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Preface

Following the meeting of the IOBC-WPRS WG "Biological and Integrated Control of Plant Pathogens" under the former name of the WG "Biological Control of Fungal and Bacterial Plant Pathogens" in Uppsala, Sweden, 15-18 June 2014, this 13th meeting of the working group will be held in Berlin, Germany, 12-15 September 2016.

The general goal of the WG is to promote cooperation between biocontrol scientists and to exchange expertise on biological control of plants diseases in the West Palaeartic Region and broader in order to support the implementation of biological control strategies in agriculture. The WG organizes workshops for scientists and extension people as well as producers and users of biological control products. These workshops focus on specific aspects of biocontrol of plant pathogens and also of particular groups of pathogens e.g. soilborne pathogens, above-ground pathogens or sclerotial pathogens, each of them differing ecologically and demanding specific control strategies. Workshops have also been oriented on specific cultivation conditions such as soilless cultures or the use of specific molecular techniques for better understanding mechanisms of interaction and establishment of the biocontrol agent.

The theme of this workshop will focus on "Biocontrol and Microbial Ecology". Microbiology made significant progress in the last decades with high importance for biological control. We need a better understanding of factors influencing biological control of plant diseases at field scale in praxis as important component in IPM. The workshop will give attention to research which discusses especially microbial ecological aspects. Contributions with new results and innovative approaches in this context will be presented on bacterial biocontrol strains – insights from omics, fungal biocontrol strains – insights from omics, biocontrol in integrated plant disease management – from the lab to field scale, microbial environments and suppressive soils, plant microbiome and biocontrol, and novel biocontrol strategies. This will facilitate the exchange of knowledge in the fields of biological control of plant pathogens, microbial ecology and integrated pest and disease management between scientists, representatives of biological control industries and users of biological control solutions. More than 170 participants from 45 countries are expected to attend the meeting with 57 oral presentations and 89 posters.

Looking forward to seeing you in Berlin, Germany, at the 13th working group meeting, and also at the follow meeting which is planned for 2018.

Jürgen Köhl
Convenor

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Biological and integrated control of plant pathogens
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Contribution of biocontrol agents to sustainable agriculture: do insights from microbiome research and BCA "omics" pay off

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Abstract: By the year 2050 there will be 9 to 11 billion people on earth to feed using the same amount or less land and water as is currently available for agricultural production. The United Nations estimated that global food production will need to increase by 70% by 2050. Currently, about one-third of all potential agricultural commodities grown worldwide are lost to diseases and other pests. Farmers are challenged to grow more, but with less fertilizer, pesticides and fumigants and more sustainable practices such as reduced tillage, precision farming and biological control. In addition, greater numbers of consumers are demanding pesticide-free food. Biocontrol via introduced or indigenous microbes is considered an essential component of sustainable agriculture. Biocontrol of plant pathogens has been studied since the early 1900s, and research on microbial inoculants during the last 40 years has yielded a growing list of commercially available products based on organisms from several different microbial groups (Fravel, 2005; Junaid *et al.*, 2013; McSpadden Gardener & Fravel, 2002; Pal *et al.*, 2006; Stockwell & Stack, 2007). However, *Bacillus* and *Trichoderma* spp. have been the microbes of choice for development into commercial biocontrol agents (BCAs) of plant diseases (Harman *et al*., 2010; Kloepper *et al.*, 2004). These microorganisms are appealing because they are easily mass produced and formulated. On the other hand, *Pseudomonas* spp. have been preferred for fundamental studies of biocontrol mechanisms because they are more amenable to genetic analysis than *Bacillus* and *Trichoderma*, and they are aggressive colonists of roots and leaves. Although Gram negative bacteria like pseudomonads are easily mass produced, they have been harder to formulate because they do not produce dormant spores like *Bacillus* spp. do. Spores of bacilli can remain viable for years. On the other hand, the non-spore forming *Agrobacterium radiobacter* strain K84 and its transfer deficient mutant K1026 have been used successfully worldwide for crown gall control for decades.

The most successful BCAs often possess multiple mechanisms of action that potentially can be active in either biocontrol or direct plant growth promotion. For example, most *Pseudomonas*, *Bacillus* and *Trichoderma* BCAs, besides having mechanisms that directly attack the target pathogen also can induce systemic resistance in the plant, and many BCAs can directly improve plant growth (Kloepper *et al*., 2004; Loper *et al*., 2012; Lorito & Woo, 2015; Lugtenberg & Kamilova, 2009; Pieterse *et al.*, 2014; Raaijmakers & Mazzola, 2012). For example, it has been known for 25 years that *P. protegens* (formerly *P. fluorescens*) Pf-5 produces a wide spectrum of antibiotics inhibitory to oomycetes and fungi, but surprisingly, genome sequencing revealed that approximately 6% of the Pf-5 genome is devoted to the production of secondary metabolites, many of which could contribute to biocontrol activity (Loper *et al.*, 2007). *Trichoderma* spp. (e.g., *T. harzianum* strain T22) have the amazing ability to: control root and foliar pathogens by directly attacking the pathogen or by inducing resistance; change the microfloral composition on the roots; enhance nutrient uptake, including uptake of nitrogen; enhance the solubilization of soil nutrients; and enhance root development

and root hair formation (Lorito & Woo, 2015). The availability of inexpensive whole-genome sequencing of biocontrol strains is revealing both a "treasure trove" of previously unknown potential biocontrol genes and traits, and perhaps more importantly, the tremendous genetic diversity among strains of the same species that appear to be morphologically and physiologically identical.

Enhancing the biocontrol activity of indigenous microbes that are part of the phytobiome is also critical to the success of 21st century sustainable agriculture. Select groups or consortia of microorganism often provide the first line of defense against both soilborne and foliar pathogens by inhibiting pathogens directly or inducing defense mechanisms in the plant. Disease-suppressive soils provide some the best examples of microbial-based defense whereby members of the soil microbiome are recruited to defend the plant against attack by a pathogen (Weller, 2105; Weller *et al*., 2002). Suppressive soils are soils in which the pathogen does not establish or persist, establishes but causes little or no damage, or establishes and causes disease for a while but thereafter the disease is less important, although the pathogen may persist in the soil (Weller, 2002). Suppressive soils occur globally and are known for many different pathogens (Mendes *et al.*, 2011; Weller *et al.*, 2002; 2007). One example is take-all decline (TAD), the spontaneous reduction in the incidence and severity of take-all and increase in yield occurring with monoculture of wheat or barley following a severe attack of the disease (Weller, 2015). TAD occurs worldwide and is highly effective for management of take-all. In the Pacific Northwest (PNW), growers rely on TAD with about 0.8 million ha of wheat suffering little damage from take-all due to the phenomenon (Weller, 2015), even though the pathogen is still present in the soil. TAD suppressiveness results from the build-up of large populations (> 10^5 CFU/g root) of fluorescent *Pseudomonas* spp. that produce 2,4-diacetylphloroglucinol (DAPG), and recent studies have demonstrated that growers can significantly enhance the robustness of take-all suppression by selecting a wheat cultivar that more favorably supports populations of DAPG producers on their roots (M-M. Yang and D. M. Weller, unpublished data).

However, despite the significant advancements in biocontrol technology, inconsistent performance and a narrow spectrum of activity by many microbial agents have been chronic problems impeding wider adoption of this technology by growers (Thomashow *et al.*, 2007; Weller *et al.*, 2007; Weller & Thomashow, 2015). For example, inoculants may perform well in one location or field season but not the next, owing to a wide range of biotic and abiotic factors that can adversely impact root and foliage colonization, expression of genes involved in biocontrol and/or activity of biocontrol metabolites (Dubuis *et al.*, 2007; Lugtenberg & Kamilova, 2009; Ownley *et al.*, 2003; Pierson & Pierson, 2010; Zamioudis *et al.*, 2013). Major knowledge gaps remain about the complex interactions among BCAs, the host, pathogens, phytobiomes and the environment. However, recent advances in DNA sequencing technologies have facilitated the rapid characterization of the composition, diversity, and functional potential of microbial communities in unprecedented detail. As a result, studies using community sequencing approaches have revealed previously unrecognized relationships among microbial consortia, plant pathogens, and disease suppression (Agler *et al*., 2016; Cha *et al*., 2016; Chapelle *et al.*, 2016; Mendes *et al.*, 2011; Nallanchakravarthula *et al.*, 2014; Penton *et al.*, 2014; Rosenzweig *et al*., 2012). These tools are proving to be a powerful means for identifying novel biocontrol targets and developing microbial community management strategies to enhance disease suppression and promote plant health (Berg, 2015; Michelsen *et al.*, 2015; Yin *et al.*, 2013; Xue *et al*., 2015). In-depth analysis of the phytobiome along with other "omics" tools including metabolomics have sparked a "renaissance" of research on and development of new biocontrol strategies and agents worldwide. The fact that multi-national agricultural companies are now testing a wide variety of biocontrol and growth-promoting agents in

thousands of test plots worldwide speaks to the important role that biological control technology is expected to play in 21st century agriculture.

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Session 1:
Bacterial biocontrol strains – insights from omics

Dissecting induced systemic resistance by *Pseudomonas* spp. from the WCS collection

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Abstract: Fluorescent *Pseudomonas* spp. were selected from potato and wheat roots in the 1980's at the Phytopathological Laboratory "Willie Commelin Scholten" in the Netherlands by Patrick Geels in the group of Bob Schippers. Several of these strains were tested for their abilities to stimulate plant growth and to suppress diseases, and strains WCS358, WCS374, and WCS417 were selected to study their modes of action. Siderophore-mediated competition for iron was first assumed to be the main mode of action, but induced systemic resistance (ISR), that is priming the defense of plants against pathogen attack, was recognized as an important mechanism by which these rhizosphere microbes protect plants against diseases. A broad spectrum of pathogens on a range of host plants is affected by ISR, but there is specificity. In most plant species tested not all three WCS strains can elicit ISR, and none of the WCS strains can elicit ISR on all the plant species tested. Thus it appears that the ISR eliciting molecules of the strains differ. Specificity in ISR elicitors was confirmed by comparing the complete genome sequences of the three strains. We now study the transcriptomes of the WCS strains in response to control plants and plants with activated ISR to elucidate communication in the rhizosphere.

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Pea broth promotes cell motility and biocontrol activity of *Lysobacter capsici* AZ78 and upregulates genes related to biogenesis of type IV pili

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Abstract: Cells of biocontrol bacteria can colonise spaces occupied by plant pathogens by using different types of motility. In spite of the ecological importance of motility, few information is available on the motility of *Lysobacter capsici*. We discover that in the biocontrol bacterium *L. capsici* AZ78 (AZ78) motility is medium-dependent, in fact it developed dendrite-like colonies when grown on jellified pea broth (PB), while no cell movement was recorded in the media commonly used in motility assays. The application of AZ78 combined with PB increased its biocontrol activity against *Plasmopara viticola* under greenhouse conditions. We demonstrated that a quantity of PB equal to that present on grapevine leaves after the treatment is sufficient to determine the AZ78cell motility. To identify the molecular mechanisms involved in the medium-dependent motility, the genome of AZ78 was mined for genes responsible for the flagellum and type IV pilus (T4P) biogenesis. The subsequent gene expression analysis revealed that genes encoding structural components and regulatory factors of T4P were upregulated in AZ78 cells grown on medium containing PB, as compared with the other tested media. To the best of our knowledge, these results represent the first hint regarding the cell motility in a *L. capsici* biocontrol strain. Furthermore, these results pave the way to the possible role of pea compounds as co-formulant, to improve the biocontrol efficacy of AZ78.

Key words: *Lysobacter*, type IV pilus, biological control

Introduction

The efficacy of bacterial biocontrol agents is related to their ability to actively colonise plant tissues before the arrival of phytopathogenic microorganisms (Compant *et al*., 2005). Therefore, the characterization of the motility mechanisms should be taken in consideration in the development of novel bacterial biocontrol agents as biopesticides. The bacterial genus *Lysobacter* is a valuable source for new biofungicides and recently, a specific strain, *L. capsic*i AZ78 (AZ78), was shown to effectively control *Plasmopara viticola* on grapevine plants (Puopolo *et al.*, 2014). The analysis of the genome of *Lysobacter* species highlighted the presence of genes related to the flagellar machinery and type IV pili (T4P) (de Bruijn *et al*., 2015). However, the characterisation of the mechanisms involved in the motility of *Lysobacter* cells is still in its infancy. In this work, the effect of a medium containing pea broth (PB) on cell motility and efficacy of the biocontrol bacterium AZ78 was characterised.

Furthermore, the availability of AZ78 genome allowed determining the molecular mechanisms involved in the motility incited by pea broth.

Material and methods

AZ78 was routinely grown on Luria Bertani Agar at 27 °C. Two-year-old *Vitis vinifera* cv. Pinot Noir grapevine plants were grown under controlled greenhouse conditions for two months. *Plasmopara viticola* was propagated onto these plants and the inoculum (2.5×10^5 sporangia/ml) was prepared according to Puopolo *et al*. (2014).

Swarming motility was evaluated according to Köhler *et al*. (2000). Agar was added to the media Swarming Agar (SWR, 0.8% Nutrient broth, 0.5% Glucose), Luria Bertani broth (LBA 0.5), and PB (PAM 0.5, 12.5% frozen peas in distilled water) to a final concentration of 0.5% (w/v). AZ78 was inoculated with a toothpick and dishes were incubated at 27 °C. After 20 h, AZ78 colonies were visualized with a Bio-Rad Geldoc system. The resulting images, were used to quantify AZ78 colony areas (mm^2) using Fiji software (ImageJ 1.49). Three Petri dishes were used as replicates and the experiment was repeated twice.

Grapevine plants were treated with distilled water (H_2O), PB, PB + AZ78 (1×10^8 cells/ml) or AZ78 (1×10^8 cells/ml) alone. The *P. viticola* inoculum, was sprayed onto the abaxial surface one day after treatment application. Seven days after inoculation, the percentage of leaves with visible sporulation (disease incidence) and the percentage of leaf area covered by sporulating lesions (disease severity) were assessed. Six plants (replicates) were used for each treatment and the experiment was repeated twice. After log transformation, the data were analysed using one-way ANOVA and means were compared using Tukey's test.

Autoclaved PB (200 ml) cooled down to room temperature was applied to the leaves of three grapevine plants and dried for one hour under greenhouse conditions. A same number of plants was treated with 200 ml of H_2O and used as control. The substances residing on ten leaves from each plant was obtained by washing for 1 h with orbital shaking (100 rpm) at room temperature in 100 ml of H_2O contained in sterile plastic boxes. The leaf-washing suspensions were filtered and collected in sterile bottles. Agar was added to each leaf-washing solution to reach a final concentration of 0.5% (w/v) and autoclaved. The obtained media were used for swarming motility assays as described above.

The genome of AZ78 (JAJA02000000; Puopolo *et al*., 2016) was mined by NCBI Blast focusing the attention on putative genes responsible for flagellum and T4P apparatus. The relative expression level of 20 genes responsible for flagellum and T4P biogenesis was evaluated for AZ78 grown on LBA 0.5, PAM 0.5 and SWR. RNA extraction and cDNA synthesis were performed. qRT-PCR reactions were carried out for two independent experiments. The data were transformed using the equation $y = \log_{10}(1+x)$ and analysed by one-way ANOVA and means were compared using Tukey's test.

Results and discussion

Motility of AZ78 cells depend on the medium composition and PB triggers cell motility in AZ78. Indeed, the colonies of AZ78 showed dendritic-like appearance on PAM 0.5 with a colony area (399.1 ± 45.7 mm^2) higher than LBA 0.5 (45.0 ± 8.7 mm^2) and SWR (77.5 ± 13.5 mm^2; Figure 1).

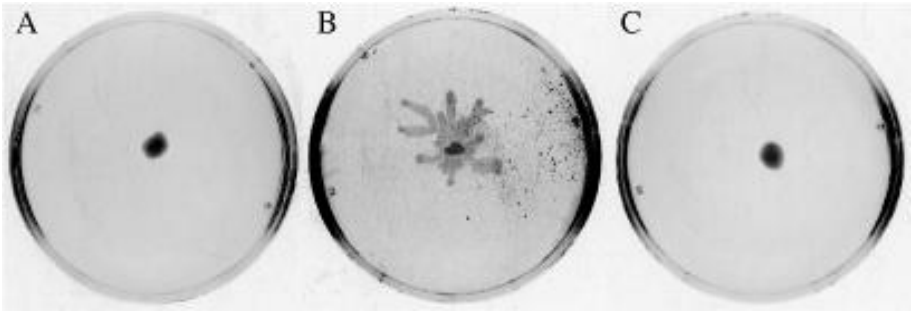

Figure 1. Swarming motility of *Lysobacter capsici* AZ78. A, LBA 0.5; B, PAM 0.5; C, SWR.

PB positively influences the level of plant protection of AZ78. In fact, the treatment of AZ78 combined with PB significantly reduced the downy mildew severity and incidence as compared with H_2O- and PB-treated plants. Particularly, the disease severity and incidence were significantly lower on plants treated with AZ78 plus PB than on AZ78 alone (Figure 2).

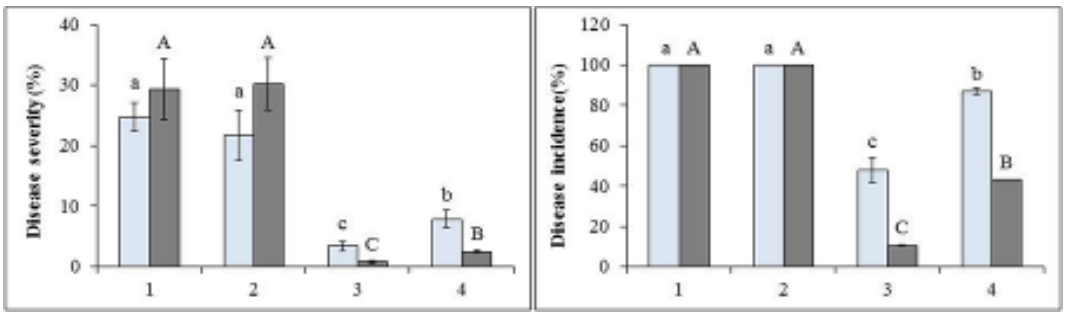

Figure 2. Effect of pea broth on the plant protection activity of *Lysobacter capsici* AZ78. Different letters indicate significant differences according to Tukey's test ($\alpha = 0.05$) of two separate experiments (grey, dark grey). 1, H_2O; 2, PB; 3, PB + AZ78; 4, AZ78.

The leaf-washing suspensions derived from plants treated with PB was sustaining the motility of AZ78 cells (85.7 ± 13.9 mm^2) significantly more than the leaf-washing suspensions derived from grapevine plants treated with H2O (6.9 ± 3.1 mm^2). The AZ78 genome mining allowed the identification of 43 genes related to T4P biogenesis, as reported for the other *Lysobacter* strains (de Bruijn *et al*., 2015). Moreover, 21 putative genes encoding components of flagellar apparatus are present in the AZ78 genome. The qRT-PCR analysis revealed a significant upregulation of the T4P biogenesis genes tested when AZ78 was grown on PAM 0.5 compared with LBA 0.5 and SWR (Figure 3). On the other hand, the absence of a transcriptional upregulation of the structural flagellar genes was observed.

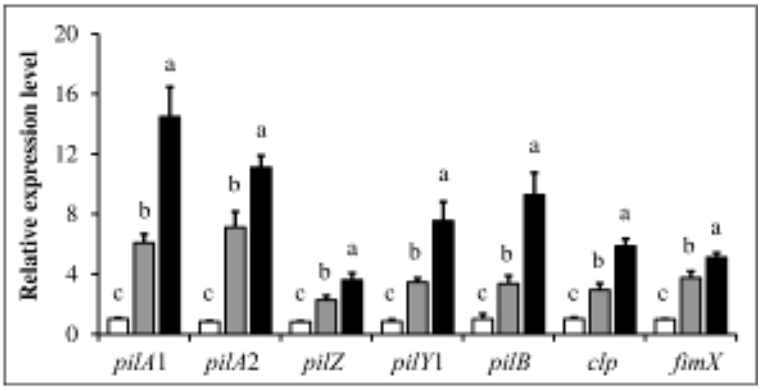

Figure 3. Expression analysis of type IV pili-related genes in *Lysobacter capsici* AZ78. Different letters indicate significant differences according to Tukey's test ($\alpha = 0.05$). LBA 0.5 (white bars), SWR (grey bars) and PAM 0.5 (black bars).

