in selected EU member states.

Country	Formulation*/**	Active substance content [g/l] or [g/kg]	Seed treatment rate [l/1000x100 kg seed]	Application rate [kg/ha] or [l/ha]	Amount of active substance [g/ha]	Ratio seed/foliar application
Austria	**Wheat**/Glume blotch (*Parastagonospora nodorum*)					
	FS	167 g/l 31.2 g/l	450 ml/100 kg seed	-	150.3 g/ha 28.08 g/ha	1:0.8 fluquinconazole
	SE	54 g/l 174 g/l	-	2.3 l/ha	124.2 g/ha 400.2 g/ha	1:14.2 prochloraz
Belgium	**Wheat**/Fusarium head blight (FHB) (*Fusarium* spp.)*;* **Barley**/ Net blotch (*Pyrenophora teres)*					
	FS	100 g/l	100 ml/100 kg seed	-	25 g/ha	1:8 prothioconazole
	EC	250 g/l	-	0.8 l/ha	200 g/ha	
	Wheat, triticale, spelt/FHB					
	FS	37.5 g/l 37.5 g/l	150 ml/100 kg seed	-	14.06 g/ha 14.06 g/ha	1:7.1 fluoxastrobin 1:14.2 prothioconazole
	EC	50 g/l 100 g/l	-	2 l/ha	100 g/ha 200 g/ha	
Czech Republic	**Wheat**/ FHB					
	FS	25 g/l	120 ml/100 kg seed	-	7.5 g/ha	1:34.4 tebuconazole
	SC	430 g/l	-	0.6 l/ha	258 g/ha	
	Wheat, barley/ FHB*;* **Barley**/ Net blotch					
	FS	250 g/l 150 g/l	20 ml/100 kg seed	-	12,5 g/ha 7.5 g/ha	1: 6.4 prothioconazole 1: 4.2
	FS	150 g/l 20 g/l	50 ml/100kg seed	-	18.75 g/ha 2.5 g/ha	1:21.3 tebuconazole
	EC	80 g/l 160 g/l	-	1 l/ha	80 g/ha 160 g/ha	1: 64
	Wheat/ Septoria leaf blotch (*Zymoseptoria tritici*), rusts (*Puccinia spp.*); **Barley**/ Powdery mildew (*Puccinia hordei*), Rhynchosporium (*Rhynchosporium commune*)					
	FS	333 g/l	150 ml/100 kg seed	-	124.87 g/ha	1:1 fluxapyroxad
	EC	62,5 g/l	-	2l/ha	125 g/ha	
	Wheat/ FHB					
	FS	350 g/l 20 g/l	100ml/100 kg seed	-	87.5 g/ha 5 g/ha	1:4.6 thiophanate methyl 1:24.5 tetraconazole
	SE	233 g/l 70 g/l	-	1.75 l/ha	407.75 g/ha 122.5 g/ha	

Finland	**Spring wheat**/Glume blotch; **Barley**/Net blotch; **Oat**/Leaf blotch and crown rot (*Helminthosporium* spp.)					
	FS	250 g/l	20 ml/100 kg seed	-	12.5 g/ha	1:10 prothioconazole
		150 g/l			7.5 g/ha	1:16.7 tebuconazole
	EC	125 g/l	-	1 l/ha	125 g/ha	
		125 g/l			125 g/ha	
	Spring wheat/Glume blotch, FHB **Barley**/FHB					
	FS	100 g/l	100 ml/100 kg seed	-	25 g/ha	1:8 prothioconazole
	EC	250 g/l	-	0.8 l/ha	200 g/ha	
Germany	**Wheat**/Glume blotch					
	FS	37.5 g/l	160 ml/100 kg seed	-	14.4 g/ha	1:10.4 fluoxastrobin
		37.5 g/l			14,4 g/ha	1:10.4 prothioconazole
	EC	100 g/l	-	1.5 l/ha	150 g/ha	
		100 g/l			150 g/ha	
Italy	**Wheat**/Septoria leaf blotch, powdery mildew, rusts **Barley**/Powery mildew, net blotch, Rhynchosporium, Ramularia (*Ramularia collo-cygni*)					
	FS	333 g/l	150 ml/100 kg seed	-	124.8 g/ha	1:1 fluxapyroxad
	EC	62.5 g/l	-	2 l/ha	125 g/ha	
	Wheat/FHB, **Barley**/FHB, net blotch					
	FS	25 g/l	120 ml/100 kg seed	-	7.5 g/ha	1:33.4 tebuconazole
	SC	43.2 g/l	-	5.8 l/ha	250.56 g/ha	
Poland	**Winter wheat**/Take-all (*Gaeumannomyces graminis var. tritici*)					
	FS	100 g/l	750 ml/100 kg seed	-	187.5 g/ha	1:0.51 fluquinconazole
		13.3 g/l			24.92 g/ha	1:12.5 prochloraz
	SE	54 g/l	-	1.8 l/ha	97.2 g/ha	
		174 g/l			313.2 g/ha	
	Spring barley/ Net blotch					
	FS	60 g/l	50 ml/100 kg seed	-	7.5 g/ha	1:33.3 tebuconazole
	EW	250 g/l	-	1 l/ha	250 g/ha	
	Winter barley/ Net blotch					
	FS	250 g/l	20 ml/100 kg seed	-	12.5 g/ha	1:10 prothioconazole
		150 g/l			7.5 g/ha	1:16.6 tebuconazole
	EC	125 g/l	-	1 l/ha	125 g/ha	
		125 g/l			125 g/ha	