Overall, these results indicated that AZ78 can move on inert surfaces in presence of PB and its combination with PB enhanced the efficacy of AZ78 against *P. viticola*. The morphology of AZ78 colonies grown on PAM 0.5 had a phenotype associable to the swarming motility observed in *P. aeruginosa* (Köhler *et al*., 2000). Likewise, the medium dependent motility of AZ78 was associated with the biogenesis of T4P, external cell appendages that play a key role in the attachment of bacterial cells to plant tissues (Mattick, 2002). Future studies will aim at identifying the factors contained in PB that trigger piliation in AZ78 in order to include them in formulations to improve the efficacy of AZ78 in field application.

Acknowledgements

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The potato microbiome and its potential impact on late blight resistance

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Abstract: In organic farming, control of late blight, caused by the oomycete *Phytophthora infestans*, relies on the use of copper-based fungicides. With the EU planning on reducing and banning the use of copper for environmental reasons by 2020, the need to develop alternative organic control methods is evident. In natural and agro-ecosystems, plant roots and shoots are colonized by a diverse community of microorganisms. In the model plant *Arabidopsis thaliana*, a protective role of this plant microbiome against a number of phytopathogens has been demonstrated. However, the putative role of the potato microbiome in protecting the plant against pathogens such as the fast evolving *P. infestans* is still under investigation. In this project, *in vitro* screening of recently isolated strains from field-grown potatoes revealed differential antagonistic activity against *P. infestans*. 16 *Pseudomonas* strains were selected and further characterized for their *in vitro* effects on mycelial growth, sporangia germination, and zoospore production and behavior, and for their effects on symptom development by means of leaf disc assays. Furthermore, 10 strains of varying anti-*Phytophthora* activity have been sequenced and *de novo* assembled in order to potentially identify the genomic determinants of this antagonistic activity through comparative genomics. In addition, in order to assess the spectrum of activity of the bacteria, 3 strains were tested against a collection of *P. infestans* strains. For this, a monitoring of the Swiss *P. infestans* population was conducted during the summer of 2015. 22 *P. infestans* isolates were successfully retrieved from infected fields and were characterized for morphological and virulence traits, as well as for fungicide resistance and compatibility to Black's potato R-gene differential set. Altogether, these findings should provide better understanding of the mechanisms involved in the anti-*Phytophthora* activity of the *Pseudomonas*, and therefore provide insight into the potential utility of *Pseudomonas* in fighting late blight.

Key words: *Pseudomonas*, comparative genomics, late blight

Antibacterial and antifungal activity of *Pseudomonas donghuensis* P482 is based on an overlapping genetic background

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Abstract: *Pseudomonas donghuensis* P482 is a tomato rhizosphere isolate, showing antagonism towards bacterial (*Pseudomonas syringae, Dickeya* spp., *Pectobacterium* spp.) and fungal (*Rhizoctonia solani*, *Fusarium culmorum*) plant pathogens. P482 strain is also able to protect plant tissue against soft rot pathogens and to colonize roots of tomato (the plant of origin), maize and potato. *In silico* analysis of the genome sequence of P482 revealed that the antagonistic activity towards various bacterial and fungal plant pathogens is not based on the production of phenazine, 2,4-diacetylphloroglucinol, pyrolnitrin, pyoluteorin or known (cyclic)-lipopeptides, as the genes involved in production of these compounds are not present in the P482 genome. To verify the genetic background of the antimicrobial activity of P482, random transposon mutagenesis was performed and the transposon mutant library was screened for the lack of antibacterial and antifungal activity. This allowed for selection of a mutant unable to inhibit growth of bacterial pathogens of *Dickeya* and *Pectobacterium* genera. At the same time *in silico* analysis of P482 genome with the use of antibiotics & Secondary Metabolite Analysis SHell platform (antiSMASH) resulted in selection of a set of hypothetical gene clusters, possibly involved in reduction of secondary metabolites with antimicrobial activity. One of these clusters comprises a gene previously inactivated through transposon mutagenesis. Site-directed mutagenesis of 5 genes of this cluster resulted in a loss of antibacterial activity of P482 towards soft rot pathogens. The same mutants were tested for their antagonistic activity towards *R. solani*, indicating an overlap between genetic background of antibacterial and antifungal activity of P482. Although we determined the genetic background of antimicrobial activity of *P. donghuensis* P482, the chemical structure of the active compound(s) remains to be solved.

This study was funded by a grant from the Polish National Science Centre (2012/07/B/NZ9/01623).

Key words: biological control, soft rot bacteria, genome mining

Different antifungal effects of *Bacillus subtilis* and *Pantoea agglomerans* on 2 grapevine cultivars against Grapevine Trunk Diseases pathogens, *Neofusicoccum parvum* and *Phaeomoniella chlamydospora*

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Abstract: Vineyards throughout the world, including Tunisia, are being attacked by Grapevine Trunk Diseases (GTDs) such as Esca and *Botryosphaeria* dieback. The bacterial microflora colonizing the wood tissues of Tunisian and French grapevines (respectively cultivars Muscat d'Italie and Cabernet Sauvignon) was investigated in order to find a suitable biological control agent (BCA) that can be applied to vineyards. Complex bacterial communities colonize wood tissues, as shown by single-strand conformation polymorphism (SSCP) analyses. Cultivable strains were isolated from the wood tissues, identified by sequencing of 16S rRNA and rpoB genes, and screened for their *in vitro* antagonistic traits. Based on the results, two bacterial strains were selected: *Bacillus subtilis* (strain B6) and *Pantoea agglomerans* (strain S5), respectively isolated from Tunisian and French grapevines. They were tested *in planta* on young vines of cv Muscat d'Italie and Cabernet Sauvignon against two fungal pathogens involved in GTDs, *i.e. Neofusicoccum parvum* and *Phaeomoniella chlamydospora*. Young vines of both cultivars were inoculated by *B. subtilis* B6 or *P. agglomerans* S5 or the combination of B6+S5 singly or in combination with *N. parvum* and *P. chlamydospora.* In term of plant protection, the most efficient condition to reduce *in planta* wood necrosis caused by the fungal pathogens in the two cultivars was the combination of the two bacteria. However, bacterial treatments were significantly more efficient to reduce wood necrosis caused by *N. parvum* or *P. chlamydospora* in Muscat d'Italie than in Cabernet Sauvignon.

Key words: *Bacillus subtilis*, Grapevine Trunk Diseases, *Neofusicoccum parvum*, *Pantoea agglomerans, Phaeomoniella chlamydospora*, varietal susceptibility

Unravelling induced immune response in grapevine by beneficial bacteria and efficacy of ISR against *Botrytis cinerea* in both controlled and vineyard conditions

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Abstract: The development of indigenous beneficial microbes adapted to vineyard environments could be a valuable tool in sustainable viticulture by improving resistance to pathogens without compromizing grape yield and wine quality. In recent years, different beneficial bacteria associated with grapevine have been identified and selected for their capacity to protect grapevine plants against the necrotrophic fungus *Botrytis cinerea*. The work performed on selected bacteria demonstrated the credibility of such a strategy to control grey mold disease in both laboratory and vineyard conditions. This biocontrol action mainly involves induced systemic resistance (ISR) by different bacteria. However, mechanisms involved in bacteria-mediated ISR remain to be deciphered. This paper summarizes our current knowledge on ISR in grapevine plants, focusing on inducible immune response, and the efficacy of ISR in both controlled and vineyard conditions. *Pseudomonas fluorescens* PTA-CT2 is one of the most effective bacteria selected for their effectiveness to control *B. cinerea*. Its application at the root level of grapevine plantlets caused differential defense responses including upregulation of an array of defense-related genes and secondary metabolite accumulation, as well as a hypersensitive response like cell death in the aboveground tissues. Data from expressed genes suggested that this bacterium activated not only jasmonic acid (JA) and ethylene (ET) signaling pathways, but also salicylic acid (SA)-regulated responses. The different defense genes induced by the bacteria are not routinely potentiated following inoculation of leaves with *B. cinerea*, but the induced resistance is clearly associated to the elicitation and potentiation of phytoalexins and to some extent to accumulation of anthocyanins in the aboveground tissues both in controlled conditions and in vineyard.

Key words: grapevine, beneficial bacteria, induced resistance

Persistence of *Salmonella* in agricultural soil is strongly influenced by its bacterial community

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Abstract: In recent years, disease outbreaks caused by *Salmonella* were increasingly associated with raw fruits and vegetables, indicating that fresh produce could be a vector for human pathogens. Contamination of fruits and vegetables can occur along the whole production chain, including the field. The survival of *Salmonella* in soil might therefore have a big impact on colonization of plants. Nonetheless, knowledge about factors influencing the persistence of human pathogens in soil is scarce.

In this study, we analyzed the impact which a change of soil bacterial community, induced either by autoclaving of the soil or the application of sludge as fertilizer, had on the survival of *Salmonella* in soil. Diverse soil microcosm experiments were performed using *Salmonella enterica* serovar Senftenberg as well as the serovar Typhimurium strains 14028s and LT2. Survival of these strains was monitored in autoclaved soil and compared to their persistence in non-autoclaved soil. Our experiments revealed that the number of cultivable *Salmonella* in autoclaved soil decreased over 14 weeks after inoculation from 10^6 to 10^4 per g of soil. In contrast, in non-autoclaved soil, *Salmonella* counts decreased from 10^6 to 10^2/g within 12 weeks and were below the detection limit 13 weeks after inoculation. Denaturing gradient gel electrophoresis (DGGE) of 16S rRNA gene fragments amplified from total community DNA showed that the structure of the bacterial community was changed directly after autoclaving.

In the following experiments, *S.* Typhimurium LT2 was inoculated into soil amended with sludge and untreated soil. Within five weeks after inoculation, the numbers of cultivable *Salmonella* decreased overall from about 10^6 to 10^3 per g dry soil. However, *Salmonella* LT2 survived at higher numbers in the untreated soil. In addition, *Salmonella* were detected in soils up to 175 dpi using the PCR-Southern blot hybridization technique or enrichment culture with subsequent plating. Furthermore, we analyzed the effect of sludge on the soil bacterial communities. DGGE and Illumina sequencing of 16S rRNA gene fragments revealed a strong influence of sludge application on the soil microbiome.

Together, our data showed that despite its rapid initial decline, *Salmonella* persists in soil for long periods. The soil bacterial community had a strong influence on *Salmonella* survival. While reduction of the bacterial community by autoclaving prolonged the survival of *Salmonella*, the fertilization of soil with sludge reduced its survival. In conclusion, our results indicate that soil could be an important reservoir for *Salmonella* posing a risk of contamination of produce in the agricultural environment and that a high microbial diversity reduced the survival of *Salmonella*.

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Study on the mechanisms of action of *Bacillus cereus* AR156: induced systemic resistance to bacterial disease

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Abstract: *Bacillus cereus* AR156 is a plant growth-promoting rhizobacterium originally isolated from the rhizosphere from garden tree, Nanjing, China, that can protect plants from different soil-borne pathogens including *Ralstonia solanacearum*, *Phytophthora capsici*, *Fusarium oxysporium*, *Verticilium dahliae* and *Meloidogyne incognita*. *Bacillus cereus* (*Bc* for short) AR156 is a sequenced and patented strains in China as a single strain's preparation and 12 countries in Europe as a combination with another 2 strains, and has been registered as a biopesticide in China against root-knot nematode in tomato. In order to explore the mechanisms of action of the *Bc* AR156 biocontrol agent, we have carried out a serials of research in recent years. We found that *Bc* AR156 induced systemic resistance to *Pseudomonas syringae* pv. *tomato* DC3000 by simultaneously activating both SA and JA/ET signaling pathway and showed NPR1 dependent (Niu *et al*., 2011). In the following experiment, two transcription factors WRKY11 and WRKY70 acted as important regulators involved in the ISR triggered by *Bc* AR156. The results revealed that AR156 treatment significantly stimulated the transcription of *WRKY70*, but suppressed that of *WRKY11* in *Arabidopsis* leaves. The epistasis analysis suggested that WRKY11 regulated AR156-triggered ISR by activating JA signaling pathway, and WRKY70 regulated the ISR by activating SA signaling pathway (Jiang *et al.*, 2016a). In addition, both WRKY11 and WRKY70 modulated AR156-triggered ISR in a NPR1-dependent manner. This study also gave us an explanation why and how a single rhizobacterium elicits ISR by simultaneously activating SA- and JA/ET-signaling pathways. Subsequently, we wanted to know how the *Bc* AR156 was percept or recognized by plant and trigger ISR in plant? In our study, we found that the extracellular polysaccharides (EPS) of *Bc* AR156 could act as a microbe-associated molecular patterns (MAMPs) and function in the early perception status of the ISR of *Bc* AR156, and induced systemic resistance against *Pst* DC3000 in *Arabidopsis* (Jiang *et al.*, 2016b). The defense-related genes *PR1*, *PR2*, and *PR5* and mitogen-activated kinases (MAPK) cascade marker gene *MPK6* were concurrently expressed in the leaves of EPS-treated plants. Although we have found some broad spectrum mechanisms of *Bc* AR156 on the biocontrol of plant disease, how it specifically controls root-knot nematode is still unclear. Since the pathogen is an animal which can obviously choose where to stay in the rhizosphere niche, we are expecting that looking for the mechanism of this process will be very interesting.

Key words: Induced systemic resistance (ISR); transcriptional factor; biocontrol mechanism; Rhizobacteria; microbe-associated molecular patterns (MAMPs)

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The rhizobacterium *Pseudomonas chlororaphis* PCL1606 doesn´t have PGPR activity as additional mechanism to biocontrol

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Abstract: *Pseudomonas chlororaphis* PCL1606 was isolated from rhizosphere of healthy avocado trees, growing in an area affected by white root rot (caused by *Rosellinia necatrix*), which is one of the most important diseases for avocado crops. PCL1606 revealed strong antagonist activity against *R. necatrix*, mainly due to the production of the antifungal compound 2-hexyl, 5-propyl resorcinol (HPR). HPR is crucial for the biocontrol activity, and is involved in bacterial colonisation of the avocado root surface. In this work, we elucidated if PCL1606 also presents plant-growth promoting (PGPR) activity as an additional mechanism involved in biological control. To study such PGPR activity, in vitro assays with tomato seedlings and commercial 6-month-old avocado plants were performed. Additionally, activities related to PGPR activity, such as 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity, indole-3-acetic acid (IAA) production, phosphate solubilization or production of siderophores, were also tested. The obtained results showed that *P. chlororaphis* strain PCL1606 does not present plant growth promoting activity as an additional trait to its biocontrol ability.

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Key words: biocontrol, PGPR, PCL1606

The impact of the outbreak of *Tuta absoluta* in tomato in Nigeria

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Abstract: Nigeria is facing the outbreak of the tomato pest *Tuta absoluta*, locally called 'tomato ebola'. This resulted to adverse scarcity of tomato supply in various markets of the affected states.

The pest is known worldwide, originates from South America far back 1912 and has ravaged tomato farms in many countries including Europe, Middle East, Asia and Africa. The tomato pest came into the country from Niger Republic and invaded farms in Nigeria, ravaging them within 48 hours, boring into fruits and stems of tomato plants. The five states affected by the tomato pest outbreak include Kastina, Kano, Kaduna, Jigawa, and Nasarawa. The pest has also spread to other tomato producing states in the North and as well as Lagos, Oyo and Ogun States. Various insecticides cannot control the pest as it easily develops resistance.

This paper studies the *T. absoluta* invasion of tomato farms in Nigeria. Farms were visited and oral interviews were conducted. The studies showed that *T. absoluta* has the capacity to reproduce 10-12 times in a year, in tranches of 250-300 larvae within 28 days. The pest spreads rapidly if there is no knowledge of containment and it is very difficult to control so that it also can affect pepper and Irish-potato. New containment packs will enable farmers to resume production activities with desired yield. The intervention programme will involve containment packs for 50,000 ha to be used among the tomato producing states. Further research will study and analyze the losses for farmers and needs of experts to support the sustainable production of fruits for local consumption and provide raw materials for processing.

Key words: tomatoes, *Tuta absoluta*, Nigeria

Biological characteristics of *Bacillus amyloliquefaciens* AK-0 and disease suppression of Korean ginseng root rot caused by *Cylindrocarpon destructans* (Zins.)

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Abstract: This study describes the characterization of *Bacillus amyloliquefaciens* AK-0 (AK-0) with respect to its ability to suppress the ginseng root rot disease caused by *Cylindrocarpon destructans*. From the total of 190 ginseng rhizosphere bacteria, the isolate AK-0 was selected for further studies based on *in vitro* screening. The morphological characters of AK-0 have been studied using a microscope. The isolate AK-0 was identified as *Bacillus amyloliquefaciens* using Biolog system and 16S rRNA gene sequence analysis, and morphological and biochemical characteristics. Bacterial population and media optimization was determined by growth curve. AK-0 cell numbers were relatively higher when the cells were cultured in BHI medium when compared with other media. AK-0 showed the strongest antagonistic activity against fungal and bacterial pathogens. The culture filtrate (CF) from the isolate AK-0 exhibited an antifungal effect on ginseng fungal pathogens, inhibition of conidia germination, and suppression of root rot disease caused by *C. destructans* on matured ginseng roots. Expression of various antibiotic biosynthesis genes using a PCR analysis suggests that the release of its antibiotic compounds might play a role in antifungal effect and suppression of ginseng root rot. The results of this study indicate that the CF from the isolate AK-0 possessed antifungal effect on ginseng fungal pathogens, and suppression of root rot disease caused by *C. destructans* on matured ginseng roots. The isolate AK-0 is considered as a potential source of novel bioactive metabolites. The CF from AK-0 exhibited an antifungal effect against *C. destructans* on ginseng roots. PCR analysis described in this study indicates that the isolate AK-0 was shown to harbor genes involved in the biosynthesis of antimicrobial compounds.

Key words: *Bacillus amyloliquefaciens* AK-0, characterization, antifungal effect, ginseng root rot, biosynthesis genes

Indigenous soil bacteria: an alternative strategy to protect against potato diseases

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Abstract: Potato has been present in Europe for more than 500 years and has become one of the major crops. Although significant improvements have been made in term of selection and agricultural practices, potato crops are still facing the burden of bacterial diseases such as tuber soft rot (*Pectobacterium carotovorum*), common scab (*Streptomyces scabies*) and fungal diseases such as tuber dry rot (*Fusarium solani*), black scurf (*Rhizoctonia solani*), early blight (*Alternaria solani*) and potato late blight (*Phytophthora infestans*), this latter being the major pathogen in terms of yield losses. Both disease pressure and exploitation profitability enforce farmers to use large amounts of pesticides that lead to important economic and environmental costs. However, the strengthening of European policies regarding the limited use of pesticides forces producers to find sustainable alternatives. A suitable solution is an Integrated Pest Management (IPM), which promotes prevention means, purposeful fertilizations and/or alternative crop protection strategies. These strategies have led to study and use microbial agents due to their antagonistic abilities and their competence to induce natural plant defenses. The present study aims at characterizing indigenous bacteria (*Bacillus* spp. and *Pseudomonas* spp.) associated with potato-agro-systems for diseases biocontrol.

From a large collection of more than 2,000 strains isolated from Belgian soils, 54 *Bacillus* spp. and 16 *Pseudomonas* spp. have been selected for further characterization. The bacteria were identified by sequencing their 16S RNA genes and, for some, their gyrase genes. PCR screenings were then performed to identify potential virulent genetic determinants and to list the genes associated with known bioactive molecules. The results indicated that none of the studied *Bacillus* strains displayed a potential pathogenicity towards vertebrates or plants. Genes associated with different bioactive molecules such as lipopeptides, bacteriocins or siderophores were also revealed. Beside preliminary genetic characterization, *in vitro* tests showed promising antagonistic activities of the bacterial collection against the following potato pathogens: *P. infestans, F. solani, A. solani, R. solani, P. carotovorum* and *S. scabies*. The most promising strains are currently further characterized through whole genome sequencing, *in vitro* antagonistic activities and field assays to assess their *bona fide* antagonistic potentials and to better understand the mechanisms involved in their antagonistic activities.

Key words: antagonistic activities, biocontrol, genetic characterization, potato diseases, soil bacteria

Biological control of *Ralstonia solanacearum* and growth promoting of chili pepper using indigenous West Sumatra isolates of *Bacillus cereus*

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Abstract: Four *Bacillus cereus* isolates acquired from previous studies are having good biocontrol and plant growth-promoting activity in plants. These studies purposed to characterize their ability in biocontrol and plant growth promoting activity. Biocontrol activity characters observed are inhibition in dual culture, HCN production, root colonization and salicylic acid production. Plant growth promoting activity characters observed are IAA production, ammonia production, and phosphate solvent. Results showed that some isolates have a good ability in both biocontrol and plant growth promoting activity. All four isolates produce IAA, some isolates produce ammonia and solve phosphate. *B. cereus* isolates show good activity in producing antibiotics shown in dual culture plating. All isolates showed activity to increase salicylic acid concentration in roots.

Key words: *Bacillus cereus* isolates, *Ralstonia solanacearum*, plant growth promotion, biocontrol

Biocontrol of *Aphanomyces euteiches* root rot in legumes by *Streptomyces* sp. Z321 isolated from Moroccan ecosystems

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Abstract: Legumes were the basis of the human nourishment for thousands of years and their use is considered as an inseparable companion of human evolution. Moreover, legumes used in pure culture or in association, play a major role in crop rotation. At international and national levels, the surfaces occupied by these crops are very important. However, legume crops can be attacked by various plant pathogens, especially *Aphanomyces euteiches*, the causal agent of root rot and seedling blight in legumes and one of the most destructive diseases especially of peas worldwide.

Despite extensive efforts to develop suitable control methods for *Aphanomyces* root rot, to date the only effective control is to avoid planting legume crops in moderately to heavily infested soils because inoculum (oospores) of the pathogen can persist in infected plant debris in the soil for many years. This persistence on soil excludes control through crop rotation and a lack of both economic fungicide control and resistant legume cultivars has resulted in unavoidable crop losses and economic disadvantage to the grower and industry. Thus, it appears to be merit in investigating the potential of Moroccan actinobacteria for biological control of *A. euteiches*. The objectives of this study were first to evaluate their ability to suppress the mycelial growth and zoospore germination of the pathogen. Thereafter, the most promising bioactive isolates were screened against many strains of Rhizobia, in order to avoid biocontrol agents that could negatively affect the symbiotic relationship with legumes. Finally, selected actinobacteria isolates were tested for their abilities to protect pea seeds and plants against *A. euteiches*. The taxonomic study by the sequencing of the 16S rDNA gene of the most promising selected strain Z321 showed that it belongs to *Streptomyces* sp.

Regarding all conducted experiments and obtained results, Moroccan actinobacteria isolates could be potential biocontrol agents of pea's root rot caused by *A. euteiches*.

Key words: Actinobacteria, biocontrol, screening, *Aphanomyces euteiches*, seed treatment, pea

Session 2:
Fungal biocontrol strains – insights from omics

Biological and integrated control of plant pathogens
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Use of the omic and NGS technologies to develop and improve biopesticides based on yeasts against postharvest diseases of fruits

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Extended abstract: Post-harvest diseases of apples, such as *Botrytis cinerea*, *Penicillium expansum* and *Gloeosporioides* group, are still provoking important economic losses which can reach 25% of the harvested fruits. There is currently an increasing demand to develop sustainable methods to control these post-harvest pathogens. Biocontrol agents are interesting candidates to answer this demand. Nevertheless, their commercial development is sometimes hampered by a low or non-reliable efficacy comparing to fungicide treatments (Droby *et al.*, 2016). Fundamental research on the mode of action of the Biological Control Agent (BCA) and of its ecological fitness could help to overcome that phenomenon. Once applied on the fruit surface, the BCA will face a complex microenvironment which is influenced by the host genotype and its physiology, by the presence and concentration of pathogens and by the microflora composition of the commensal microorganisms. Multiple interactions between them occur at the site of action and may influence the BCA efficacy. Moreover, this site is also under the influence of environmental parameters like humidity, temperature, UV light. Progresses have been made during two decades to understand the modes of action and the ecological niche of *Pichia anomala* strain K. These advances required the combination of various methodologies (*in vitro* and *in situ*) and techniques (microbiology, microscopy, genome characterization, transcriptome, proteome, gene disruption…).

Concerning the modes of action of *P. anomala* strain K, preliminary biochemical studies suggested an involvement of exo-β-1,3-glucanase in the biocontrol properties. During a first targeted genomic approach, a sequential disruption method was used to inactivate PAEXG1 and/or PAEXG2, two genes coding for strain K exo-β-1,3-glucanase. The biocontrol properties of the strain were affected by single inactivation of PAEXG1 or PAEXG2 gene and by the double inactivation of both genes compared to the parental strain. The relative contribution of exo-β-1,3-glucanase was strongly depend on the quantity of applied strain in the wound and the maturity of apples (Friel *et al*., 2007). An "open" strategy complementary to the targeted approach was applied to identify other genes involved in biocontrol properties. *P. anomala* strain Kh5 (haploid derived from strain K) was grown *in vitro* on a medium containing glucose or cell wall preparation of *B. cinerea* as the sole carbon source. Eleven candidate genes were identified. Their differential expression was confirmed independently by real-time PCR. These genes corresponded to β-glucosidase, transmembrane transport, citrate synthase, and external amino acid sensing and transport. Some of these functions could be related to cell wall metabolism and potentially involved in mycoparasitic properties (Massart *et al.*, 2015b).

The proteomic approach also corresponds to an open strategy studying the cell protein contents and highlighting the variations in the proteome according to the different conditions tested. In the exponential phase of strain Kh6 (another haploid derived from strain K) at the wound site of the apple, most of the proteins influenced by the presence of *B. cinerea* were involved in the energetic metabolism and in the protein synthesis (Kwasiborsky *et al.*, 2014).

In the absence of the pathogen, strain Kh6 produces energy through the glycolysis pathway while the presence of the pathogen oriented the energetic metabolism to the oxidative phosphorylation. More specifically, the BCA activates the pentose phosphate pathway. In addition, the presence of the pathogen led to an overexpression of proteins involved in nucleotides synthesis and transcription. These adaptations suggested that strain Kh6 modified its metabolism to optimize energy and nucleic acids production in order to colonize the wound as fast as in the absence of the pathogen.

Ecological studies are focused on the influence of environmental parameters on the growth and biocontrol properties of a BCA. These ecological studies on the BCA can also be completed by similar studies on the targeted pathogens to allow a better understanding of the complex relationship between both microorganisms. For a post-harvest application, the ecological studies evaluate if the BCA is well adapted to the existing storage conditions. Moreover, the comparison with pathogen's niche allow the selection of the most appropriate storage condition to control the pathogen, favouring BCA growth and limiting pathogen's growth. A high relative humidity in storage room was able to favour *P. anomala* strain K, while the development of *P. expansum* symptoms decreased. For a pre-harvest application, ecological studies highlight the adverse environmental parameters hampering the establishment and the growth of the BCA. Being sensitive to UV radiations and low HR (Lahlali *et al*., 2011), a formulation limiting the negative influences of both parameters was developed.

Finally, the advent of high-throughput sequencing (or next-generation sequencing – NGS) technologies is now driving a paradigm change that allows researchers to integrate microbial community studies into the traditional biocontrol approach which has long been focused on the study of single strains of BCAs and on their interaction with pathogens and host plants (Massart *et al*., 2015a). Thanks to NGS, the integration of plant-associated microbial communities to the research will open new biocontrol hypotheses. A better understanding of these interactions will provide unexpected opportunities to develop innovative biocontrol methods against plant pathogens. Working on the microbial community of apple surface in presence or in absence of strain K, first results will be also present on the constitution and characterization of contrasted microbiota. In case of favourable microbiota for biocontrol, it is expected to select ''helper'' microbial strains or molecules driving the microbiota to a pathogen-resistant composition (''prebiotic'' approach).

Key words: Biocontrol, *Pichia anomala*, apples, modes of action, microbial ecology, omics, NGS

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Approaches to the development of biocontrol agent *Penicillium frequentans* isolate 909 for brown rot control on stone fruit

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Abstract: Microbial biocontrol agents have to be registered following the rules of the EC Regulation 1107/2009 (EC2009), which demands information on ecophysiological properties and on the risks and safety of biological products. *Penicillium frequentans* survive actively over a wide range of environmental conditions. The probability of risks to human and animal health is considered to be remote in terms of mycotoxic production, and there is commercial active material capable to inhibit fungus growth.

Key words: mycotoxins, survival, physical environment

Introduction

The use of BCAs as an alternative to synthetic, chemical fungicides has many constraints and obstacles that make it difficult to implement their use as a practical control strategy (Kiewnick, 2007). Many microorganism with good antagonistic potential, however fail to be developed for practical use, primary due to inconsistent performance associated with field applications and often adverse environmental conditions (Cartwright & Benson, 1995). The EU emphasizes the necessity to know crop safety and evaluate the production of secondary metabolites on fruit surface together with their risk on human (Turner *et al.*, 2012). Mycotoxins from *Penicillium* species can be a serious contaminant problem in storage fruit (Cole & Cox, 1981). The toxins are naturally produced by a variety of plant pathogen, such as *Penicillium* species (*P. expansum*, *P. citrinum*, *P. digitatum*), and can be formed rapidly during production, transportation and storage, and also from non-plant pathogenic *Penicillium* spp.

Pf 909 is used as a BCA against brown rot in stone fruit caused by *Monilinia* spp. Pf 909 efficacy is very dependent on its survival and persistence on flower and fruit surfaces, which depend on the climate (Guijarro *et al.*, 2007).

This study tries to achieve part of the registration requirements necessary for authorization of BCA Pf909 in EC countries. Identifying areas of research that still need to be addressed to demonstrate that Pf909 is sufficiently stable on field conditions and crop safe for registration purposes. This work is divided into three major sections: (1) effect of physical factors on Pf909 development, (2) spectrum of resistance of Pf909 to commercial products use for conventional animal and human fungi control, (3) presence of the main mycotoxins produced by *Penicillium* spp., evaluated during the interaction process where Pf909 is on fruit surface.

Material and methods

Experiments for determining the physical properties effects on Pf909

A monosporic isolate of Pf909, which was originally obtained from the phyllophere of peach twigs (Melgarejo & Sagasta, 1984) was used. The specific objectives for physical characterization were to determine the influence of water activity (a_w, 0.999-0.900), pH (3-11), temperature (T 4-37 °C) and light intensity (LI 63-123 lm) on mycelial growth and sporulation of Pf909 *in vitro* on a potato dextrose basal medium (BM). For determining the effect of pH the BM (pH 5.6) was amended with either 1N HCl to acidify the BM to pH 3 and 4 or 2N NaOH to alkalize the BM to pH 8, 9, 10 or 11. For a_w, the BM (a_w = 0.999) was osmotically modified using a glycerol solution so that the a_w of the resultant BM was 0.999, 0.992, 0.990, 0.980, 0.970, 0.964, 0.930, and 0.900. All plates were incubated at 22 °C in the dark. For determining the effect of temperature, the BM inoculated Petri dishes were incubated at 4, 10, 15, 22, 25, 32, 36, or 37 °C in the dark. For determining the effect of light intensity (LI), BM were incubated at 22 °C under continuous light (180 and 360 lumens) or in the dark (0 lumen).

Determination of effect of fungicides on Pf909

Antifungal activity of seven fungicides (Table 1) was measured by a quantitative micro spectrophotometric assay. The antifungal materials were serially diluted, ranging from 1/2 up to a 1/100 dilution in 0.1% acetone solution from the recommended used dose. Mycelia growth inhibition was measured in 10^5 conida/ml czapeck solution 96-well microtiter plates at 490 nm wavelength. Half maximal effective concentration (EC_{50}) value, defined as the concentration of a fungicide that inhibited mycelia growth by 50%, was estimated by linear regression of the log of the absorbance OD_{490} versus the antifungal concentration.

Table 1. Characteristics of antifungal materials used and their EC_{50} on Pf909. EC50 = Half maximal effective concentration; R^2 = linear estimation coefficient.

Active material	**Commercial Dose (Co)**	**Regression curve**	**R^2**	**EC50 (µg /µl)**
Flucocinolone acetonide	0.52 µg/g	**y = -18.34x + 1.56**	**0.47**	**0.21**
Dexametazone	0.02 µg/g	**y = -27.23x + 2.09**	**0.45**	**0.19**
Fluconazol	0.05 µg/g	**y = -18x + 0.73**	**0.43**	**0.09**
Ketoconazol	0.014 µg/g	**y = -32.03x + 0.80**	**0.82**	**0.02**
Triamcinolone acetonide	0.25 µg/g	**y = -21.56x + 1.87**	**0.52**	**0.22**
Clotrimazole	0.10 µg/g	**y = -0.41x + 1.15**	**0.45**	**623.69**
Triamcinolone	0.36 µg/g	**y = -12.09x + 0.87**	**0.08**	**0.18**

Characterization of relevant metabolites produced by Pf909 on fruit surface
Healthy nectarines were sprayed with 5 ml of Pf909 (10^6 conidia/ml) dry conidia suspension with a 95% viability. The presence or absence on fruit of the four main non-volatile mycotoxins (citrinin, patulin, ocratoxin, and penicillic acid) produced by *Penicillium* spp. was evaluated by HPLC-MS just after inoculation and after the postharvest storage period (7 days) at Technische Universitaet Graz (Austria).

Phylogenetic relationship among* Penicillium *spp. mycotoxin producers and Pf909
To study phylogenetic relationship between Pf909 and the main *Penicillium* species producer mycotoxins in fruit, Pf909 is compared to a selection of twelve different *Penicillium* species, sixty two corresponding sequences of *Penicillium* spp. producing bioactive metabolites from different hosts and locations retrieved from Gen Bank database, presented in Figure 1. The phylogenetic marker used was part of the genome region loci RPB2 gene (RNA polymerase II gene-RPB2). It was amplified and sequenced for Pf909 and compared with published strain sequences.

Results and discussion

Overall, the results showed maximum growth rates and sporulation at 22 °C and 25 °C, 0.999 a_w, pH 5.6 to 9, and darkness (Figure 2). Mycelial growth rate were optimal with more than 0.964 a_w and T between 15 and 25 °C, with very little growth at 32 °C or less than 10 °C at any a_w. Sporulation was optimum at 25 °C and at 0.999 a_w and was fastest at 0.997-0.999 a_w, with the slowest growth occurred at the lower a_w. Light intensity conditions had a significant impact on the fungal growth and sporulation (Figure 2). Fruit samples treated with Pf909 did not present any mycotoxins in all the analyzed samples. Pf909 is a good potential BCA to brown rot control because it should be able to adapt to different environmental stress conditions, such as extreme pH, UV rays, long period of exposes to sun light, low and high temperatures and dry conditions. Effective colonization, a large population size, and the viability of a BCA on plant surfaces are all essential for successful biocontrol of plant diseases (Cartwright & Benson, 1995).

There is no evidence of mycotoxins production on fruit surface treated with Pf909 conidia just before application and 7 days after. Phylogenetical studies have resulted in narrowed mycotoxin species producers in all 13 *Penicillium* species present on fruit but there is a good correlation between these species and mycotoxins production. It was possible to differentiate Pf909 from the rest of *Penicillium* mycotoxin producers present on fruits using RPB2 gene.

Pf909 is an innocuous microorganism, which can be controlled by six of the seven commercial antimycotic products tested and which does not produce any mycotoxins on treated fruit. There is no evidence of mycotoxins' production on fruit surface by Pf909 conidia after 10 days.

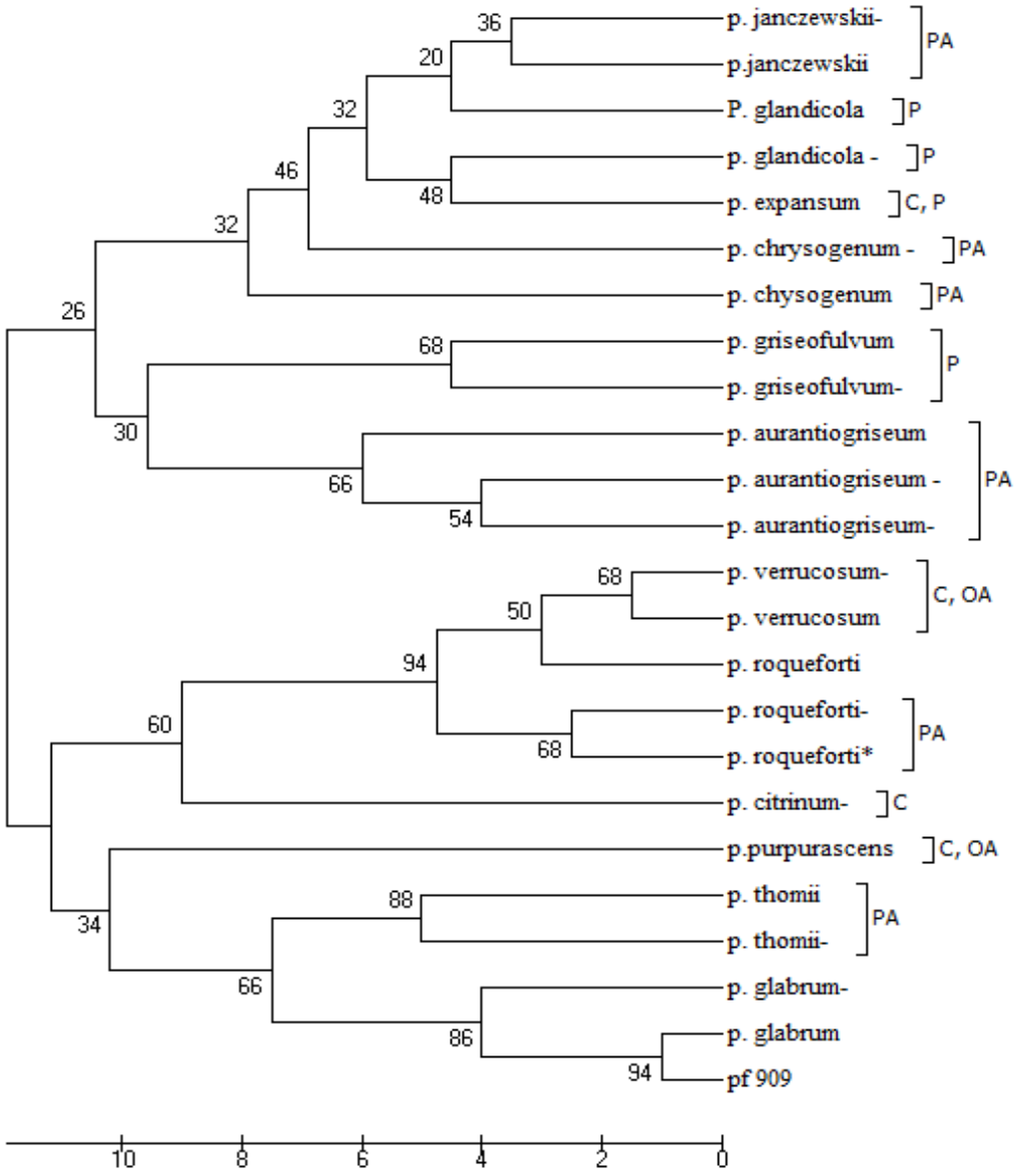

Figure 1. Molecular phylogenetic relationships analysis by Maximum Likelihood method based on the Tamura-Nei model, among the main toxin-producing species of *Penicillium* spp. on fruit based on an heuristic search of aligned RPB2 sequence, with *P. glabrum* used as an outgroup. Species producing the toxins of interest have the toxin name in brackets nearby (OA = ochratoxin A, PA = penicillic acid, C = citrinin, P = patulin).