Slovakia	**Barley**/ Net blotch					
	FS	250 g/l	20 ml/ 100 kg seed	-	12,5 g/ha	1:10 prothioconazole
		150 g/l			7,5 g/ha	1:5
	FS	150 g/l	66,7 ml/100 kg seed	-	24,9 g/ha	1:16,6 tebuconazole
		20 g/l			3,32 g/ha	1:37,6
	EC	125 g/l	-	1 l/ha	125 g/ha	
		125 g/l			125 g/ha	
	Wheat/ FHB					
	ES	60 g/l	200 ml/100 kg seed	-	30 g/ha	1:8,9 prochloraz
		15 g/l			7,5 g/ha	1:17,7 tebuconazole
	EW	267 g/l	-	1 l/ha	267 g/ha	
		133 g/l			133 g/ha	
Spain	**Chickpeas**/Ascochyta blight (*Didymella rabiei*)					
	DS	16% (160 g/kg)	200 g/100 kg seed	-	44.8 g/ha	1: 26.8 copper oxychloride
	WG, WP	50% (500 g/l)	-	2,4 l/ha	1200 g/ha	
	Barley, wheat/Leaf blotch and crown rot					
	FS	2,5% (25 g/l)	150 ml/100 kg seed	-	9,37 g/ha	1: 26.7 tebuconazole
	EW	20% (200 g/l)	-	1,25 l/ha	250 g/ha	
	Cereals/Septoria leaf and glume blotch					
	DS	48% (480 g/kg)	200 g/100 kg seed	-	240 g/ha	1:13.3 mancozeb
	WP	80% (800 g/kg)	-	4 kg/ha	3200 g/ha	

*Sometimes several different formulations were registered; ** A key to the formulation codes (in alphabetical order): DS - Powder for dry seed treatment; EC - Emulsifiable concentrate; EW - Emulsion, oil-in-water; FS - Flowable concentrate for seed treatment; SC - Suspension concentrate; SE - Suspo-emulsion; WG - Water dispersible granule; WP - Wettable powder;

Source: Official websites of registration authorities of European Union member states

Gisi U, Resistance risk for DMI fungicides in *Aspergillus fumigatus*: potential hot spots. In: Deising HB; Fraaije B; Mehl A; Oerke EC; Sierotzki H; Stammler G (Eds), "Modern Fungicides and Antifungal Compounds", Vol. IX, pp. 295-300. © 2020. Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-16-7

Resistance risk for DMI fungicides in *Aspergillus fumigatus*: potential hot spots

Gisi U

Consultant in Agricultural Sciences; Leimenweg 81; CH-4493 Wenslingen, Switzerland
Email: u.gisi@bluewin.ch

ABSTRACT

A risk assessment model for selection of resistance to DMIs in *Aspergillus fumigatus* was developed for all known DMI application types in crop, seed and material protection as well as for treatments in animals and humans. The resistance risk matrix was used to identify so called «hot spots» in which growing fungal populations co-exist with effective concentrations of an effective DMI. A strong correlation of cross resistance between all DMI compounds used in agriculture, medicine and material protection was observed for the majority of *A. fumigatus* isolates. The *A. fumigatus* genome was found to be quite variable acquiring many mutations in the *cyp51A* gene already present in «wild type» compost isolates. Both mating types may co-exist in many substrates. Although *A. fumigatus* frequency can be rather high in self heating substrates like compost and corn silage, DMI resistance seems to be mostly absent. In addition to treatments of patients, a high risk of resistance selection was assigned to flower bulb and wood treatments. The left overs are often major components of compost, and resistant isolates were detected there especially in the Netherlands and France. The identification of «hot spots» must be done separately for every type of DMI application in each country. The dissemination of *A. fumigatus* spores to the air should be avoided as much as possible through adequate handling processes of organic material in order to reduce the spread of DMI resistance and allow the use of DMIs in agriculture, material protection and medicine as long as possible.

INTRODUCTION

The thermotolerant, saprophytic fungus *Aspergillus fumigatus* Fresenius (teleomorph *Neosartorya* sp., Trichocomaceae, Ascomycetes) can be isolated from air and different organic substrates in almost any corner of global environment. It does not attack living plant material but can be an opportunistic pathogen of animal and human tissue causing respiratory allergies and invasive aspergillosis especially in immuno-compromised patients who are then treated mostly with demethylation inhibitor fungicides (DMIs), chemically named «azoles» (triazoles, imidazoles). Since many years, *A. fumigatus* isolates resistant to DMIs have been reported originating from treated but also from DMI-naive patients. In addition, it was suggested that

DMI resistant isolates might originate also from the environment, e.g. plants and soil that have been treated previously by chemically related DMIs used to control plant pathogens in agricultural crops, seeds, bulbs, fruits and for material protection potentially selecting for resistance also in *A. fumigatus* either directly as «collateral effect» during plant treatments or indirectly through residues in (mostly dead) organic material. On the basis of theoretical considerations and practical experience, an assessment was made to estimate the risk of selection of resistance to DMIs during different treatment practices in agriculture and medicine (Gisi 2014) to identify so called «hot spots» where *A. fumigatus* can develop and might come in contact with DMIs. A «hot spot» for selection of DMI resistance is defined as an area in which growing fungal populations (allowing high biomass and genetic diversity, high competitiveness and dispersal, resulting all together in high fitness) co-exist with effective concentrations of an effective DMI over an extended time period and coherent area. The major elements for assessing resistance risk are biological and molecular features of the fungus on one hand and the fungicide mode of activity and application practices.

BIOLOGICAL AND MOLECULAR FEATURES OF *ASPERGILLUS FUMIGATUS*

The abundance of *A. fumigatus* in commercial growing substrates and composts (from Germany, the Netherlands, Italy, Spain, Hungary) was generally rather high (frequencies from 10^3 to 10^5 cfu/g), whereas in soils from meadows, agricultural land and forests it was below 10^3 cfu/g (Franceschini *et al.* 2016; Santoro *et al.* 2017). In «green» compost (made from plant material) and some municipal compost, the frequency was mostly higher than in «brown» compost made from animal manure. Corn plant material harvested in Italy and conserved anaerobically in silage for five months contained very low concentrations (below 10^2 cfu/g) of *A. fumigatus*, whereas one to two weeks after silo opening the concentration increased drastically to 10^5 to 10^8 cfu/g (Spadaro *et al.* 2019). Two DMI applications (tebuconazole based) of corn per season did not influence the sensitivity of *A. fumigatus* isolates in fresh and deteriorated silage (Spadaro *et al.* 2019). In the majority of cases, both mating types were present in each single plate during isolation of *A. fumigatus* from corn silage. An unequal mating type distribution was detected with mat-2 being more frequent than mat-1 (Spadaro *et al.* 2019). In compost produced from oranges treated with imazalil in Italy, both mating types were found, and all isolates were sensitive (Pugliese *et al.* 2018). In contrast, DMI resistant *A. fumigatus* isolates were detected in compost heaps produced from left overs of flower bulbs (e.g. tulips, daffodils) having been treated with DMIs (e.g. tebuconazole) in the Netherlands (pers. comm., CLM report 2017). It is an open question whether they have been selected during bulb treatment or by residues in the compost. Also in France, DMI resistant isolates were discovered at low frequencies (below 3% of isolates) in wood having been processed by tank dipping with propiconazole (at 1000 mg/L); resistant isolates were mostly detected at propiconazole concentrations of 0.3 to 60 mg per kg wood, whereas at lower and higher concentrations, isolates were generally sensitive (Jeanvoine *et al.* 2017) suggesting that «effective» DMI concentrations are responsible for selection rather than «residue or over-dosed» concentrations. In soil samples from agricultural land, *A.*

fumigatus abundance is low and DMI resistant isolates were mostly absent (Santoro *et al.* 2017, B. Fraaije *et al.* 2019 pers. comm., *Aspergillus fumigatus* workshop, Amsterdam).

The intrinsic activity of several agricultural and medical DMIs was measured against *A. fumigatus* compost isolates *in vitro*: EC50 values increased in the following order: imazalil (most active DMI) < posaconazole < voriconazole < difenoconazole < epoxiconazole (least active DMI). The sensitivity distribution of isolates was continuous with a range of 8-30 fold, the resistant reference isolates (TR_{34}+L98H, TR_{46}+Y121F+T289A) being clearly outside the sensitive population (Santoro *et al.* 2017). All tested DMI fungicides (agricultural and medical) were cross-resistant for the vast majority of isolates with a strong correlation ($r^2 = 0.65–0.95$) (Santoro *et al.* 2017). No *cyp51A* target site mutations linked to azole resistance were detected in the isolates. However, a range of *cyp51A* alterations were found in the sensitive isolates (see below) which obviously did not influence their sensitivity status.

The genetic diversity of *A. fumigatus* compost isolates was investigated using SSR markers (simple sequence repeats, microsatellites, non-coding molecular markers) and *cyp51A* SNPs (single nucleotide polymorphisms in *cyp51A* gene, number and type of mutations, coding markers) (Santoro *et al.* 2017). According to SSR analysis, a high genetic diversity was detected in populations, each isolate being different from one another even when originating from the same compost sample. These results suggest frequent sexual and/or parasexual processes to occur in compost, a finding that has been confirmed experimentally in compost by Zhang *et al.* (2017). Clustering of SSR genotypes was according to geographic origin (e.g. Spain, Italy, Hungary). Based on *cyp51A* genotypes, the phylogenetic relatedness and clustering of isolates were according to number and type of mutations: Out of 61 isolates, 42 were wild type, 19 carried one to 13 SNPs per isolate, and a total of 29 different SNPs were found suggesting that there are «simple» and «complex» genotypes, and that the *cyp51A* gene is quite «flexible»; no promotor inserts and SNPs confering DMI resistance (e.g. TR_{34}+L98H) were detected (Franceschini *et al.* 2016, Santoro *et al.* 2017, Pugliese *et al.* 2018; Table 1).