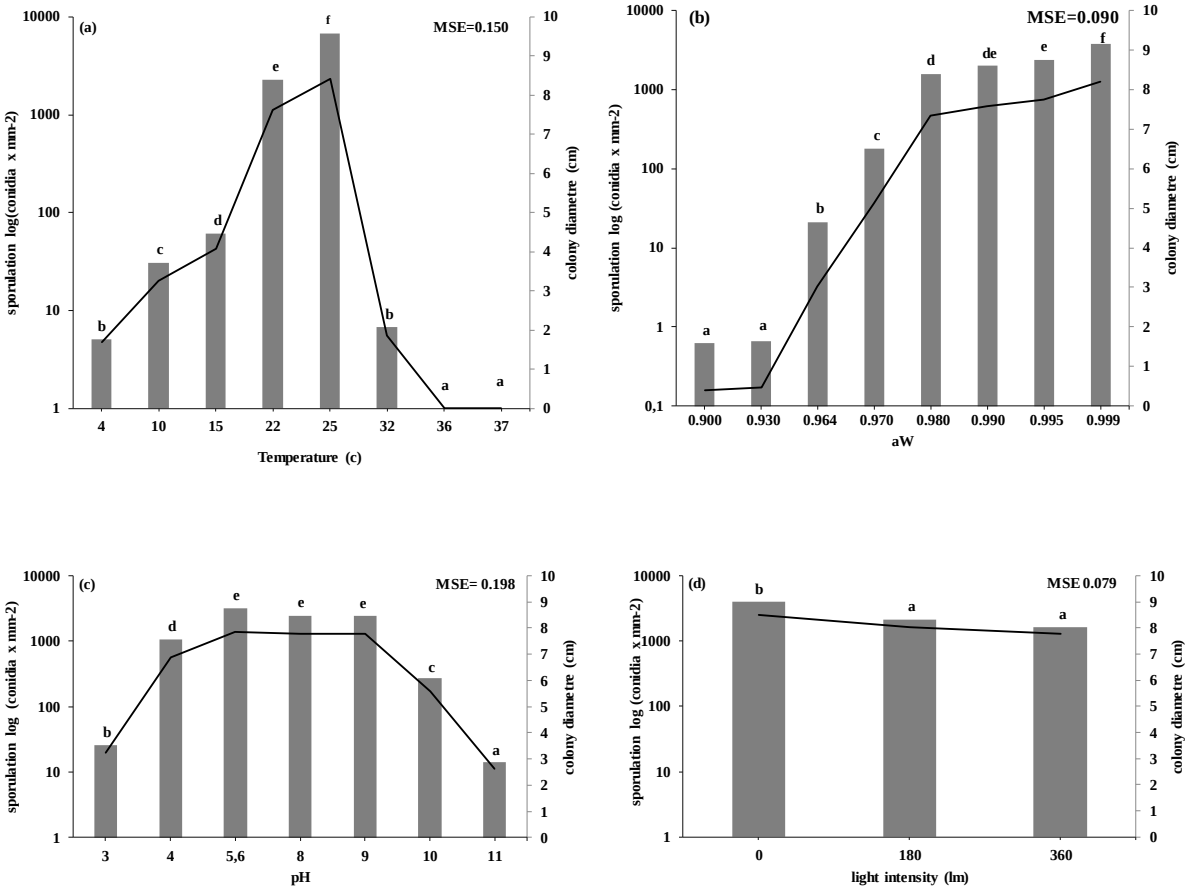

Figure 2. The effect of different incubation (a) temperatures, (b) water activity (a_W), (c) pH, and (d) Light intensities (LI) on the growth of Pf909. Values of colony diameter associated with different letters are significantly different from each other according to Student-Newman-Keuls multiple range test ($p < 0.05$). MSE = mean square error. Colony sporulation (__)

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Genome and transcriptome analyses of the mycoparasite *Clonostachys rosea* highlights mycotoxin tolerance as a key biocontrol trait

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Abstract: The mycoparasitic fungus *Clonostachys rosea* is an efficient biological control agent under field conditions for a variety of plant diseases on agricultural crops. The genome of *C. rosea* strain IK726 was determined using Illumina/SOLiD technology, and shown to contain high numbers of ABC-transporters (86 genes) and MFS-transporters (620 genes). Interestingly, the increase of ABC-transporter gene number in *C. rosea* was associated with phylogenetic subgroup G (pleiotropic drug resistance transporters) and subgroup B (multidrug resistance transporters), possibly involved in protecting *C. rosea* against exogenous toxins. Transcriptomes from *C. rosea* interacting with *Botrytis cinerea* and *Fusarium graminearum* showed that 61% of all induced genes were predicted to encode ABC- and MFS-transporters. Four and seventeen transporter genes were specifically induced during interaction with *B. cinerea* and *F. graminearum*, respectively. In summary, our data suggest that mycotoxin tolerance is an important component of the biocontrol ability of *C. rosea*.

Key words: biological control, membrane transporter, mycoparasitism

Introduction

Certain species of opportunistic, mycoparasitic fungi from the genera *Clonostachys* spp. and *Trichoderma* spp. are currently used as biological control agents (BCAs) to control plant pathogenic fungi. *Clonostachys rosea* is an ascomycete fungus that is reported to control diseases caused by a wide range of plant pathogenic fungi, including *Botrytis cinerea* (Sutton *et al*., 1997), *Fusarium culmorum* (Jensen *et al*., 2000) and *F. graminearum* (Xue *et al*., 2009). Several biocontrol mechanisms are reported in *C. rosea*, including direct parasitism of pathogenic fungi, antibiosis, production of fungal cell wall degrading enzymes, induction of plant defence and plant growth promotion (Jensen *et al*., 2007). We recently determine the genome sequence of *C. rosea* strain IK726 (Karlsson *et al*., 2015). A better understanding of the mechanisms that determine the outcome of biocontrol interactions is necessary for science-based improvements of biocontrol applications in agriculture.

The aim with the current work is to identify *C. rosea* genes that are specifically induced during interaction with *B. cinerea* or *F. graminearum*, and genes induced in response to both pathogens, using an RNA-Seq transcriptome approach. We hypothesize that the transcriptomic response in *C. rosea* towards the two pathogens differs due to their intrinsic differences, such as cell wall composition and secondary metabolite spectra.

Material and methods

Isolate and culture conditions
The *C. rosea* isolate IK726 was used for genome and transcriptome sequencing. Confrontation assays between *C. rosea* and *B. cinerea* strain B05.10, *F. graminearum* strain PH-1, and *C. rosea* itself (control) were performed on solid Vogel's minimal medium with 1% (w/v) sucrose (Vogel, 1956) and RNA was extracted from the interaction zone 24 hours after hyphal contact, using the RNeasy Plant Mini Kit (Qiagen, Germany).

Comparative analysis of ABC-transporter genes
The program CAFE v. 3 (Han *et al*., 2013) was used to compare the predicted ABC-transporter gene content in *C. rosea* with *Trichoderma atroviride, T. hamatum, T. longibrachiatum, T. reesei* and *T. virens*, and to identify lineages with accelerated rates of gene gain or loss.

Transcriptome and gene expression analysis
RNA libraries were sequenced with Illumina HiSeq. After quality filtration, reads were aligned to the *C. rosea* genome sequence using Tophat. Subsequent transcript assembly and differential gene expression analysis were performed using the Cufflinks package. Reverse transcription quantitative PCR (RT-qPCR) was carried out as described previously (Kosawang *et al*., 2014).

Results and discussion

Analysis of ABC-transporters in mycoparasitic C. rosea and Trichoderma spp.
Previously, the *C. rosea* genome was shown to contain high numbers of ABC-transporter genes (90) and MFS-transporter genes (620) (Karlsson *et al*., 2015). Table 1 show that the increase of ABC-transporter gene number in *C. rosea* is specifically associated with phylogenetic subgroup G (pleiotropic drug resistance transporters, PDR) and subgroup B (multidrug resistance transporters, MDR), predicted to be involved in cell protection.

Table 1. ABC-transporter subgroup gene numbers in *C. rosea* and *Trichoderma* spp.

Species	ABC-transporter subgroups[1]									
	A	B	C	D	E	F	G	I	H	Total
C. rosea	2	27	18	2	1	5	33	1	1	90
T. atroviride	2	13	13	2	1	5	10	1	1	48
T. hamatum	2	12	16	2	0	2	11	1	1	47
T. longibrachiatum	2	13	12	2	0	4	11	1	1	46
T. reesei	2	12	13	2	1	5	9	1	1	46
T. virens	2	15	23	2	1	5	12	1	1	62

[1] ABC-transporter subgroup classification according to Kovalchuk & Driessen (2010).

Transcriptomic analysis of C. rosea during fungal interactions

The transcriptomic analysis of hyphal interaction between *C. rosea* and *B. cinerea* or *F. graminearum* identified 41 differentially expressed genes. Functional annotation revealed that 61% of all induced genes were predicted to encode membrane ABC- and MFS-transporters, while 12% encoded proteins involved in biosynthesis of secondary metabolites and 7% encoded carbohydrate-active enzymes (Figure 1).

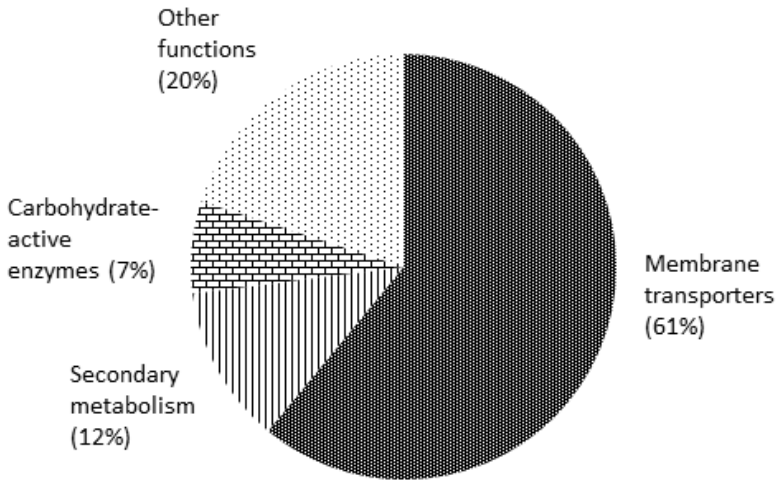

Figure 1. Functional annotation of differentially expressed genes in *C. rosea* during interaction with *B. cinerea* and *F. graminearum*.

Four membrane transporter genes were induced in interactions with both *B. cinerea* and *F. graminearum*. However, most membrane transporter genes were specifically induced towards either *B. cinerea* (4 genes) or *F. graminearum* (17 genes). This specific expression pattern was confirmed for 8 selected genes using RT-qPCR. Most differentially expressed membrane transporter genes encoded MFS-transporters, despite the fact that they require a proton gradient and are therefore considered to be induced more slowly than ABC-transporters. This suggests extensive transcriptional changes in *C. rosea* during the first 24 hours after hyphal contact and even before contact. One subgroup G (PDR) ABC-transporter gene (*abcG18*) was specifically induced during interaction with *F. graminearum*, while one subgroup C (MDR) ABC-transporter gene (*abcC8*) was induced specifically during interaction with *B. cinerea*. Previously, the *C. rosea* PDR transporter genes *abcG5* and *abcG29* were shown to be induced 170- and 16000-fold after 2 h exposure to the *Fusarium* mycotoxin zearalenone (Kosawang *et al*., 2014). Gene deletion mutants for *abcG5* or *abcG29* were not able to protect barley seedlings against fusarium foot and root rot disease in climate chamber experiments (Dubey *et al*., 2014; Dubey *et al*., 2016). In conclusion, our data suggest that mycotoxin tolerance governed by membrane transporter pumps is an important component of the biocontrol ability of *C. rosea*.

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Transcriptomic responses of the biocontrol yeast *Pichia anomala* to aflatoxigenic *Aspergillus flavus*

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Abstract: *Pichia anomala* (*Wickerhamomyces anomalus*) WRL-076 is a biocontrol yeast which has been shown to inhibit growth and aflatoxin production of *Aspergillus flavus*. The molecular mechanism of biological control was further characterized by the temporal transcriptome response of *P. anomala* to *A. flavus* in a liquid growth medium. Total RNA was extracted and processed using an Illumina TruSeq RNA Sample Prep kit. RNA-seq reads were mapped to the *W. anomalus* genome using tophat2 with default settings. Differential expression analysis was performed using edgeR. Gene ontology (GO) annotation of *P. anomala* was retrieved from http://genome.jgi.doe.gov/. Enrichment of GO categories in differentially regulated genes was determined using Fisher's exact test in the R environment. In comparison of yeast gene expression with and without *A. flavus*, a large number of genes were differentially expressed. At 24 h, 662 genes and 679 genes out of a total of 6423 genes were up- and down-regulated respectively, specifically genes involved in protein phosphorylation, protein kinase, DNA-templated regulation of transcription, and microtubule-based movement. They were enriched in the down-regulated genes at 24 h, but in up-regulation at later time points. This suggests that *P. anomala* was recuperating from the competition of *A. flavus*. Transport was enriched in up-regulated genes at 48 h, which implies that *P. anomala* was actively utilizing nutrients from the environment to build its biocontrol activities.

Key words: transcriptome, mycotoxins, biocontrol, aflatoxin, *Wickerhamomyces anomalus*

Introduction

Mycotoxins are naturally occurring toxins produced by filamentous fungi that affect many agricultural crops. Over 300 mycotoxins have been identified, of which about 20 have been shown to occur naturally in food at sufficient levels posing food safety concerns (Bennett & Klich, 2003). The most commonly occurring mycotoxins are aflatoxins (B_1, B_2, G_1, G_2, and M_1), ochratoxin A (OTA), patulin, citrinin and fumonisins (B_1, B_2 and B_3). Among them, aflatoxin B_1 (AFB_1) poses greatest threat to human health. The majority of these toxins are produced by fungi in the genera *Aspergillus*, *Penicillium* and *Fusarium*. Mycotoxin content in food is monitored and regulated in many countries around the world. Undoubtedly mycotoxins are a unique challenge to food safety worldwide. Consequently, the development of biological control methods based on ecological parameters is an environmental compatible approach to reduce mycotoxins in food chain.

Microorganisms naturally present in agricultural ecosystems have been studied as alternatives to traditional chemical methods for controlling plant diseases and fungi associated with mycotoxin production. Yeast species are promising biocontrol agents because they do

not produce allergenic spores and they are usually non-pathogenic. Yeasts are easy to cultivate, fast growing and are present in a variety of environmental niches (Droby *et al*., 2009). Two hundred strains of yeasts were initially screened for their ability to prevent the growth of *A. flavus* by a visual bioassay (Hua *et al*., 1999). One isolate in particular, *Pichia anomala* WRL-076 is greatly effective in inhibiting both the growth and aflatoxin production of *A. flavus*.

RNA-seq is an important approach to interpreting the functional elements of the genome. We applied this technology to study the interactions of *P. anomala* and *A. flavus* for identifying the genes play important roles in biocontrol mechanism. The information is essential for developing appropriate formulation and methods of application.

Material and methods

Experimental design

Yeast strain, *Pichia anomala* WRL-076 and *Aspergillus flavus* strain CA14 were maintained on potato dextrose agar (PDA). Suspensions of yeast or fungal spores were prepared in 0.05% Tween 80 solution and counted using a hemocytometer. The molecular mechanism of biological control was further characterized by the temporal transcriptome response of *P. anomala* to *A. flavus* in a liquid growth medium at ratio of 1:1 during 72 h experimental period.

RNA-seq analysis

Total RNA was extracted and processed using an Illumina TruSeq RNA Sample Prep kit. RNA-seq reads were mapped to the *W. anomalus* genome using tophat2 with default settings. Differential expression analysis was performed using edgeR. Gene ontology (GO) annotation of *P. anomala* was retrieved from http://genome.jgi.doe.gov/. Enrichment of GO categories in differentially regulated genes was determined by using Fisher's exact test.

Results and discussion

Bioactive volatile compound produced by P. anomala

The major volatile from *P. anomala* WRL-076 was identified by SPME-GC/MS analysis to be 2-phenylethanol. The major volatile compound produced by *P. anomala* WRL-076 is 2-phenylethanol (Hua *et al*., 2014)). It inhibited spore germination and aflatoxin (AF) production of *A. flavus.* We found that 2-PE also altered the expression patterns of chromatin modifying genes *MYST1*, *MYST2*, *MYST3*, *gcn5*, *hdaA* and *rpdA*. We further characterized the temporal transcriptome response of *A. flavus* to 2-PE at a subinhibitory level (1 µl/ml). A total of 131 of the 13,485 *A. flavus* genes were significantly impacted during the 72 h experimental period at False Discovery Rate < 0.05. Eighty-two of these genes exhibited decreased expression including those that encode conidiation proteins. All genes in the aflatoxin gene cluster were significantly decreased during the first 48 h treatment. Gene Ontology (GO) analyses showed that GO terms related to metabolism of propionate and branched-chain amino acids were significantly enriched in the down-regulated gene group, while those associated with ribosome biogenesis, translation, and biosynthesis of α-amino acids were over-represented among the up-regulated genes. Encyclopedia of Genes and Genomes (KEGG) pathway analysis revealed that metabolic pathways negatively impacted among the down-regulated genes were those involved in valine, leucine and isoleucine degradation,

propanoate metabolism, and tryptophan metabolism. These degradation and metabolic pathways most likely are required for aflatoxin biosynthesis by providing building blocks and energy. A better understanding of the mode of action of 2-PE at low concentrations, a scenario likely to be encountered in field applications of the biocontrol yeast, is critical to the development of an effective biocontrol strategy (Chang *et al*., 2015).

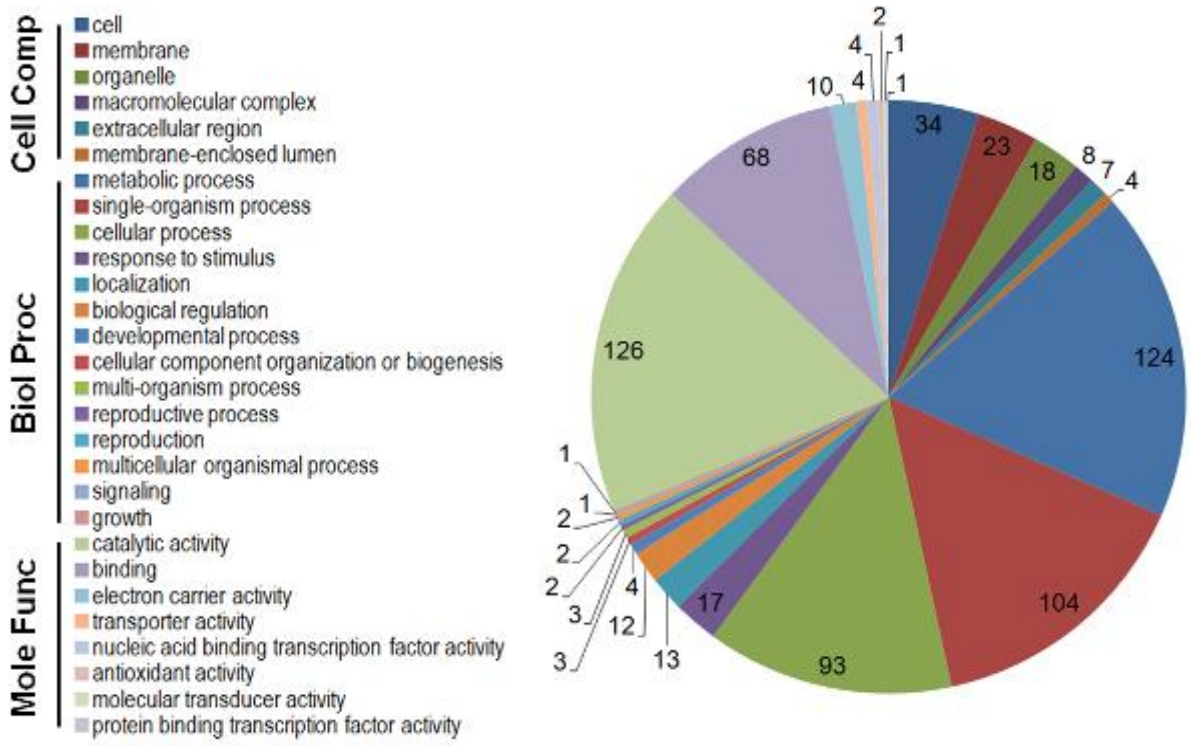

Figure 1. GO functional enrichment analyses of the differentially expressed genes (Chang *et al*., 2015)

Comparison of yeast gene expression with and without A. flavus

A large number of genes were differentially expressed. At 24 h, 662 genes and 679 genes out of a total of 6423 genes were up- and down-regulated respectively, specifically genes involved in protein phosphorylation, protein kinase, DNA-templated regulation of transcription, and microtubule-based movement. They were enriched in the down-regulated genes at 24 h, but in up-regulation at later time points. This suggests that *P. anomala* was recuperating from the competition of *A. flavus*. Transport was enriched in up-regulated genes at 48 h, which implied that *P. anomala* was actively utilizing nutrients from the environment to build its biocontrol activities. In conclusion**,** *A. flavus* has a significant influence on *P. anomala* gene expression. We applied GO annotations to identify major categories of genes that were involved in biological control processes. A group of genes in defense function, such as signal transduction were up-regulated. The lytic enzymes, exo-beta-1,3-glucanase 1 and 2 were slightly higher at 48 and 72 h. Further analysis is warranted for a better understanding of biocontrol efficacy.