FUNGICIDE APPLICATION, EMERGENCE AND SELECTION OF RESISTANCE

For the emergence of resistance to DMIs in individual isolates of *A. fumigatus*, several mechanisms have to be considered: The major resistance mechanism is based on the presence of SNPs in the *cyp51A* gene arising spontaneously at a specific (mutation) frequency. Whether exposure to DMIs will increase and/or direct this frequency is debatable. An additional resistance mechanism is the presence of tandem repeat (TR) based nucleotide insertions (34 and 46 bp) in the promotor of the *cyp51A* gene. TR_{34} and TR_{46} increase the expression of *cyp51A* generating more *cyp51A* transcripts *«allowing the fungus to better cope with azole stress»* (Zhang *et al.* 2017). This potentially contributes to the emergence of certain SNPs and resistant phenotypes even at low DMI concentrations. *Cyp51A* tandem repeats and SNPs are often found in combinations (e.g. TR_{34}/L98H and TR_{46}/Y121F/T289A).

Table 1 Presence and frequency of different SNPs in *cyp51A* gene of 61 *A. fumigatus* compost isolates.

aa region 1-64		**aa region 65-128**		**aa region 129-290**		**aa region 291-515**	
1x	V24D	3x	I71L	1x	E130G	3x	E427K
10x	L27F	*0x*	*L98H*	2x	S142P	1x	F495L
3x	N33S/G	2x	K99R	1x	E145Q	1x	S496T
3x	P38S	1x	E105Q	2x	E152G		
3x	P45Q	11x	F115V	2x	E154D		
2x	F46Y	11x	V120G	3x	S169T		
10x	L47V	*0x*	*Y121F*	2x	M172V		
2x	S49T	3x	K127Q	1x	I182S		
12x	S52T			3x	A204D		
				2x	P216A		
				0x	*T289A*		

In addition, resistance can also be caused by overexpression of ABC/MDR transporter (AfuMDR, MFS) genes in *A. fumigatus*. In that case, cross resistance is not always obvious even not among medical DMIs. For the selection and distribution of resistance to DMIs in (local) populations, the following aspects have to be considered. Medical and agricultural DMIs have basically the same biochemical mode of action, although the binding modes to the CYP51 enzymes may vary. All DMIs exhibit cross-resistance for the majority of *A. fumigatus* isolates. Mutations in the *cyp51A* gene (e.g. L98H) may appear through preferential selection, i.e. selection of certain mutations by a certain but not by another DMI (as it is known to occur e.g. in *Mycosphaerella graminicola*). The mutations at position M220 in *A. fumigatus* confer resistance specifically against itraconazole, however L98H against all (medical and agricultural) azoles (Gisi 2014). It is generally assumed that emergence and selection of resistance are driven by local processes (e.g. during patient treatment; in a specific field during flower production). However, translocation of conidia by air and contaminated material by trade (e.g. seeds, flower bulbs) can occur over long distances (e.g. on treated bulbs from the Netherlands to Ireland; Dunne *et al.* 2017). Generally, it is assumed that a high number of applications and long lasting selection will accelerated resistance development in plant pathogen populations. Whether lower than recommended rates (e.g. half rates, residues) may influence the selection process, is debatable. Also in medicine, it is accepted that the selection of resistant *A. fumigatus* isolates can happen during the extended azole treatment of patients resulting in resistance factors (a shift of sensitivity) of 10-20 after treatment (Snelders *et al.* 2008).

RESISTANCE RISK MATRIX AND "HOT SPOTS"

Based on theoretical considerations and the information presented above, the assumed selection risk (ASR) was assessed for different DMI application types in *A. fumigatus*. The matrix published in 2014 (Gisi) was challenged and modified (Figure 1). Basically, the maximal exposure concentration (MEC) the fungus (pathogen) is exposed to (calculated values according to recommended application rates) is plotted against the pathogen exposure risk (PER) expressing the likelihood of the pathogen to be present and multiply in the treated substrate (details see Gisi 2014). The product MEC x PER results in the ASR (scale from 1 to 16), with 16 being the highest risk and greatest likelihood of a «hot spot». The ASR remains unchanged for most appliction types compared to the previous version with medical treatments being highest (9-12, in body) and spray applications of field crops lowest (1, in soil). However, the ASR for wood preservation should be moved from 4 to 8 (in wood) and for flower bulb tretments from 8 to 12 because treated wood and bulbs as well as the composted left-overs still contain considerable DMI residues and the substrate favours *A. fumigatus* growth and reproduction. The ASR for material protection is still unknown but probably rather high although no reliable data are available to assess the risk. The ASR for post harvest treatments of citrus fruits is reduced from 6 to 4 because no resistant isolates have been detected so far.

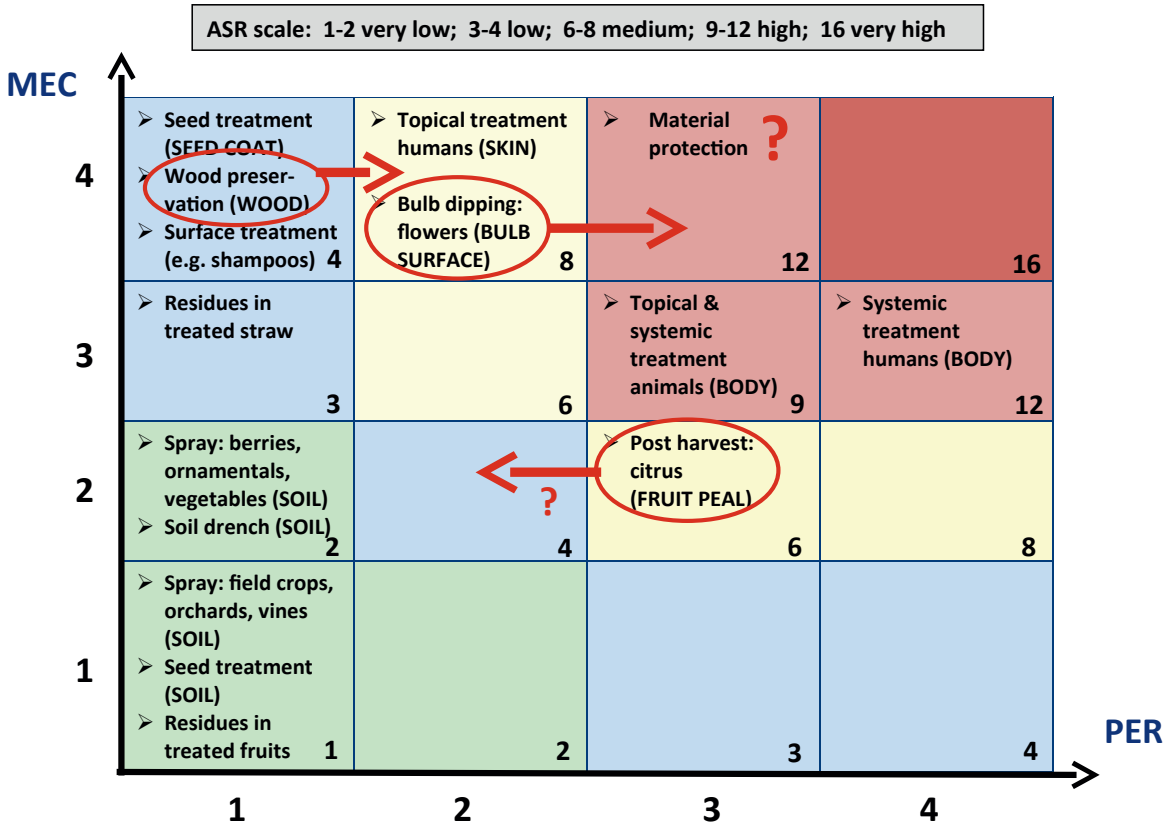


Figure 1 ASR (Assumed Selection Risk) matrix for different DMI application types against *A. fumigatus* to identify «hot spots» (modifications compared to version of Gisi 2014). MEC: Maximal Exposure Concentration; PER: Pathogen Exposure Risk.

CONCLUSIONS AND OUTLOOK

A. fumigatus is especially competitive and frequent in dead organic substrates in which high temperatures prevailed (e.g. upon self-heating) eliminating many saprophytic competitors, such as in compost and corn silage after opening, whereas its abundance is low on plants, plant litter of meadows and forests and in (agronomical) soils. Although compost may be the major source of *A. fumigatus*, not every compost type contains high numbers of *A. fumigatus* propagules. Important features during composting are the origin of organic material, previous treatments, composting processes and local conditions. More attention should be given to DMI treatments in flower bulbs and material (e.g. wood) protection (high use concentrations and residues). Resistance risk has to be assessed separately for each «hot spot» in every country.

REFERENCES

Dunne K; Hagen F; Pomeroy N; Meis JF; Rogers TR (2017). Intercountry transfer of triazole-resistant *Aspergillus fumigatus* on plant bulbs. *Clin Inf Dis* 65, 147-149.

Franceschini S; Chitarra W; Pugliese M; Gisi U; Garibaldi A; Gullino ML (2016). Quantification of *Aspergillus fumigatus* and enteric bacteria in European compost and biochar. *Compost Sci Util* 24, 20-29.

Gisi U (2014). Assessment of selection and resistance risk for demethylation inhibitor fungicides in *Aspergillus fumigatus* in agriculture and medicine: a critical review. *Pest Manag Sci* 70, 352-364.

Jeanvoine A; Rocchi S; Reboux G; Crini N; Crini G; Millon L (2017). Azole-resistant *Aspergillus fumigatus* in sawmills of Eastern France. *J Appl Microbiol* 123, 172-184.

Pugliese M; Matić S; Prethi S; Gisi U; Gullino ML (2018). Molecular characterization and sensitivity to demethylation inhibitor fungicides of *Aspergillus fumigatus* from orange-based compost. *PLoS ONE* 13 (7) https://doi.org/10.1371/journal/.pone.0200569.

Santoro K; Matić S; Gisi U; Spadaro D; Pugliese M; Gullino ML (2017). Abundance, genetic diversity and sensitivity to demethylation inhibitor fungicides of *Aspergillus fumigatus* isolates from organic substrates with special emphasis on compost. *Pest Manag Sci* 73, 2481-2494.