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Effect of two mitoviruses (FcMV1 and FcMV2-2) on the virulence of *Fusarium circinatum* and laccase activity

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Abstract: Laccase enzymes (benzenediol: oxygen oxidoreductase, EC 1.10.3.2) play a major role in the degradation of phenolic compounds such as lignin. They are common in fungi and have been suggested to participate in host colonization by pathogenic fungi. Three putative mycoviruses have recently been isolated from the causal agent of pine pitch canker disease, *Fusarium circinatum* Nirenberg & O´Donell. In this study, the effects of single and double mycoviral infections (i.e. Fusarium circinatum mitovirus 1 and 2-2) on laccase activity, growth rate and pathogenicity were investigated in fourteen *F. circinatum* monosporic cultures. Extracellular laccase activity was analyzed by the Bavendamm test, image processing and a spectrophotometric method. Mycelial growth, *in vivo* pathogenicity and seedling survival probability were also determined in Monterrey pine (*Pinus radiata* D. Don) seedlings. The findings showed that (i) mycelial growth of isolates from the same fungal population was homogeneous, (ii) the presence of mycovirus appears to increase the virulence of fungal isolates, (iii) co-infection (with two mycoviruses) caused cryptic effects in fungal isolates, and (iv) laccases embody a possible auxiliary tool in fungal infection.

Key words: biocontrol, image analysis, pine pitch canker disease

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Effects of arbuscular mycorrhizal fungi (AMF) on the growth and health status of tomato plants (*Lycopersicon esculentum* Miller)

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Key words: arbuscular mycorrhizal fungi (AMF), tomato, disease index

Introduction

Mycorrhizal fungi (MF) are soil microorganisms that have establish mutual symbiosis with the majority of higher plants roots. It has widely been accepted that MF have increasing effect on their host plants growth through nutrients. MF can stimulate plant growth especially in soils with low nutrient uptake. MF also have been reported to protect plant roots from some root infecting.The aim of the work was to assess the effect of selected arbuscular mycorrhizal fungi (AMF) on the growth and health status of tomato plants cultivated in a plastic tunnel.

Material and methods

The experiment was carried out during 2015 in the horticultural farm situated in Grądy (N51°20', E22°49'), Lublin province, in eastern Poland. Tomato plants ('Esmira F_1', 'Antalya F_1' 'Pelikan F_1'cvs) were the objects of research. The seedlings were planted in the plastic tunnel in the first decade of May at spacing of 0.80 m x 0.50 m. This one-factorial experiment was set up in 5 replications (25 plants were grown in each plot). Two species of arbuscular mycorrhizal fungi: *Glomus etunicatum* W. N. Becker & Gerd (GE) and *Glomus intraradices* N. C. Schenck & G. S. Sm. (GI) were used for the inoculation of each cultivar seedlings. AMF spores were provided by the Institute of Soil Science and Plant Cultivation in Puławy, Poland. Before planting the tomato seedlings, all plants were inoculated with GE and GI. AMF was introduced to a depth of about 5 cm in the rhizosphere zone of tested plants. Each inoculum contained about 25-30 spores per plant. The control treatment was plants without inoculum of AMF. Plants were grown in a tunnel in a grow cycle (6 clusters) until the beginning of October, using a drip irrigation system.

Fruit yield

The harvest took place successively, every 7-10 days, at the phase of technological maturity of the fruits. The yielding parameters were calculated for 2 plants from each combination on the basis of 5 replications (10 plants for each combination). The yield was calculated in kg/m^2. The total yield (T) included marketable fruits (M), diseased fruits (DS) and undergrown (U) fruits (with the weight of less than 30 g).

Fresh and dry weight of roots
At the stage of full ripening of fruits (88 BBCH) the fresh and dry weight of roots was measured. Ten plants from each combination were carefully harvested. Whole roots were washed under running water to remove soil particles and theirs fresh weight (kg) was measured. Cleaned roots were dried at room temperature (25-27 °C) for 3 weeks and dry weight was measured (kg).

Disease index (DI)
The disease index (DI) of tomato plants was determined at two stages: leaf development (19 BBCH) and full ripening of fruits (89 BBCH). Ten plants from each combination were assessed. Disease index was estimated visually by assessing yellow and necrotic spots on the leaves using a rating scale of 0-5: 0° – no disease symptoms, 1° – small yellow spots on the leaves up to 25% of the leaf area, 2° – yellowing leaves and small necrotic spots on the leaves up to 50% of the leaf area, 3° – necrotic spots on the leaves on from 50% to 75% of the leaf area, 4° – extensive necrotic spots on the leaves above 75% of the area, wilting, dying plants. The values obtained in degrees were converted into the Disease Index (DI) according to Agamy *et al*. (2013).

Mycological analysis of plants
The mycological analyses were conducted at the full ripening of fruits (19 BBCH). Five plants were taken for a laboratory analysis at random from each experimental combination. Stems, leaves and roots of tomato were analyzed according to the method described by Jamiołkowska (2007). For each experimental treatment 20 dishes with the plant material, 10 plant fragments per dish, were prepared and incubated at 20-22 °C for 7 days in dark. The obtained colonies of fungi were transferred to potato-dextrose medium (PDA-Difco) and species were identified using the available monographs.

Results and discussion

The highest total (T) and marketable yield (M) was obtained from tomato plants of cv. ‘Antalya’ inoculated with AMF (T: 13.2-13.4 kg, M: 12.7-12.9 kg) and from cv. ‘Esmira’ in control combination (T: 14.6 kg; M: 12.4 kg). The lowest yield was achieved from cvs ‘Pelikan’ and ‘Esmira’ inoculated with GE (respectively T: 10.6-11.1 kg, M: 9.3-10.5 kg). Analysis of results obtained in present study showed no significant influence of AMF inoculation on the total and marketable yield of tomato fruits. The lowest weight of fruits with diseases symptoms (DS) was obtained from ‘Antalya’ (GE 0.07 kg; GI 0.15 kg) and ‘Esmira’ (GI 0.75 kg; GE 0.37 kg) inoculated with AMF. The highest weight of fruits with diseases symptoms were from control combination of ‘Antalya’ (0.88 kg) and ‘Esmira’ (1.0 kg). Application of AMF had a positively impact on the decreasing of weight of fruits with diseases symptoms.

The present study showed the influence of AMF inoculation on the fresh weight of tomato roots. The fresh weight of roots inoculated with AMF in cvs ‘Antalya’ and ‘Esmira’ were significantly highest (‘Antalya’: 128.5 g (GE) - 102.8 g (GI), ‘Esmira’: 56.6 g (GE) - 53.8 g (GI)) compared to control combinations (‘Antalya’ 84.8 g, ‘Esmira’ 50.0 g) and compared to cv. ‘Pelican’ inoculated with AMF and in control (55.8 g and 66.8 g, respectively). Dry weight of roots from cv. ‘Antalya’ inoculated with GE was significant higher (30.8 g) compared to other cultivars and other experimental combinations. There was no significant difference in dry weight of roots between ‘Esmira’ and ‘Pelikan’. Positive

effect of AMF inoculation on the dry weigh of roots was obtained only for ‘Antalya’ inoculated with AMF. For other tested cultivars there was no influence. Present study showed the influence of AMF inoculation on the fresh weight of tomato roots.

DI of tomato plants varied depending on the stage of plant growth, cultivar and AMF inoculum. The lowest DI was recorded for the plants in the stage of development of shoots (8.83-29.3%) and the highest in full ripening of fruits (12.44-53.02%). DI lowest values were recorded for plants inoculated with AMF (‘Antalya’ GI 11.1-22.95%, GE 12.32-12.44%), and the highest in the control combinations (‘Pelican’ 16.35-53.02%). Application of AMF decreased the value of DI of plants in the growing cycle. Mycorrhiza had a positive influence on health status of tomato plants cultivated in plastic tunnel.

Biodiversity of fungi colonizing tomato plants

Mycological analysis of tomato leaves and stems showed the most important fungi colonizing these parts of tomato were *Alternaria* spp., *Cladosporium* spp. and *Fusarium* spp. *Alternaria* spp. were dominant of tomato leaves. The number of isolates of this fungus was different and depended on an experimental combination (from 31 to 166 colonies). Least colonies of *Alternaria* spp. were obtained from plants with mycorrhiza (mainly ‘Pelikan’ cv. GI – 31 colonies, GE – 33 colonies), as compared to the control. *Fusarium* spp. occurred in greater numbers on tomato stems (from 9 to 111 isolates). The frequency of *Fusarium* spp. was higher for cultivars from control combination and smaller for all cultivars with mycorrhiza. Exception was ‘Pelikan’ cv. inoculated with *G. etunicatum* (111 colonies). *Cladosporium* species colonized more numerous stems of tomatoes with mycorrhiza than stems of the controls (exception ‘Pelikan’ cv. inoculated with GE). Mycological analysis of tomato plants suggests that mycorrhiza reduces the numbers of *Alternaria* spp. and *Fusarium* spp. colonies on aboveground part of tomato, but does not reduce the number of colonies of *Cladosporium* spp .

After mycological analysis of tomato roots *Fusarium* spp. (mainly *F. oxysporum*), *Trichoderma* spp. and *Penicillium* spp. colonies were numerously obtained. Colonies of *Fusarium* spp. were isolated especially from ‘Esmira’ cv. and ‘Pelican’ cv. without mycorrhiza, but they were not numerous on tomato roots inoculated with GE. *Trichoderma* spp. occurred in large numbers of colonies on the roots with mycorrhiza, more numerous than in the control. Mycological analysis of the roots indicated that application of GI and GE reduces the number of fungi *Fusarium* spp. on tomato roots and increases the number of antagonistic fungi of *Trichoderma* spp.

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Management of chickpea wilt (*Fusarium oxysporum* f. sp. *ciceris*) using *Trichoderma* spp.

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Abstract: Thirty-eight *Trichoderma* isolates were collected from fields grown with chickpea in six districts of Northwestern Ethiopia during two seasons, and purified. Some isolates were identified to be *Trichoderma harzianum*, *T. koningii* and *T. pseudokoningii*. The effect of *Trichoderma* isolates on *in vitro* colony growth of *Fusarium oxysporum* f. sp. *ciceris* and the effect of seed treatment with *Trichoderma* isolates against chickpea wilt in glasshouse were studied at Amhara Region Agricultural Institute. In glasshouse experiment the treatments were arranged in randomized complete block design in three replications. Data concerning *in vitro* colony diameter, seedling emergence, wilt incidence, fresh and dry shoot weight were collected and ANOVA were realized using the SAS system for windows V8. *In vitro* tests revealed that *Trichoderma* isolates showed differences in their colony growth and antagonistic potential. Sixteen isolates showed competition potential, seventeen mycoparasitic and five lysis effects on *F. oxysporum* f. sp. *ciceris*. In glasshouse experiment, out of five *Trichoderma* isolates, two had shown mycroparasitic effect, two competition effect and one lysis effect. These isolates were tested as seed treatment on two chickpea varieties namely *Adet* local and Shasho against *Fusarium* wilt as compared with untreated control. Significant differences were observed among the treatments in reducing wilt incidence on *Adet* local and Shasho varieties. However, Shasho showed low levels of disease incidence compared to the *Adet* local. Lower incidence was recorded on *Adet* local with *Trichoderma* isolate Tr6 (mycoparasitic ability) and for Shasho with *Trichoderma* isolate Tr5 (competition ability), respectively. Significant differences were recorded in fresh and dry weight of shoots in the *Trichoderma* treated *Adet* local over the control. *Trichoderma* isolates improved the plant growth also. Highest fresh and dry shoot weight of Adet local was recorded with the isolate Tr3 (competition ability) followed by Tr6 (mycoparasitic). Significant differences were also recorded in fresh and dry shoot weight in the *Trichoderma* treated Shasho variety over the control. However the highest record was for Tr6 (mycoparasitic) followed by Tr7 (mycoparasitic). The result showed that *Trichoderma* isolates have the potential to reduce wilt incidence and delay disease onset. Our study revealed that biological control agents such as *Trichoderma* can be a useful component of integrated chickpea fusarium wilt management and further study in field conditions is now required.

Key words: Biological control, Chickpea wilt; *Fusarium oxysporum* f. sp. *ciceris; Trichoderma*; Northwestern Ethiopia

The antifungal activity of *Artemisia herba-alba* aqueous extract and essential oils against mycotoxigenic fungus

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Abstract: The aim of the study was to evaluate the antifungal activity of extracts of aqueous extracts and essential oils of *Artemisia herba-alba* against the mycotoxigenic fungus *Fusarium* spp. and *Alternaria* spp. The plant was selected on the basis of their reported ethnobotanical uses. Different concentration of aqueous extracts (20, 25 and 30%) and essential oil (0.15, 0.175, 0.200 and 0.250%) were screened *in vitro* for their antifungal activity against *Fusarium* spp. and *Alternaria* spp. For the antifungal evaluation we used direct concoct method. The inhibitory concentration (IC) and fungistatic concentration (FSC) of the aqueous extracts and essential oils were determined. 30% of the extracts inhibited growth of *Alternaria* and the 0.025% of essential oils presents a good antifungal activity. Aqueous extracts were more effective than essential oils against *Fusarium* spp. and *Alternaria* spp. The IC values of the extracts were determined ranging between 25 and 30% of aqueous extracts, and the FSC were determined at 0.025% of essential oil. The *Artemisia herba-alba* aqueous extracts can be considered as potential sources of antifungal compounds for treating diseases in plants because this extract is a source of antifungal components: tannins, alkaloids, flavonoids, saponins and steroids. We conclude from this that these extracts exhibit remarkable fungicidal properties that support their traditional use as antiseptics.

Key words: *Artemisia herba-alba*, aqueous extract, essential oils, *Fusarium*, *Alternaria*, antifungal activity, phytochemical test

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Effect of *Penicillium rubens* strain 212 proteins on controlling *Fusarium* wilt in tomato plants

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Abstract: *Penicillium rubens* strain 212 (PO212) (formerly *P. oxalicum*) is an effective biocontrol agent (BCA) in the control of a wide range of horticultural plant pathogens. Evidence of the role proteins play in disease reduction will be presented. We proposed to look for PO212 putative protein candidates that are involved in disease reduction, in order to improve the PO212 efficacy to reduce vascular wilt of tomato. We have demonstrated that extracts of submerged culture free-conidia PO212 were as effective as dried conidia in the control of *Fusarium oxysporum* f. sp. *lycopersici* (FOL) in tomato. There were no negative effects on host or pathogen. However, only treatments with viable and dried conidia improved plant growth. PO212 showed the highest mycelial growth and sporulation on medium with 1% xylane. In addition, commercial xylanases were as effective as dried PO212 conidia against Fusarium wilt of tomato.

Key words: enzyme activity, biocontrol agent, mode of action, *Fusarium oxysporum* f. sp. *lycopersici*

Introduction

We have previously developed a BCA, PO212, which is effective against vascular wilting tomato caused by FOL and different species of the genus *Verticillium* (Larena *et al*., 2003). It has been shown that living conidia of PO212 act as "elicitors" in *Arabidopsis thaliana*, activating the induction of genes related to plant defense at concentrations of 1 x 10^6 conidia/ml (Villarino *et al*., 2013). The induction of the natural defense mechanisms of plants to provide resistance against pathogens is an approach currently developed in plant protection. Although the mechanism of action of these elicitors has still not been fully defined, the application the aqueous extracts of cultures from some *Penicillium* spp. has been effective in reducing the severity of vascular diseases caused by pathogens such as *F. oxysporum* f. sp. *vasinfectum, Verticillium dahliae* and *F. oxysporum* f. sp. *melonis* in cotton and melon plants (Dong *et al*., 2003; Gotlieb *et al*., 2003), without being toxic to the pathogens. It can be considered that part of the elicitors responsible for the induction of resistance are released to the liquid medium and have a soluble nature. Therefore, we proposed to look for thermolabil and soluble substances involved in the mode of action of PO212 in order to improve its efficacy to reduce vascular wilt of tomato. We focused on fungal proteins with enzyme activity, such as cellulases, xylanases and pectinases, associated with diseases reduction caused by fungal pathogens in plants.

Material and methods

Extracts of submerged culture of PO212

Conidia and extracts of PO212 submerged culture were obtained from Morton medium. Flasks with Morton A medium were inoculated with a conidial suspension of PO212 (10^6 conidia/ml), and shaken for 24 h at 150 rpm in the dark at 20-25 °C. Then, the mycelium was transferred to Morton B medium and incubated in the same conditions during 7 and 14 days. After incubation, the cultures were filtered and centrifuged. The supernatant was called submerged culture extracts of 7 and 14 days (CE7d and CE14d, respectively). A third volume of each CE was autoclaved for 30 min at 121 °C to check the involvement of labile compounds in the mode of action of PO212.

Biocontrol efficacy assays with PO212

Tomato seeds (cv. San Pedro) were sown in trays (1200 cm^2) containing an autoclaved mixture of vermiculite (Termita, Asfaltex, S.A.) and peat (Gebr. BRILL substrate GmbH & Co. KG) (1:1, v:v), and maintained in a growth chamber at 22-28 °C with fluorescent light (100 $\mu E/m^2$ s, 16 h photoperiod) and 80-100% humidity for 3 weeks. Seedlings in seedbeds were watered 7 days before transplanting with different treatments: i) a conidial suspension of PO212 (10^6 conidia/g substrate); ii) CE7d; iii) CE14d; iv) a conidial suspension of C7d (10^6 conidia/g substrate); v) a conidial suspension of C14d (10^6 conidia/g substrate); vi) an autoclaved conidial suspension of PO212 (aPO212, 10^6 conidia/g substrate); vii) aCE7d (CE7d autoclaved); viii) aCE14d (CE14d autoclaved); ix) sterile distilled water; and x) Morton B medium. Seven days after treatments, tomato seedlings were transplanted from seedbeds into flasks containing sterile Hoagland solution as described by De Cal *et al*. (1997). FOL (10^4 conidia/ml) was added to the flasks just before transplanting. Treated and non-inoculated plants were used as control. The flasks were placed in a growth chamber for 35 days under the conditions described above. The experiment was carried out twice. Disease development caused by FOL in tomato plants and toxicity on tomato plants were evaluated each week up to 35 days after transplanting.

Enzyme activities of PO212

In order to determine the capacity of PO212 to metabolize different enzyme substrates, the PO212 growth and sporulation on culture medium (yeast nitrogen base medium) supplemented with 1% of different enzyme substrates were studied: i) Citrus Pectin (Sigma); ii) Xylan (Sigma); iii) Carboxymethyl cellulose (Sigma); and iv) Polygalacturonic acid (Sigma citrus fruit). Plates without enzyme substrates were used as control. Five plates were prepared for each substrate and incubated at 22 °C in the dark, until colonies in the control plates covered the plates. Every day, PO212 mycelial growth was measured, and sporulation rate was calculated at the end of 7 days of incubation. The assay was repeated twice.

Then toxicity to plants and efficacy against FOL in tomato plants were evaluated using commercial xylanases as described above. The roots were submerged in a solution of Xylanases (*Thermomyces lanuginosus*, Sigma, E.C.N. 253.439.7) at a concentration of 50 and 100 U/ml for 30 min. Seedlings treated with sterile distilled water, a suspension of PO212 dried conidia (10^7 conidia/ml) or CE14d were used as control. Seven days after treatments, seedlings were transplanted and inoculated with FOL as described above.

Results and discussion

Biocontrol efficacy assays with PO212

There was a significant reduction in disease development (AUDPC) caused by FOL only after treatments with PO212 dried conidia (PO212), and PO212 submerged culture extracts of 7 and 14 days (CE7d and CE14d, respectively) (Figure 1).

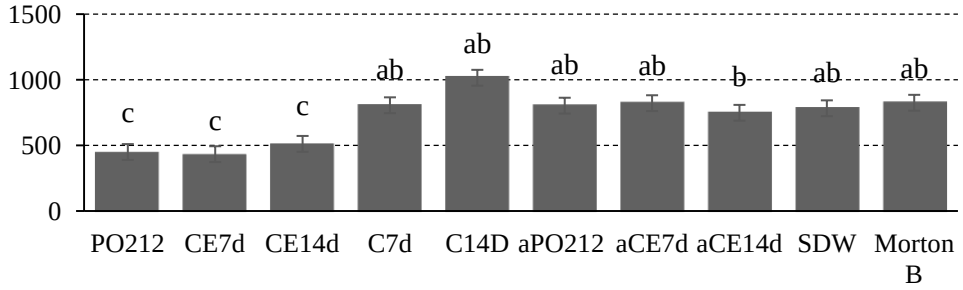

Figure 1. Effect of conidia and extracts of PO212 submerged culture on disease development (AUDPC) caused by FOL on tomato plants in controlled environment at 35 days after transplanting. Data are the mean of five flasks with four plants per flask. Means followed by the same letter are not significantly different ($P \leq 0.05$) according to Student-Newman-Keuls multiple range tests.

Only plants treated with dried conidia of PO212 showed a significant increase ($P \leq 0.05$) in the stem and root weight and the number of leaves per plant at the end of the assay (Table 1). None of the treatments had toxic effects on plants compared to the untreated and non-inoculated plants (data not showed). None of the treatments significantly reduced the growth and sporulation of pathogen in petri dishes on culture medium (data not shown).