Snelders E; Van der Lee HAL; Kuijpers J; Rijs AJMM; Varga J; Samson RA; Mellado E; Donders ART; Melchers WJG; Verweij PE (2008). Emergence of azole resistance in *Aspergillus fumigatus* and spread of a single resistance mechanism. *PLoS Med* 5(11): e219. https://doi.org/10.1371/journal.pmed.0050219

Spadaro D; Matić S; Prencipe S; Ferrero F; Borreani G; Gisi U; Gullino ML (2019). *Aspergillus fumigatus* population dynamics and sensitivity to demethylation inhibitor fungicides in whole-crop corn, high moisture corn and wet grain corn silages. Pest Manag Sci, https://doi.org/10.1002/ps.5566.

Zhang J; Snelders E; Zwaan BJ; Schoustra SE; Meis JF; van Dijk K; Hagen F; van der Beek MT; Kampinga GA; Zoll J; Melchers WJG; Verweij PE; Debets AJM (2017). A novel environmental azole resistance mutation in *Aspergillus fumigatus* and a possible role of sexual reproduction in its emergence. *mBio* 8, https://doi.org/10.1128/mBio.00791-17.

R4P network, Proposal for a simple and unified classification of pesticides. In: Deising HB; Fraaije B; Mehl A; Oerke EC; Sierotzki H; Stammler G (Eds), "Modern Fungicides and Antifungal Compounds", Vol. IX, pp. 301-306. © 2020. Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-16-7

Proposal for a simple and unified classification of pesticides

Research and Reflection Ring on Pesticide Resistance (R4P), France; c/o Anne-Sophie Walker
INRA UMR 1290 BIOGER, Avenue Lucien Brétignières, 78850 Thiverval-Grignon FRANCE
Email: contact-r4p@inra.fr

INTRODUCTION

Improving knowledge of the modes of action of pesticides in order to better manage their unintended effects

Regulations to promote the reduction and better management of chemical inputs in agriculture, and more recently agroecology, have in common that they aim to reduce the unintended effects of plant protection products (PPPs), whether of natural or synthetic origin.

A first unintended effect of the use of plant protection products is the selection of resistant individuals within populations of pests and diseases, *i.e.* capable of surviving concentrations of PPPs that usually control the development of sensitive genotypes of the same species. In some cases, the presence of these resistant individuals leads to a total or partial loss of efficacy of PPPs in the field. This leads to the use of catch-up treatments with PPPs with sometimes less favorable ecotoxicological profiles or dose increases. Paradoxically, the loss of diversity in the approved modes of action and some practices to reduce the quantities of PPPs used (e.g., under-dosing, split applications) can facilitate resistance selection, particularly those associated with the most complex mechanisms to study and predict. An important challenge of integrated protection is therefore to optimize PPP use strategies in order to limit the evolution of resistance. This optimization is based on the characterization of the selection pressures associated with the active substances, and therefore implies a precise knowledge of their modes of action. The organisation and promotion of knowledge on PPPs' mode of action is then a prerequisite to improve resistance management.

A second unintended effect, related to the use of PPPs, is their impact on human health and the environment. Many studies reveal such negative effects, which are becoming better known and more and more criticized. The "One Health" concept, emerged in 2004 at the initiative of the WCS (Wildlife Conservation Society) and suggests that the protection of human health requires the protection of animal and plant health and their interactions with the environment. It encourages collaboration, synergy and cross-fertilization between all sectors and actors whose activities can have an impact on health and well-being, through risk prevention and mitigation of the effects of crises that arise from the interface between humans, plants, animals and their ecosystems. One of the levers to prevent the risks to human health and the environment associated with the use of PPPs in agriculture is to measure overall exposure to these products,

all uses, and natural compartments combined. This objective measurement requires, as with PPP resistance phenomena, a detailed knowledge of the modes of action and active substances, whatever their uses.

In this context of predicting and managing unintended effects related to the use of PPPs in agriculture, or more broadly biocides, it therefore seems essential to have access to classification tools that simply and reliably identify their different modes of action for all possible uses. These classifications may also be used to educate users and promote sustainable use of PPPs.

The skill and style of classifying plant protection products

Several independent classifications of PPPs are available and specifically present molecules intended for a type of use (fungicides, by the FRAC - Fungicide Resistance Action Committee; acaricides-insecticides by the IRAC - Insecticide Resistance Action Committee; herbicides by the HRAC - Herbicide Resistance Action Committee, or the WSSA - Weed Science Society of America). These classifications are established by scientists and all have in common that each active substance is classified first by its biochemical mode of action, and secondly by its chemical class. These criteria are most relevant, considering that in most cases, the toxicity of a PPP results from its interaction with a protein, which is its primary target. In some cases, for the same target, it is possible to distinguish several classes of inhibitors, depending on the interaction or binding modalities. Acquired resistance to plant protection substances often results from changes in their targets. These alterations lead in many situations to positive cross-resistance to all active substances associated with the same primary target, regardless of the chemical class, which is why this criterion is used as a secondary sorting key. However, in the case of non-target site resistance (e.g. lack of PPP penetration, increased compensatory metabolism or increased efflux outside the intracellular space), the cross-resistance spectrum may extend to different modes of action or concern only certain active substances within the same chemical class. This requires specific recommendations for each situation.

Depending on the classification, active substances are associated with a code represented by a number, a letter, or an association of a number and a letter. A common mode of action for several uses (fungicide, insecticide, acaricide, herbicide) can thus be associated with different codes, depending on the classification used. For example, mitochondrial II complex inhibitor acaricides or succinate dehydrogenase (SDHI) are coded 25B (IRAC), while they are coded C2 or 7 (FRAC) when used as a fungicide. Similarly, acetyl-coenzyme A carboxylase (ACCase) lipid inhibitors are either herbicides used on grasses, coded A or 1 (HRAC and WSSA, respectively), or acaricides or insecticides, coded 23 (IRAC). This is probably the result of historical reasons, linked to the successive discoveries of the active substances and the asynchronous and independent development of their different uses. Considering these independent classifications, this nomenclature may lead to some code redundancy, between independent modes of action.

MATERIALS AND METHODS

In the context described above, and given recent advances in knowledge of PPP modes of action, we propose to unify current classifications to take into account the fact that some pests, that are sometimes very distant from a taxonomic point of view (such as a weed, insect, mite or pathogen), nevertheless have common biochemical targets that can be inhibited by common modes of action. This may lead to a unique PPP classification. It is understood, however, that sharing a biochemical target does not augur well for cross-efficiency – or cross-resistance selection – between PPPs.

Our proposal combines the existing classifications, presented by pesticide use, into a single nomenclature. It includes all PPP active substances authorised in France or abroad as well as products withdrawn or not yet placed on the market, retrieved from the actual classification and international pesticide databases. It gives identical codes to common modes of action, regardless of the type of pest targeted. This unified classification uses the same criteria (*i.e.* biochemical mode of action and then chemical class) as the current classifications, by pesticide use, which allows an easy match between the codes associated with the active substances. This classification prevents codes redundancy.

The R4P codes consist of the letter U, followed by 2 to 3 descriptors. The first descriptor (in the form of a capital letter) refers to the major vital processes affected by the active substance (Figure 1). The characterization of the primary target, and possibly the target-inhibitor interaction modalities, are the subject of the second descriptor (in the form of a number) of the R4P classification. Active substances associated with the same primary target descriptor (R4P code number), regardless of their chemical class, generally, but not systematically, have positive cross-resistance profiles in the event of target-site resistance. In addition to their classification by biochemical mode of action, PPPs are then grouped according to their chemical structure. A deliberately simplified name for these chemical structures was chosen. A breakdown by "main" chemical class is proposed for all of them and sometimes a subdivision by "secondary" chemical class is also provided. Depending on the information available on acquired resistance phenomena (including cross-resistance spectra), a chemical structure descriptor (lowercase letter, corresponding to the third descriptor) is sometimes proposed within certain classes of modes of action. Note that for multisite inhibitors (class W) and substances with unknown modes of action (class X), classification is based solely on their chemical structures. To avoid confusion between this new and existing nomenclature, the letter "U" (for "Unified" nomenclature) is added before all codes.

RESULTS AND DISCUSSION

The unified classification is available online: http://inra-r4p.fr and https://osf.io/UBHR5. A second comprehensive update should be released in 2020. It includes biocontrol solutions that are often partially presented in classifications, while many are or will be authorised in Europe