PO212 had the highest mycelial growth and sporulation when it was grown on medium containing 1% xylan as enzyme substrates (data no shown). Treatment with 50 and 100 U/ml xylanase significantly reduced ($P \leq 0.05$) AUDPC compared to untreated control and PO212 (Table 2). However, only plants treated with PO212 showed an significant increase ($P \leq 0.05$) on stem weight (Table 2).

To conclude, we showed that PO212 produce one or more soluble and labile substances in submerged cultures, which seem to play an important role in disease reduction caused by FOL in tomato plants although plant growth promotion was not observed. Xylanases reduced disease caused by FOL in the same degree as PO212.

Table 1. Effect of conidia and extracts of PO212 submerged culture on number of leaves, and stem and root weight on tomato plants in growth chambers at 35 days after transplanting. Data are the mean of five flasks with four plants per flask. Means followed by the same letter in each column are not significantly different ($P \leq 0.05$) according to Student-Newman-Keuls multiple range tests. SDW: Sterile distilled water; MSE, mean squared error of analysis of variance

Treatment	Stem Weight (g)	Root weight (g)	Nº leaves/plant
PO212	6.4 a	4.9 a	7.3 a
CE7d	1.9 bc	3.7 b	5.1 bc
CE14d	2.4 b	2.5 bc	5.8 b
C7d	1.6 bc	2.1 c	3.5 c
C14D	0.4 c	2.5 bc	3.5 c
aPO212	1.6 bc	3.1 bc	5.2 bc
aCE7d	1.5 bc	2.6 bc	4.0 bc
aCE14d	2.6 b	2.9 bc	4.6 bc
SDW	2.3 b	3.0 bc	4.9 bc
Morton B	1.6 bc	3.3bc	4.4 bc
MSE	18.59	2.86	6.20

Table 2. Effect of xylanase at 50 and 100 U/ml on development disease (AUDPC) caused by FOL on tomato plants and on stem and root weight per plant in growth chambers at 35 days after transplanting. Data are the mean of four flasks with three plants per flask. Means followed by the same letter in each column are not significantly different ($P \leq 0.05$) according to Student-Newman-Keuls multiple range tests.

Treatment	AUDPC	Stem weight (g)	Root weight (g)
PO212	218.8 c	2.6 a	1.8 a
Xylanase 50U/ml	149.5 d	1.5 b	1.3 ab
Xylanase 100U/ml	165.6 cd	1.4 b	1.0 b
CE14d	285.4 b	1.6 b	1.3 ab
SDW	390.6 a	1.6 b	1.2 ab
MSE	38.908	1.60	0.57

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Investigating the biocontrol potential of dark septate endophytes against plant fungal pathogens

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Abstract: Soil-borne fungal pathogens are crucial factors causing substantial losses to agricultural and horticultural products worldwide. Controlling the diseases caused these fungi is a very challenging task due to their ability to survive many years in soils and their wide host range. There is a growing need for developing environmental friendly strategies to control soil-borne diseases. The use of fungal endophytes in plant protection showed to be a very promising approach to control soil-borne pathogens.

Dark septate endophytes (DSEs) are a group of widespread root-colonizing fungi. The impact of these endophytes on plant performance is not well understood. In our current study, we assessed the biocontrol potential of the two model DSEs *Periconia macrospinosa* and *Cadophora* sp. against the tomato pathogens *Rhizoctonia solani, Pythium aphanidermatum* and *Verticillium dahliae*. To investigate the interaction between DSEs and fungal pathogens we performed *in vitro* assays followed by a greenhouse experiment in which tomato plants, either inoculated with one of the DSEs or control, were challenged by one of the three pathogens. Plant growth parameters were measured to analyze the impact of DSEs on plant growth. Furthermore, the pathogens were quantified in the roots using qRT-PCR assays.

Both DSE species inhibited the growth of the three fungal pathogens on agar plates but not in the roots. The presence of *Rhizoctonia solani* and *Pythium aphanidermatum* in the roots was not reduced in the treatments where DSE inocula were applied. On the contrary, the root colonization by *Verticillium dahliae* was increased in *Cadophora* sp.-inoculated plants.

Key words: dark septate endophytes, fungal pathogens, biocontrol

Session 3:
From the lab to field scale –
Biocontrol in integrated plant disease management

Biological and integrated control of plant pathogens
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Safe crops, better health and higher income: Ground-truths from the development and scaling up of aflatoxin biocontrol in Africa

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Abstract: Aflatoxin contamination of food crops due to *Aspergillus flavus* is frequent and widespread harming health and trade in Africa. Competitive exclusion of aflatoxin-producing isolates by endemic atoxigenic vegetative compatibility groups (VCGs) of *A. flavus* is a proven biological control tool for aflatoxin management. We describe progress made with the development of biocontrol of aflatoxins in Africa, the current status, and prospects for further scaling-up in maize and groundnut value chains.

Key words: aflasafe, *Aspergillus*, atoxigenic, maize, groundnut

Introduction

Food quality and safety issues resulting from aflatoxin contamination are significant obstacles for improving nutrition and agricultural production while linking smallholder farmers to markets. Aflatoxin exposure is frequent and widespread in most African countries where the key staples maize and groundnuts are particularly vulnerable to aflatoxin contamination. Aflatoxin poses a significant public health risk in many tropical developing countries and is also a barrier to the growth of domestic, regional and international markets for food and feed. The most documented health impact of chronic exposure to aflatoxins is liver cancer while that of acute exposure is death. Broader health effects such as child stunting and immune suppression with higher rates of illness have also been associated with aflatoxin exposure. Aflatoxin contaminated feed decreases livestock productivity. Aflatoxin contamination has also led to the destruction of hundreds of thousands of tons of grains, leading to volatility in food prices as well as huge losses of much-needed income, food, and trade with health and food security consequences.

There is tremendous diversity within the aflatoxin-producing *Apsergillus* fungi that produce aflatoxins and not all strains produce toxins. Introducing endemic atoxigenic (non-toxic) vegetative compatibility groups (VCGs) of *Aspergillus flavus* into farmers' fields alters the composition of fungal communities and decrease both the frequencies of aflatoxin producers and the quantities of aflatoxins in crops. Competitive exclusion of aflatoxin-producing isolates by endemic atoxigenic VCGs is a proven non-toxic biological control technology that reduces aflatoxins during both crop development and post-harvest storage, and throughout the value chain in the United States. IITA, USDA-ARS and national partners have successfully adapted this competitive displacement technology for use on maize and groundnut in various African countries using native micro-flora, developing biocontrol products called Aflasafe. We provide an account of the Aflasafe development process, our current status of work and future directions for making the product available to millions of African farmers to make their crops safe.

Material and methods

Selection of VCGs to constitute biocontrol products started in 2003 with examining about 4.500 isolates of *A. flavus* obtained from crop samples collected from widely dispersed locations in Nigeria. Similar numbers of isolates were collected from another 12 African nations over the years. More than 50,000 isolates have been screened using various selection criteria related to safety, distribution, adaptation and efficacy. One type isolate each from the four most effective and widely distributed native atoxigenic VCGs were selected to constitute the final biocontrol product in a country. In collaboration with national institutions, the products were then tested in hundreds of farmers‘ fields for 2 to 5 seasons to gather efficacy data. A dossier containing efficacy, toxicological and ecotoxicological information was prepared for registering the biocontrol product with biopesticides regulators in each country. We are now developing regional products containing atoxigenic VCGs co-occurring in several countries in the region. A mix of technological, policy and institutional innovations were used to scale up and scale out aflasafe in various parts of sub-Saharan Africa.

Results and discussion

The aflatoxin biocontrol technology was adapted in Africa, starting in Nigeria where the safe and cost-effective biocontrol product aflasafe® consistently cuts aflatoxin contamination by 70-95% in maize and groundnut. Country-specific aflatoxin biocontrol products were then developed for Senegal, Kenya and Burkina Faso. The VCGs in the Senegal biocontrol product are also found in neighbouring The Gambia enabling the use of the product in both countries dependent on groundnut exports. The Kenyan product is highly effective (up to 98% aflatoxin reduction in farmers’ fields) in aflatoxin-prone areas of Kenya where people have died of aflatoxicoses.

The excellent efficacy of biocontrol in reducing aflatoxin in Nigeria, Senegal and Kenya has led to the expansion of the program to other African nations. As of June 2016, one Aflasafe product each are registered by national biopesticides regulators for use in Nigeria and Kenya, while registration of a third product by a regional registration agency has enabled its use in Senegal and The Gambia. A product is ready for registration in Burkina Faso while products are under testing in Ghana, Tanzania, Mozambique, Malawi and Zambia. Strain identification for product formulation continues in Uganda, Burundi and Rwanda.

The biocontrol strains carry over through the value chain, discouraging contamination in storage and transport even when conditions favor fungal growth. Positive influences of atoxigenic strain applications carry over between crops and provide multi-year benefits. A single application of atoxigenic strains may benefit not only the treated crop but also rotation crops and second season crops that miss a treatment. Additionally, because fungi can spread, as the safety of fungal communities within treated fields improves, so does the safety of fungal communities in areas neighboring treated fields.

To make the biocontrol product available to farmers and other end-users, a functional manufacturing plant (capacity 5 tons/hour; see Figure 1) was built in 2014 in IITA campus in Nigeria. A small-scale modular manufacturing plant is under construction in Kenya. A model for creating sustainable market demand for Aflasafe in maize value chain is being piloted under the AgResults Aflasafe Initiative in Nigeria where farmers have purchased aflasafe® to treat about 30,000 ha (application rate: 10 kg/ha; cost of the product: $18.75/ha). The product is cost-effective since Aflasafe users are able to sell their aflatoxin-reduced maize to quality conscious food and feed industries at 13-17% premium achieving 200 to 480% return on

investment on Aflasafe. The Kenyan government is providing the biocontrol product Aflasafe KE01® to smallholder farmers to treat almost 23,000 ha in aflatoxin-prone areas as a public good in public health interest and to improve the marketability of maize grains. A Senegalese agribusiness firm provided 40 tons of Aflasafe SN01® to its contract growers in 2016 to improve the safety and marketability of groundnuts procured from the farmers.

In order to encourage Aflasafe use and commercialization, licensing mechanisms for manufacturing/marketing/distribution are being put in place. The Bill & Melinda Gates Foundation and USAID have recently funded a technology transfer and commercialization initiative to scale-up use of Aflasafe in 500,000 ha in 11 African nations through private, public, or public-private partnerships.

Figure 1. The manufacturing plant of Aflasafe, a biocontrol product for aflatoxin mitigation, located at the IITA campus in Ibadan, Nigeria.

Smallholder farmers harvest, store and consume home-grown crops. The deployment of Aflasafe can profoundly improve the safety of food of smallholder farm families and reduce postharvest losses since the technology dramatically reduces the source of contamination in the field before harvest. Reduced crop contamination could also translate into improved food security and better access to domestic, regional and international markets that pay a premium for aflatoxin standard abiding maize and groundnuts. Scaling-up of biocontrol has also the potential to revitalize exports and to increase smallholder's opportunity to access premium export markets. For health and income improvements to happen, the biocontrol technology must be scaled up to reach the various players in the maize and groundnut value chains by developing proper product manufacturing and delivery mechanisms.

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Bacillus subtilis CH13: a highly effective biocontrol agent for the integrated management of plant diseases

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Abstract: *Bacillus subtilis* CH13 is a commercially important bacterium with proven plant growth promoting and biocontrol abilities. Here we report the results of the 2-year biocontrol trials with CH13 on different cultivars of cabbage and potato grown in fields naturally infected with phytopathogens. Application of *B. subtilis* CH13 at cell concentration of 10^8 CFU/ml throughout the growing season resulted in a significant reduction of disease incidence and a yield increase, compared to untreated control. Analysis of the CH13 genome sequence revealed that this strain is genetically closely related (99.98% identity) to *B. amyloliquefaciens* FZB42. Similarly to FZB42, CH13 has a remarkable genomic potential for synthesizing numerous biocontrol metabolites, including lipopeptide antibiotics, siderophores and volatiles. Overall, our data suggests that incorporating CH13 into conventional agricultural system provides an effective, environmentally sound approach for the control of phytopathogens and the improvement of agricultural productivity.

Key words: *Bacillus*, biocontrol, genome sequencing, field trials, yield increase

Introduction

The effectiveness of bacterial agents in the biocontrol of bacterial and fungal phytopathogens has been well documented in numerous studies. Moreover, the number of commercially available products based on such biocontrol strains is continuously increasing. The vast majority of biofungicides contains species of *Bacillus* (Borriss, 2015). Among them, the best-known commercial bacilli are those belonging to *Bacillus subtilis* and *Bacillus amyloliquefaciens*. These species are ubiquitous soil and plant-associated bacteria which are able to protect plants from pathogens through a variety of mechanisms. The well-established biocontrol mechanisms rely on the synthesis of volatiles, such as acetoin and 2,3-butanediol, and the secretion of a wide range of potent antibiotics, including lipopeptides and polyketides (Pérez-García *et al*., 2011; Borriss, 2015).

Bacillus subtilis strain CH13 (*Bacillus amyloliquefaciens*, see below) is a commercially important plant growth promoting and biocontrol agent in Russia. This strain was originally isolated from chernozem soil of Moldova Republic and identified based on physiological and biochemical analyses as well as its 16S rRNA gene sequencing. When applied as seed/plant treatment, CH13 can significantly enhance plant growth and yield, as well as protect plants against biotic and abiotic stresses. *In vitro* studies aimed at elucidating the potential plant-beneficial metabolites of CH13 indicate that this strain produces IAA and its derivatives, volatiles, hydrolytic enzymes and a mix of antibiotic compounds (Chebotar *et al*., 2009).

Previously, we showed that CH13 is an effective tool for wheat grain disinfection, especially against various *Fusarium* spp. It is likely that biocontrol of these phytopathogens by CH13 is based on the production of a wide range of lipopeptide antibiotics which are known to be highly effective against filamentous fungi. However, nothing is known about the underlying genetic determinants associated with the production of these metabolites. Moreover, it has never been tested whether CH13 exerts biocontrol activity also on other phytopathogens and plants, especially under realistic agricultural conditions. Therefore, the aim of this study was 1) to evaluate the ability of CH13 to control bacterial and fungal pathogens of potato and cabbage in large-scale field trials, and 2) to provide an insight into the genetic basis for biocontrol ability of CH13 based on the analysis of its draft genome sequence.

Material and methods

Biocontrol field trials

Biocontrol trials were conducted on cabbage and potato grown in research fields naturally infected with phytopathogens. These include: 1) In cabbage: *Rhizoctonia solani* + *Olpidium brassicae* + *Pythium debaryanum* (which together cause blackleg), *Alternaria brassicae* (gray leaf spot), and *Xanthomonas campestris pv. campestris* (black rot); 2) In potato: *Rhizoctonia solani* (stem canker and black scurf) and *Phytophthora infestans* (late blight). The severity of plant infection was estimated according to the methods recommended by All-Russia Research Institute for Plant Protection (Dolzhenko, 2009).

Details of the field trial programs are given in Table 1. For cabbage, the number of treatments with the CH13 suspension was 1+1+2 (one seed treatment + one transplant treatment + 2 foliar treatments) with an application rate of 2 ml/kg + 0.2% bacterial suspension + 2.0 l/ha. For potato, the number of treatments was 1+2 (one tuber treatment + two foliar treatments) with an application rate of 2.0 l/ton + 2.0 l/ha. The concentration of the bacterial suspension was adjusted to 10^8 CFU/ml. Control plants were left untreated.

Table 1. Field trial program.

Plant	Cultivar	Time and Location	Growth program	№ of treatments	Application rate
Cabbage	Parus	25.04.13-15.09.13 Moscow reg.	Herbicide, 1.6 l/ha; Insecticide, 0,1 l/ha; NPK fertilizer, 300 kg/ha	1+1+2	2.0+0.2%+2.0
	Kolobok	19.04.14-15.10.14 St.Petersburg reg.	NPK fertilizer, 85 kg/ha	1+1+2	2.0+0.2%+2.0
Potato	Rozara	28.04.13-30.08.13 Tambov reg.	Herbicide, 0.7 kg/ha; Insecticide, 0.1 l/ha	1+2	2+2
	Zhukovskiy	27.04.14-13.08.14 Tambov reg.	Insecticide, 0.1 l/ha	1+2	2+2

Genome sequencing and analysis of B. subtilis CH13

The genomic DNA isolated from strain CH13 was sequenced using Illumina technology. Genome assembly and annotation of CH13 were performed using A5 (Tritt *et al*., 2012) and Prokka (Seemann, 2014) software, respectively. One type "*plantarum*" strain *B. amyloliquefaciens* FZB42 and one type "*amyloliquefaciens*" strain DSM7 were used as reference genomes for comparative analyses. The average nucleotide identity between strains was calculated using JSpecies (Richter *et al*., 2009).

Results and discussion

Biocontrol trial results

All variants, in which CH13 was added showed significant biocontrol (Table 2). Application of CH13 allowed to reduce disease incidence by 2-5 times and to increase plant yield by 9-24%. This strain proved to be especially active against soil-borne pathogens, namely blackleg of cabbage (4.2-5.3 times reduction in disease symptoms), followed by *Rhizoctonia solani* (4.2-4.5 times reduction) and *Phytophthora infestans* (3.6-3.7 times reduction) in potato. For seed-borne *Alternaria brassicae* and *Xanthomonas campestris* these values were somewhat lower – 1.5-2.2 and 2.5 times, respectively. These results highlight the importance of early preventative measures against seed-borne pathogens, e.g. through both pre- and post-harvest treatment. Another approach, which is receiving increasing interest from the scientific community, could be seed biopriming with beneficial endophytic microorganisms.

In general, our past and present data suggests, that the beneficial action of CH13 is not limited to a single plant species or soil condition. Moreover, CH13 proved to be compatible with conventional agrochemicals including fungicides, insecticides and herbicides. These facts indicate that application of CH13 can be easily incorporated in existing farming programs, thereby providing an effective, environmentally sound approach for the control of phytopathogens and the improvement in agricultural productivity.

Genome analysis of CH13

Whole genome alignment of CH13 versus reference genomes indicated that the genome of CH13 was very similar to that of FZB42 with an average nucleotide identity of 99.98%. This suggests that strain CH13 should be assigned as *Bacillus amyloliquefaciens* rather than *B. subtilis.* Similarly to FZB42, we found six biosynthetic gene clusters directing the synthesis of non-ribosomal peptides (surfactin, fengycin, bacillomycin D, bacillibactin, bacilisyn and the putative product of the *nrs* cluster), three gene clusters involved in polyketide synthesis (difficidin, bacillaene, and macrolactin) and two gene clusters for the production of ribosomally synthesized small peptides. Additionally, gene clusters for the synthesis of the volatiles acetoin and 2,3-butanediol have been located in both strains. It is tempting to speculate that production of antibiotics, siderophores and volatiles in the rhizosphere of plants is a major factor contributing to the excellent biocontrol properties of CH13 and other closely related strains.

Table 2. Effectiveness of CH13 against several cabbage and potato pathogens in 2-year field trials.

Plant	**Pathogen**	**Disease incidence, %**		**Yield, ton/ha**	
Cabbage		Control	CH13	Control	CH13
cv. Parus'13	*Rhizoctonia solani* + *Olpidium brassicae* + *Pythium debaryanum*	11.8	2.2	56.8	67.5 (+18.8%)
	Alternaria brassicae	2.4	1.6		
	Xanthomonas campestris pv.campestris	9.4	3.7		
				LSD_{05} = 6.1 ton/ha	
cv. Kolobok'14	*Rhizoctonia solani* + *Olpidium brassicae* + *Pythium debaryanum*	12.1	3.0	44.4	48.0 (+ 9.3%)
	Alternaria brassicae	12.8	5.7		
	Xanthomonas campestris pv.campestris	13.8	5.6		
Potato				*LSD_{05} = 3.43 ton/ha*	
cv. Rozara'13	*Rhizoctonia solani*	3.6	0.8	26.8	33.4 (+24.6%)
	Phytophthora infestans	14.6	4.0		
				LSD_{05} = 1.76 ton/ha	
cv. Zhukovskij'14	*Rhizoctonia solani*	8.1	1.9	18.2	20.5 (+12.2%)
	Phytophthora infestans	7.0	1.8		
				LSD_{05} = 1.72 ton/ha	

Acknowledgements

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From bioassays to field trials: screening and selection of microbes for control of *Rhizoctonia* root rot on wheat

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Abstract: *Rhizoctonia* root rot is the major fungal root disease of cereals in low to medium rainfall areas with limited chemical and agronomic control options. Disease is influenced by soil microbes and this project successfully developed and used new screening methods to allow rapid initial evaluation of microorganisms in bioassays and in the field to identify strains which are candidates for development as commercial inoculants. From 2310 strains initially assessed in a high throughput plant-pathogen-soil tube bioassay, 43 strains (1.9%) reduced disease. Of these, 21 strains had > 20% survival on seeds over 7 days and were then assessed in hand planted microplots to provide initial evidence of efficacy in the field. The eleven strains which reduced disease were then assessed in 20 m field plots. From this screening methodology, one strain was identified that increased grain yield in *Rhizoctonia* infested fields between 3.8 to 4.2% and performed better than current chemical controls.