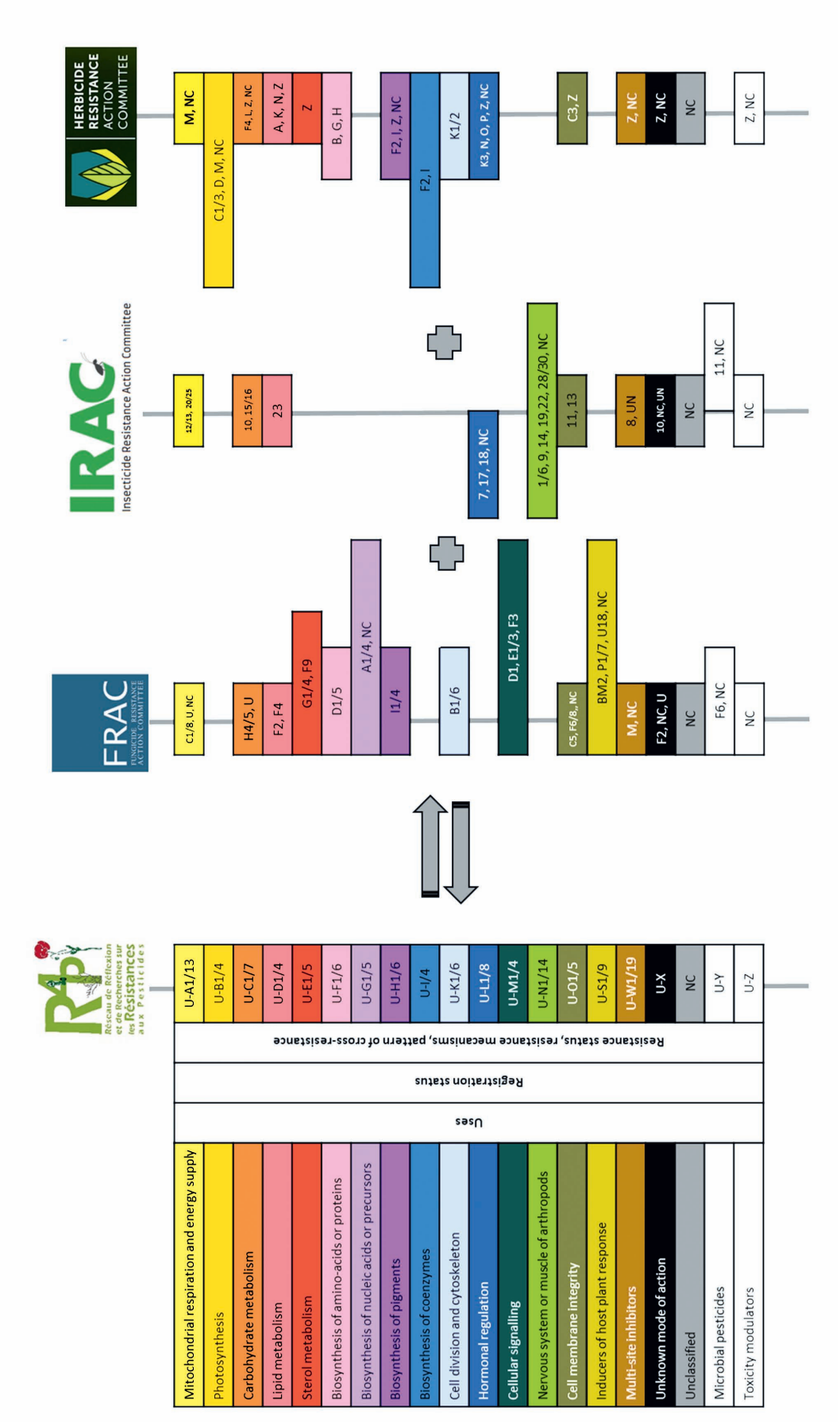


Figure 1 Unified classification of pesticides, according to their biochemical mode of action and independent the existing classifications.

and their use will gradually increase in the coming years. Some of them may be, as much as synthetic PPPs, confronted with the evolution of resistance in targeted pests and diseases, which justifies their inclusion in this PPP nomenclature (for example, within classes S or Y).

This universal classification of PPPs is intuitive, evolutionary and has been designed to integrate the issue of better resistance management from the outset. Indeed, a good knowledge of the modes of action associated with active substances, and by extension, commercial products, is essential to reflect their sustainable deployment, in space and time. In the regulatory framework, anti-resistance strategies are based on knowledge of the modes of action on the one hand, and on knowledge of the status of associated resistance on the other. In addition to the information specific to the classification of PPPs, the R4P nomenclature brings information to each active substance or chemical family together in order to improve their rational use. These characteristics concern:

- Target organisms of active substances: descriptions of the groups of pests and diseases on which they have a known activity (*e.g.* for herbicides, known activity towards broadleaf weeds, grasses or other plant groups)
- The status of PPPs usage in France: it is specified whether the active substance is approved, as well as its year of approval for the first registered use and, possibly, its date of withdrawal. This section could easily be adapted for countries other than France.
- Resistance status: it is specified whether at least one case of resistance to the active substance or mode of action has been observed in at least one pest in France and abroad. However, this does not necessarily mean that the active substance is ineffective (resistance in practice).
- Resistance mechanisms: it is specified whether resistance is related to the target, or associated with another mechanism, according to a recently published categorization of mechanisms (R4P, 2016). Each resistance mechanism is also associated with a description of the cross-resistance spectra.

ACKNOWLEDGEMENT

R4P network in France consists of the following members: Dr Benoit Barrès (Directeur de l'USC CASPER Anses Lyon), Dr Marie-France Coiro-Costet (UMR INRA SAVE Bordeaux), Dr Danièle Debieu (UMR BIOGER Thiverval-Grignon), Dr Christophe Délye (UMR Agroécologie INRA Dijon), Dr Sabine Fillinger (UMR BIOGER INRA Thiverval-Grignon), Dr Bertrand Gauffre (UMR PSH INRA Avignon), Jacques Grosman (expert Résistances DGAl), Dr Mourad Hannachi (UMR SADAPT INRA Thiverval-Grignon), Dr Gaëlle Le Goff (Institut Sophia Agrobiotech INRA Antibes), Dr Christophe Plantamp (USC CASPER Anses Lyon), Myriam Siegwart (UMR PSH INRA Avignon) and Dr Anne-Sophie Walker (UMR BIOGER INRA Thiverval-Grignon). The classification was established with the help of Pierre Leroux, Christian Gauvrit and Robert Delorme, and colleagues from Anses.

REFERENCES

R4P classification: http://inra-r4p.fr; https://osf.io/ubhr5/

FRAC classification: http://www.frac.info/

IRAC classification : http://www.irac-online.org/

HRAC classification: http://www.hracglobal.com/

WSSA classification: http://wssa.net/

E-Phy database: https://ephy.anses.fr/

Index phytosanitaire ACTA 2017, Ed. ACTA : http://acta-publications.com/index-phytosanitaire-acta-2017.html

The Compendium of Pesticide Common Names: http://www.alanwood.net/pesticides/index.html

MatPhyto Database : http://matphyto.acta-informatique.fr/Accueil

Compendium of Pesticides Common Names: http://www.alanwood.net/pesticides/index.html

The Pesticide Manual 17th Edition, Ed. British Crop Production Council: https://www.bcpc.org/product/bcpc-online-pesticide-manual-latest-version

R4P (2016). "Trends and Challenges in Pesticide Resistance Detection." Trends in Plant Science 21(10): 834-853.