Key words: *Rhizoctonia solani*, wheat, screening, biocontrol

Introduction

Root rot caused by *Rhizoctonia solani* Kühn AG8 is the major fungal root disease in cereal cropping systems in low to medium rainfall areas in Southern Australia. *Rhizoctonia solani* is also a major problem in other dryland cereal cropping systems as well as in vegetable crops. *Rhizoctonia* is difficult to control with a wide host range, no genetic resistance and control with fungicides is limited.

Rhizoctonia root rot is known to be influenced by soil microorganisms, and examples of using microbial strains to reduce disease have been reported. There are numerous obstacles in the development of biocontrol agents for commercial use (Köhl *et al*., 2011), with two of the major constraints being the time and cost of *in planta* bioassays and field trials. However, Campbell's 1994 critique of biocontrol research stated that screening methods need to be based on controlling disease on plants and the need to screen thousands of strains that were adapted to the environment they were required to operate in.

Incorporating the ideas of Köhl *et al*. (2011) and Campbell (1994), we developed a methodology to rapidly screen large numbers of strains directly into a plant-pathogen-soil system to provide initial evidence of efficacy in reducing *Rhizoctonia* root rot on wheat. In addition a microplot system was developed to enable multiple strains to be assessed at low cost for evidence of efficacy in reducing disease in the field prior to selecting strains to be assessed in larger scale field plots.

Material and methods

Microbial strains were screened for control of *Rhizoctonia* root rot in a stepwise procedure. Strains were first assessed in the primary high throughput 2 week seedling tube bioassay. Strains that showed a positive effect were then assessed in a 4 week pot bioassay to confirm disease control efficacy and selected strains then assessed at different inoculum levels in a tertiary pot bioassay. Strains were then identified by 16S rRNA gene sequencing and their survival on seeds was quantified. Selected strains were then assessed for disease control in field microplots and then in 20 m field trial plots.

Source and culture of microbes

Rhizoctonia solani AG8 strain W19 is an aggressive strain isolated from wheat and added to bioassays as colonised millet seed. Isolates screened for *Rhizoctonia* control came from two sources, (1) a culture collection of spore forming endophytic actinobacteria (Kaewkla & Franco, 2013) and (2) newly isolated strains from wheat roots. New strains were isolated from wheat seedlings grown in cropping soils with an intractable *Rhizoctonia* problem. Strains were isolated from the rhizosheath or from well washed roots based on the method of Fall *et al*. (2004). Baker's yeast agar (BYA, Christensen & Cook, 1978) was used as the base isolation medium in combination with antibiotics to select for different microbial groups.

Tube and pot bioassays

The primary high throughput assay consisted of a 50 ml tube with field soil, added *R. solani* and two wheat seedlings grown for two weeks, two replicates per strain. Strains were added as a 150 µl suspension directly to the seeds at planting. New strains were taken directly from isolation plates, resuspended in 96 well plates and used to inoculate seeds. Culture collection strains were grown on mannitol soy agar, and added as for new strains. Plants were assessed by shoot growth and number of roots reaching the bottom of the tube.

The secondary and tertiary pot assays consisted of 300 g/pot of field soil, added *R. solani* and 5 wheat seedlings grown for 4 weeks with 4 replicates. Microbes were applied to seeds as suspensions before planting. Effective strains in the secondary assay were then applied at three rates in the tertiary assays. Plants were assessed for plant growth and root disease levels (0-5 scale). Strains were selected for further development based on better disease control than our current 'benchmark' *Rhizoctonia* control strains which were included in each assay. Soils with an intractable *Rhizoctonia* problem were used for all bioassays.

Candidate strains were then identified by 16S rRNA gene sequencing and survival as a seed coating in 0.3% (w/v) xanthan gum sticker solution was quantified over 7 days.

Field trials

Microplots and field trials were carried out on commercial cereal paddocks with moderate to high levels of indigenous *Rhizoctonia* inoculum (> 100 pg *R. solani* AG8 DNA/g soil). Microbes were applied to wheat seed in a 0.3% (w/v) xanthan gum sticker solution between 10^4-10^6 cfu/seed. All field experiments were set up in a split-plot randomised complete block design, with an untreated row or plot next to every microbe treated row or plot with 6 replicates. Registered chemical controls for *Rhizoctonia* were also included for comparison.

Microplots consisted of a 1 m row with seeds hand planted at 5 cm spacing, 2 cm depth using a planting template. A machine seeder was put through the trial prior to planting to cultivate, form rows and add fertiliser, except no seed planted. At 8-11 wks, 10 plants were dug up and assessed for *Rhizoctonia* disease on seminal and nodal roots (0-5 disease scale, 0 = healthy roots, 5 = highly diseased roots), and shoot and root dry weights.

Field trials consisted of 20 m x 6 row plots, with 3 rows planted with microbe-treated seeds and 3 rows with untreated seeds. Plots were machine planted. Plants (21) were assessed at 8-11 wks as for microplots and plots harvested at maturity for grain yield.

Results and discussion

The aim of this project was to identify microbial strains that would be suitable for development as commercial inoculants for control of *Rhizoctonia* root rot in dryland cereal cropping systems. The methodology developed was informed by the ideas of Campbell (1994) and Köhl *et al*. (2011) in which a large number of strains were screened directly into a wheat-*Rhizoctonia* pathosystem using disease conducive field soils.

In the primary assay, 2310 strains were assessed, 185 in the second assay and 43 in the tertiary pot bioassay. This equates to 1.9% of the strains tested showing a disease control response. The primary assay was designed to be rapid and low cost to set up and evaluate in order to maximise the number of strains assessed. There is a direct trade-off between quality and quantity in this system and we wanted to maximise quantity of strains assessed. Of the 43 strains assessed in the third bioassay, 19 were from the actinobacterial collection (predominantly *Streptomyces* spp.) and 24 newly isolated strains, included fungi, (*Trichoderma*, *Aspergillus* and *Cylindrocarpon*) and bacteria from 4 phyla encompassing the genera *Streptomyces*, *Actinomadura*, *Microbacterium* (Actinobacteria), *Bacillus*, *Brevibacterium*, *Paenibacillus* (Firmicutes), *Pseudomonas*, *Pandoraea*, *Phyllobacterium* (Proteobacteria) and *Chryseobacterium* (Bacteroidetes). This indicates there is a diversity of taxa able to reduce *Rhizoctonia* root rot. The most likely commercial application method of inoculants in the field is by seed coating. Strains with less than 20% survival over 7 days included the fungi, the Gram negative bacteria and microbacteria were rejected.

Field assessment of strains is costly and time consuming and measuring a response to microbial inoculation for *Rhizoctonia* control is difficult using traditional plot trials due to the patchy nature of the disease and high variation between replicate plots. To overcome this, all field assessments were undertaken in a split-plot design with treated and untreated seeds in the same disease space. Twenty one strains were assessed in microplots and 11 strains which reduced disease in microplots were assessed in 20m field trial plots.

To test the microplot system, 10 strains were assessed at two sites in 2012. The pooled results indicated that seed treatment significantly increased plant growth and reduced disease on roots (Table 1). When compared to two chemical seed treatments (Vibrance®, Syngenta and EverGol® Prime, Bayer), 12 of 13 strains in 2013 produced a greater reduction in root disease compared to the chemical seed treatments. This validates the *in planta* screening method as being successfully able to select strains that can reduce disease in the field.

Strains (11) that individually produced a significant reduction in disease or increase in plant growth in microplots were assessed in 20 m field trial plots to assess yield response. One *Paenibacillus* strain was identified that produced between a 3.8 to 4.2% grain yield increase over three sites compared to a 3% increase using a registered in-furrow chemical treatment (Uniform®, Syngenta), currently the best chemical control option for *Rhizoctonia* control. See Table 2 for abbreviated results from the 2014 trial. Not all strains that reduced root rot at 11 weeks resulted in a yield response e.g. *Bacillus* strain in Table 2.

Table 1. Pooled results (10 strains, 6 replicates at 2 sites) from the first microplot assessment (2012) at 8 wks comparing microbe treated and untreated plants. Plants (10) were assessed for dry weight (DW) and disease score (DS. 0-5 scale) on seminal and nodal roots. N = 120.

Variate	Microbe Treated	Un-treated	Fprob	% change from untreated
Shoot DW g	2.829	2.563	< 0.001	10
Root DW g	0.5537	0.5196	0.003	7
Seminal DS	0.828	1.224	< 0.001	-32
Nodal DS	1.092	1.508	< 0.001	-28

Table 2. Abbreviated field trial results (2014) for three microbial strains compared to chemical control (Uniform®) showing seminal root disease score at 11 weeks and grain yield between treated and untreated split-plots. % change indicates percent change with microbe treated seeds compared to untreated seeds. *indicates significant difference at $P = 0.05$, n = 6.

Strain	**Seminal Root Disease Score (0-5)**			**Grain Yield t/ha**		
	Microbe Treated	Un-treated	% change	Microbe Treated	Un-treated	% change
Paenibacillus	2.1*	2.5	-17	2.68*	2.57	4.2
Bacillus	2.3*	2.9	-20	2.49	2.49	0.0
Streptomyces	1.9*	2.8	-32	2.60	2.52	2.8
Uniform®	1.4*	2.2	-35	2.56	2.49	3.0

These results show that rapid *in-planta* screening can be used as a first step to identify prospective control agents, and combined with field microplots provide a relatively cheap assessment of field performance prior to assessment in costly field trials. The use of split-plot design field experiments to compare treated and untreated seeds in the same disease space provided a superior method for assessing strains in a heterogeneously distributed disease.

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Bumble bees & *Gliocladium*: Potential partners in the biocontrol of internal fruit rot in sweet pepper

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Abstract: Sweet pepper (*Capsicum annuum* L.) is an important crop worldwide. It is grown hydroponically in greenhouses in several countries, such as Belgium, the Netherlands, UK and Canada. Since the beginning of this decade, internal fruit rot has been causing yield losses of 10 to 40% per year in greenhouses in Canada, and more recently, in Europe. The disease is caused by several *Fusarium* species, mainly the *Fusarium lactis* species complex. The infection occurs via the flower, but the pathogen develops when the fruit is ripe and the symptoms are visible mostly during commercialization or at consumer. The disease negatively affects the crop reputation, leading to low-price formation. Neither chemical control nor biological control options are currently available. Sustainable control measures are being investigated in Belgium. Research at semi-commercial conditions at the experimental centers PSKW and PCH has shown that the beneficial fungus *Clonostachys rosea* f. *catenulata* strain J1446 (syn. *Gliocladium catenulatum*) can reduce incidence of internal fruit rot when the antagonist is directly applied to the flower. Furthermore, under semi-commercial conditions, promising control has been achieved using bumble bees (*Bombus terrestris*) as vectors of *Gliocladium* J1446. This system is particularly promising as it allows continuous delivery of the antagonist to the flowers and it substantially reduces the use of the antagonist compared to spraying. In the present study we investigated the joint introduction of these partners in commercial sweet pepper greenhouses with a history of internal fruit rot. In 2015, we introduced bumble bee hives specially developed to deliver antagonists (Flying doctors®) in commercial sweet pepper greenhouses in 3 locations. Prestop 4B, a commercial formulation of *G. catenulatum* J1446, was supplied twice a week to the hives. Hive activity, incidence of *Gliocladium* on flowers and disease incidence were monitored. The incidence of *Gliocladium* during the trials varied from 7 to 63%. *Gliocladium* incidence was higher in the greenhouse zones located close to the hives. Disease incidence was lower in the greenhouse zones with higher incidence of *Gliocladium*. Reductions of 30% (2 locations) and 60% (1 location) were found. Currently, we further investigate the effect of hive positioning within the greenhouse on *Gliocladium* distribution and disease incidence.

Key words: entomovectoring, *Clonostachys*, *Fusarium lactis*

Developing a new biocontrol strategy against brown rot in stone fruit in Europe

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Abstract: Brown rot caused by *Monilinia* spp. is one of the most economically important fungal diseases of stone fruit (peaches, nectarines, plums, apricots and cherries) in Europe, and the disease is responsible for substantial pre-harvest and postharvest losses. Each year, 4.2 million tons of stone fruits are being produced (727,700 ha). Italy, Spain, Greece and France are main producers. The economic losses may reach up to 80% of production depending on the cultivar (early or late season variety) and on weather conditions of the season. Currently, effective brown rot control depends on an integrated strategy based on orchard fungicide spray programs and cultural practices. Biocontrol agents (BCAs) commercially available are needed to respond to social awareness and increasingly strict legislation concerning the sustainable use of pesticides and the presence of residues on fruit.

Bacillus subtilis (CPA-8), reclassified as *B. amyloliquefaciens* (CPA-8) and *Penicillium frequentans 909* (Pf909) are common constituent species of the resident microbiota of peaches and both have previously demonstrated good efficacy to control brown rot in stone fruits. The objective of the present work was to study the possibilities of using these BCAs to control brown rot by preharvest treatments. The CPA-8 formulation was optimized and subsequently formulated BCAs were tested in commercial field trials.

Fluid-bed spray-drying system demonstrated to be the best method to formulate CPA-8 although reasonable results have also been obtained with freeze-drying. Carriers, protectants, binders and process conditions of the fluid-bed spray-drying system have been optimized for CPA-8. BCAs formulates were used alone or in combination, and were applied by calendar (four applications) or following the advice of a prediction model developed by IRTA and INIA. Both BCAs, applied by calendar or according to the prediction model, showed a good efficacy, comparable to those of chemicals, under a standard level of *Monilinia* spp. However, high disease pressure reduced the efficacy of biocontrol strategies. It was also concluded that the combination of BCAs did not improve the efficacy in comparison with separate application.

This research was supported by the European project BIOCOMES FP7-612713 and by the Catalan government (Generalitat de Catalunya) for the PhD grant 2015-FI-B100166 (Amparo Gotor).

Key words: *Monilinia*, formulation, preharvest

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Incorporation of a microbial fungicide into a chemical fungicide program for the control of black sigatoka disease in banana plants

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Abstract: Black Sigatoka (BS) disease is caused by the fungus *Mycosphaerella fijiensis* Morelet, one of the most devastating diseases of banana crops worldwide. This study evaluated the effect of incorporating a microbial fungicide (MF) based on *Bacillus subtilis* EA-CB0015 and its metabolites, into a chemical fungicide program regularly used for the control of BS in field conditions. Results showed that the incorporation of the MF into the fungicide program did not have any significant effect on the state of evolution of the disease (SED), although it did have in the disease severity (DS), suggesting that the MF could be used instead of the protectant fungicide mancozeb under certain circumstances.

Key words: *Mycosphaerella fijiensis*, *Bacillus subtilis*, biological control

Introduction

The increasing number of fungicide applications has brought economic and environmental concerns in many crops. Specifically, among the diseases that reduce banana yields, BS disease caused by the hemibiotrophic fungus *Mycosphaerella fijiensis* Morelet (Stover, 1980) is one of the most damaging. Its control is mostly based in recurrent applications of protectant and systemic fungicides which are aerially applied all year around in export banana plantations. This has eventually lead to fungicide resistance in the pathogen populations. Consequently, more sustainable control strategies such as biological control have become necessary.

We previously described the isolation and selection of the strain *B. subtilis* EA-CB0015 which produces lipopeptides (Villegas-Escobar *et al*., 2013; Ceballos *et al*., 2012), we optimized a culture medium for biomass and lipopeptide production (Mosquera *et al*., 2014) and developed a liquid formulation that enhances shelf life, activity and product delivery (Villegas-Escobar *et al.*, 2014). Finally we evaluated the effect of the microbial fungicide (MF) in greenhouse and field conditions obtaining promissing results (Gutierrez-Monsalve *et al.*, 2015). Here, we evaluated the effect of incorporation this formulation into a chemical fungicide program regularly used for the control of BS in field conditions.

Material and methods

Microbial fungicide

The bacterium *B. subtilis* EA-CB0015 and its metabolites were produced according to Gutierrez-Monsalve *et al*. (2015) and formulated according to the submitted patent application number PCT/IB2014/061167 (Villegas-Escobar *et al*., 2014). The concentration of the formulation wich was designated microbial fungicide (MF), was adjusted to $2.0 \pm 1.0 \times 10^9$ CFU/ml.

Field trial

The effect of applying two different fungicide programs (A, B) on the control of BS was evaluated in the experimental field of Cenibanano (Colombia, 7º 46'22"N, 76°40'22"W) from 11/2014 to 01/2015. Program A consisted on 7 cycles, each consisting of: mineral oil (7 l/ha); protectant fungicide mancozeb (2 l/ha); emulsifying Pegal (6.2 l/ha); one of the systemic fungicides difeconazole (0.5 l/ha), fenpropidin (0.6 l/ha), epoxiconazole (0.8 l/ha), fenpropimorf (0.7 l/ha); tridemorph (0.5 l/ha), pirimetanil (0.5 l/ha); and water. While program B consisted of replacing the protectant fungicide mancozeb in program A by the MF (1.5 l/ha). Each cycle was applied at 19 l/ha by aerial spraying every 11 to 15 days. Program A was applied to 21.63 ha (plots 1 to 7) , while program B was applied to 6.81 ha (plots 8 to 11).

In each plot, the stage of evolution of the disease (SED) (Foure and Ganry, 2008) of ten plants and disease severity (DS) (Gauhl, 1989) of five plants were measured every 7 days. DS was recorded by measuring the youngest leaf with infection (YLI), youngest leaf with spots (YLS), weighted average of infection (WAI), and number of leaves per plants (L/P). The DS was determined using the Stover (1971) scale modified by Gauhl (1989).

The area under the curve (AUC) of YLI; YLS, L/P, WAI and SED were analyzed during the trial as well as before the trial (09/2014 to 11/2014; 09/2013 to 11/2013; 11/2013 to 01/2014; 09/2012 to 11/2012; and 11/2012 to 01/2013).

Analysis of variance (ANOVA) and LSD multiple comparison tests were used to analyze the AUC of each variable with a confidence level of 95% in StatGraphics Centurion XVI.

Results and discussion

Stage of evolution of the disease (SED)

Replacing the protectant fungicide mancozeb by the MF in the fungicide program did not have an effect in the SED of BS (Figure 1). Considering that we could not find differences in the SED of BS before the trial from 09/2014 to 11/2014 (*P value* = 0.934, data not shown), these results suggest that MF could be used instead of fungicide mancozeb without affecting the SED.

Disease severity (DS)

During the trial, we observed an increase in the severity of BS disease when the protectant fungicide mancozeb was replaced by the MF in the fungicide program (Figure 2A, Figure 3), suggesting that the MF increased the severity of BS. Therefore, in order to determine if the areas selected for the application of both programs had natural occurring differences, the AUC of the different severity variables were analyzed before the trial and during the same time period of previous years.

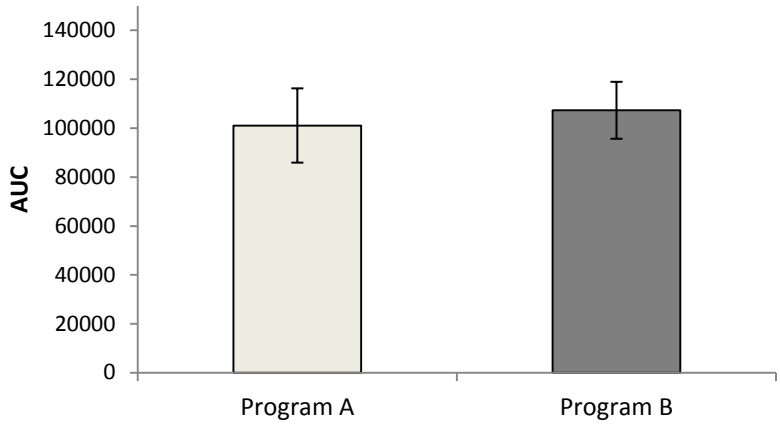

Figure 1. Effect of two fungicide programs on the state of evolution of the disease of black Sigatoka in banana plants during application of MF (*P value* = 0.499). AUC: area under the curve of SED, Program A: chemical fungicide program, Program B: incorporation of MF instead of mancozeb in the chemical fungicide program.

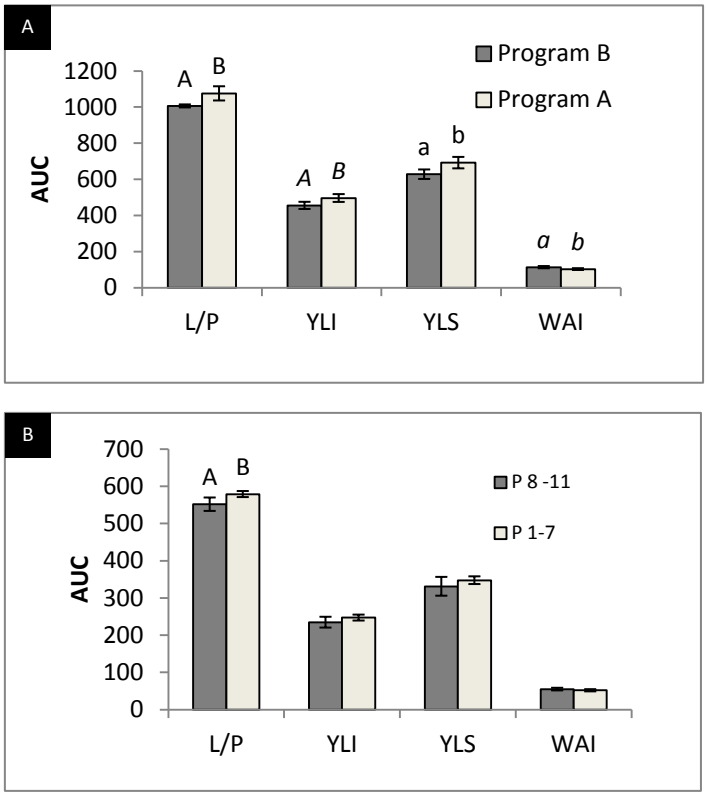

Figure 2. Effect of two fungicide programs on the disease severity of black Sigatoka in banana plants. A) During application of MF, (L/P, *P value* = 0.001; YLI, *P value* = 0.0119; YLS, *P value* = 0.0057; WAI, *P value* = 0.0185). B) before the application of MF from 09/2014 to 11/2014 (*P value* = 0.0088), AUC: area under the curve of DS, Program A and Program B as stated in Figure 1. Different letters indicate significant difference between the treatments.

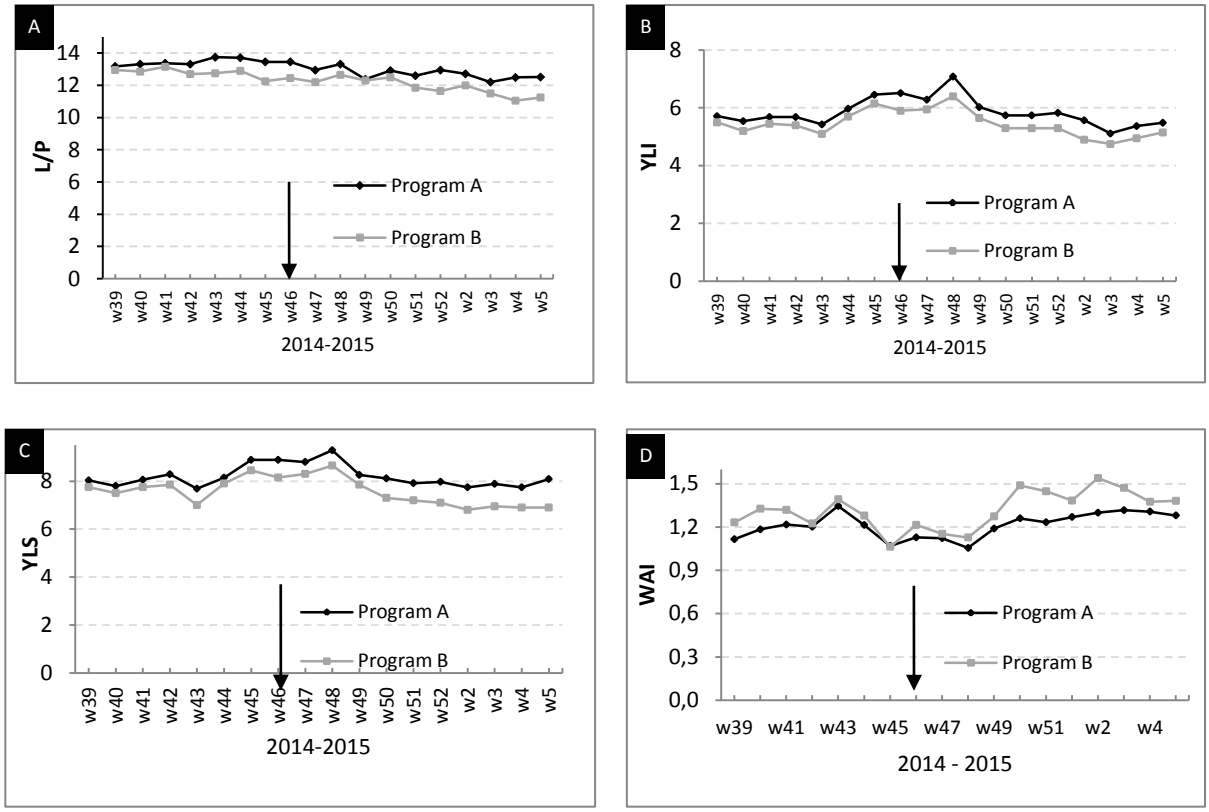

Figure 3. Disease severity of black Sigatoka on banana plants during September 2014 to January 2015. A) L/P, B) YLI, C) YLS, D) WAI. Program A and Program B as stated in Figure 1. w: week of the year. Black arrow: week where the MF was incorporated.

We could not find differences for the variables YLI, YLS and WAI between the two areas prior the trial, however we found that L/P differed (Figure 2B). For years before, we found that in 2012 the evaluated variables differed for both periods, but in 2013 they did not differ (data not shown). These results suggest that other environmental factors also affect the severity of BS in the two areas selected in addition to the fungicide programs evaluated.

Finally the Colombian Agricultural Institute (ICA) states that the minimal value acceptable to control BS should be 8 for L/P, 5 for YLI and 8 for YLS. During the evaluation of both programs none of them were below the minimal values of L/P and YLI; although both programs had YLS below 8 in some weeks of the evaluation (Figure 3).

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Effect of integrating fungicide and biocontrol foliar sprays on maize grain yield and fumonisin content

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Abstract: The adoption of biocontrol is low in maize production although its synergistic action with fungicides on the grain content of fumonisin is reported. The objective of this work was evaluate the potential effect of *Bacillus* sp. (BIOUFLA2) and *Streptomyces araujoniae* (BIOUFLA1) combined or not with fungicide (azoxystrobin + ciproconazol) on grain yield, *Fusarium verticillioides*, incidence and total fumonisin content. Maize plants were sprayed two times, at the phenological stages V9 and R1 with water, BIOUFLA1, BIOUFLA2, fungicide alone or in different combinations two by two. All plants were inoculated with *F. verticillioides*. Upon harvest, treatments were evaluated for total yield, *F. verticillioides* grain contamination (blotter test) and total fumonisin (B1 and B2) contents. The treatment combination fungicide at V9-V10 combined to *S. araujoniae* at R1 resulted in increased yield in three out of the four field trials, while all the other treatment combinations resulted in increased yield in two out of the four trials. All treatment combinations less the two sprays of fungicide reduced *F. verticillioides* incidence in grains compared to the water control. None of the treatments contributed to the reduction on the fumonisin content compared to the control but the two sprays of fungicides or *S. araujoniae* and the combination *S.araujoniae* (V9-V10) + Fungicide (R1) resulted in higher mycotoxin contents compared to the control. The use of the fungicide at V9 followed by *S. araujoniae* (R1) resulted in more consistent yield increases, reduced grain contamination by *F. verticillioides* and did not increase the fumonisin content compared to the control.

Key words: Fumonisins, biological control, integrated management

Introduction

Fumonisins B1 (FB1) and B2 (FB2) are main mycotoxins in maize in post-harvest and is mostly produced by *F. verticillioides* (Shephard *et al*., 1996).

Different methods for reduction of fumonisins in maize are applied pre-harvest or during harvesting and processing (Wild & Gong, 2010) but the presently used fungicide spray program has a strong contribution on the reduction of the mycotoxin content in maize (Paul *et al*., 2011).

On the other hand, biocontrol agents such as *Bacillus subtilis* occupies the same ecological niche as *F. verticillioides* and hamper growth of the fungus through competitive exclusion (Bacon *et al.*, 2001) but little is known about the exclusive use or the use in combination of biocontrol agents and fungicides on maize yield and grain quality.

Material and methods

A total of four field trials were carried out with maize hybrid DKB390 pro2 sprayed with different combinations of two biocontrol agents (*Bacillus* sp. BIOUFLA2 and *Streptomyces araujoniae* BIOUFLA1) and the fungicide PRIORIXTRA (Syngenta), a combination of azoxistrabin + ciproconazole at the phenological stages V9 and R1 at different treatment combinations.

The biocontrol agents were grown in liquid YPD medium for 72 hours and diluted at a ratio of 1:1 (ca. 10^8cfu/ml) for use in the field trials. The fungicide was diluted according to the manufacturer's recommendation (250 ml/ha) and all treatments were sprayed over plants at 200 l/ha. All plants were inoculated with *F. verticillioides* (CML 2823) of 10^5 conidia/ml, 10 days after the stigma style onset with 5 ml of the pathogen suspension per ear. Upon harvest total grain yield was determined per plot and part of the grains was used for blotter tests with 200 grains of each treatment per plot and four replicates per treatment, and the incidence of *F. verticillioides* per plot was recorded.

Part of the grains were also used for fumonisin quantification by pooling grain samples of all four replicates per field, powdering 10 g of the composite samples, extracting the mycotoxin using an acetonitrile:water (1:1) solution, diluted to 1:1 with formic acid (1%) and qualified in HPLC for fumonisins B1 and B2. The total yield, incidence of *F. verticillioides* and total fumonisin content (FB1 + FB2) were analysed for significant effects and means compared by Scott-Knott grouping test ($P < 0.05$).

Results and discussion

There was significant yield increase in three out of the four field trials (Figure 1) and the only treatment combination that assured consistent yield increase compared to the control was the combination of fungicide at V9 and *S. araujoniae* at R1, all the other treatment combinations assured yield increase in two of the four trials.

The use of fungicides has been reported with beneficial effects on corn plants, leading to growth promotion and consequently productivity (Paul *et al*., 2011), but these results are variable, and when used alone these active ingredients become inefficient and cannot ensure high productivity.

In regard to *F. verticillioides* incidence in grains, all treatment combinations less fungicide sprayed two times, contributed to healthier grains. However, none of them contributed to the reduction in the fumonisin content and three of them, fungicide or *S. araujoniae* and the combination of *S. araujoniae* at V9 and fungicide at R1 resulted in higher fumonisin content.

Although a high incidence of grains contaminated with *F. verticillioides* (50-66%), the fumonisin content is mostly below the maximum tolerated fumonisin content regulated by FDA (4 ppm) (Shephard & Marasas, 2004) and only the treatments of two sprays with the fungicide and two sprays of *S. araujoniae* resulted in fumonisin content above that allowed level.

Actually, the grain contamination by *F. verticillioides* does not clearly impact yield and fungicide is important to assure leaf protection against other pathogens that directly impact yield. On the other hand, the exclusive use of the fungicide or *S. araujoniae* does not last long enough to protect the grains. These treatments may have created a biological vacuum that resulted in the reduction of competitors of *F. verticillioides* and increased grain contamination and fumonisin content. The combination of those two control strategies which likely act through different mode of actions, resulted in the most consistent benefit for all evaluated quantitative and qualitative grain-related variables.

Therefore, to assure both quantity and quality of maize kernels the combination of a first spray of fungicide at V9 and a second spray of the biocontrol agent *S. araujoniae* has been the most promising treatment combinations.

A **Production (ton/ha)**

	Treatments		**Area 1**	**Area 2**	**Area 3**	**Area 4**
	At V9	At R1				
T1	Water	Water	9.99 **c**	3.15 **a**	3.55 **b**	5.26 **c**
T2	Fungicide	Water	10.98 **b**	3.55 **a**	4.00 **b**	6.18 **b**
T3	Fungicide	Fungicide	11.53 **a**	4.03 **a**	3.60 **b**	6.55 **b**
T4	Fungicide	*Bacillus* sp.	10.99 **b**	3.70 **a**	4.44 **a**	5.68 **c**
T5	Fungicide	*S. araujoniae*	10.99 **b**	3.99 **a**	4.68 **a**	7.06 **a**
T6	*Bacillus* sp.	Fungicide	10.95 **b**	3.51 **a**	3.42 **b**	6.52 **b**
T7	*S. araujoniae*	Fungicide	11.98 **a**	4.14 **a**	3.81 **b**	6.31 **b**
T8	*Bacillus* sp.	*Bacillus* sp.	10.98 **b**	3.64 **a**	4.56 **a**	5.38 **c**
T9	*S. araujoniae*	*S. araujoniae*	11.10 **b**	3.70 **a**	3.40 **b**	7.23 **a**

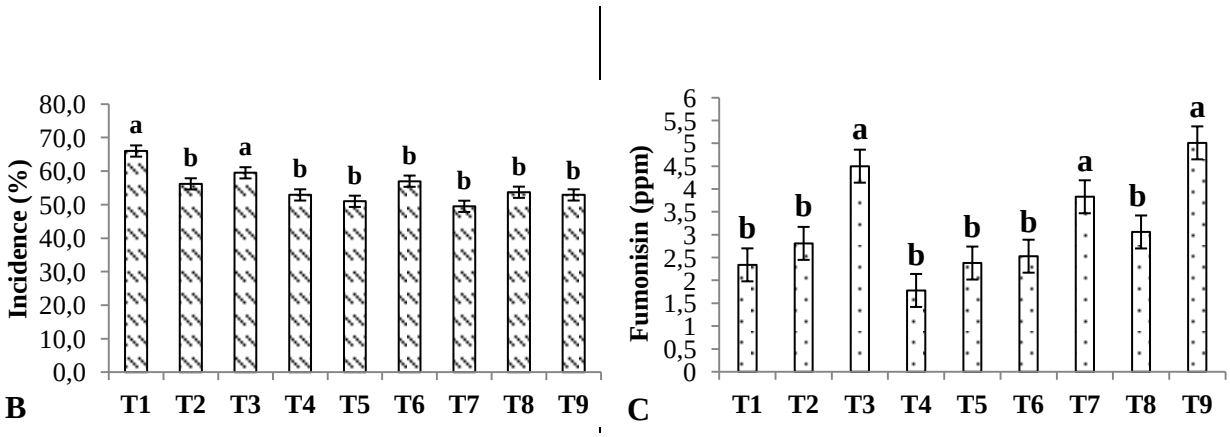

Figure 1. Evaluation of the isolated and combined effect of biological control and fungicide (azoxistrobin+ciproconazole) on: (A) Total productivity at four fields; (B) Incidence of *F. verticillioides* in kernels and (C) fumonisins content (FB1 + FB2). Means followed by the same letter do not differ statistically (Scott-Knott test; $P < 0.05$). For figures B and C, bars represent average of four field trials and four replicates per treatment.

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Are there regional differences in the susceptibility of *Sclerotinia sclerotiorum* strains to *Coniothyrium minitans*?

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Abstract: In an attempt to understand regional differences in the efficacy of biocontrol against *Sclerotinia sclerotiorum* in France, strains of the pathogen were collected from different locations and tested for their susceptibility to the biocontrol fungus *Coniothyrium minitans*. Based on results obtained for the first 22 strains examined, wide and highly significant differences were observed, suggesting that the efficacy of this biocontrol method could vary locally depending on the frequency of susceptible *vs* less susceptible strains of *S. sclerotiorum*. However, the differences in susceptibility observed so far for strains from the North and the South of France cannot explain global regional differences in the efficacy of this biocontrol method. If confirmed by ongoing work on additional strains of *S. sclerotiorum*, these results will point to other hypotheses examined in the framework of national project "ScleroLeg".

Key words: *Sclerotinia sclerotiorum*, *Coniothyrium minitans*, biological control

Introduction

Since the registration of *Coniothyrium minitans* strain CON/M/91-08 in France (commercial product Contans®), biological control against *Sclerotinia sclerotiorum* has increasingly been used on various crops, with overall good results. However, returns from growers suggest regional differences in the efficacy of biocontrol, with better results in the North than in the South of France in open fields. Several possible environmental and agronomic hypotheses could be proposed to explain this situation, including differences in the pedoclimatic conditions of the farms (temperature, composition, structure, chemical and microbial properties of the soil, for example). Another possible hypothesis could be the existence of differences in the susceptibility to *C. minitans* among strains of *S. sclerotiorum*. Although little information is available, a few reports have pointed to the possibility that plant pathogens could possess or develop reduced susceptibility to biocontrol agents (Bardin *et al*., 2015).

The present study was initiated in the framework of national project "ScleroLeg" (https://www.picleg.fr/Les-Projets-en-cours/Scleroleg) to compare the susceptibility to *C. minitans* of strains of *S. sclerotiorum* collected from different different regions of France.

Material and methods

Strains of* S. sclerotiorum *and production of sclerotia

Strains were collected by project partners as mature sclerotia taken from diseased plants in commercial fields from several regions of France. The sclerotia received in the laboratory were systematically surface-sterilized and subjected to single-hypha culturing. The strains were then stored at -20 °C. Prior to its use in tests with *C. minitans*, each strain was grown for 3 weeks on PDA medium at 22 °C. The sclerotia were then collected on the Petri dishes and used immediately as described below.

Inoculum of* C. minitans *and inoculation of sclerotia

The inoculum of *C. minitans* consisted of spore suspensions adjusted to a concentration of 10^8 spores/ml. For each strain of *S. sclerotiorum*, 4 batches of 20 sclerotia were prepared in sterile tubes and mixted with 2 ml of either *C. minitans* inoculum (3 inoculated batches) or sterile water (one control batch). Each batch of sclerotia was then mixted into 150 g of sterile sand and incubated in the dark at 22 °C.

***Assessing the susceptibility of* S. sclerotiorum *to* C. minitans**

After 3 weeks of incubation, the sclerotia were disinfested for 3 minutes in sodium hypochlorite and rinsed in sterile water to remove *C. minitans* from their surface. Each sclerotium was then cut in half and the two fragments were placed on PDA medium and incubated for one week at 22 °C. To assess the susceptibility of *S. sclerotorium* to sclerotial colonisation by *C. minitans*, we examined each half sclerotium for presence and growth of *C. minitans* and of *S. sclerotium* after 3 and 7 days of incubation. For each strain of *S. sclerotiorum*, a total of 80 half-sclerotia were plated on PDA, 60 from the batches of inoculated sclerotia and 20 from non-inoculated control sclerotia.

To account for possible intrinsic differences in growth rate among strains of *S. sclerotiorum*, regardless of the effect of *C. minitans* on sclerotia, we computed an "index of growth reduction" as:

$$I = 100*(D_{control} - D_{inoculated})/D_{control},$$

where $D_{inoculated}$ was the diameter of the *S. sclerotiorum* colonies after 3 days of incubation of half sclerotia inoculated with *C. minitans* and $D_{control}$ was that for non-inoculated control sclerotia.

Results and discussion

Among strains of *S. sclerotiorum* collected by partners of the ScleroLeg project, over 70 are to be tested for their susceptibility to sclerotium colonization by *C. minitans*. To date, results have been obtained and analysed for only a subsample of these strains (10 from the North and 12 from the South of France). They will be presented below.

***Growth of* C. minitans *from sclerotia of* S. sclerotiorum**

Development of *C. minitans* colonies on the PDA medium was never observed from non-inoculated sclerotia of *S. sclerotiorum*. The mycoparasite developed from most but not all inoculated sclerotia, suggesting that for some of them, the extent of internal colonization by the mycoparasite was not sufficient to allow detectable growth within 7 days after the half-

sclerotia were deposited on PDA. The average diameter of the *C. minitans* colonies after 7 days varied widely depending on the strains of *S. sclerotiorum* (Figure 1), presumably reflecting differences in the amounts of *C. minitans* biomass present in the half-sclerotia at the time they were deposited on PDA. The effect of the *S. sclerotiorum* strain on this diameter was highly significant ($P < 0.001$).

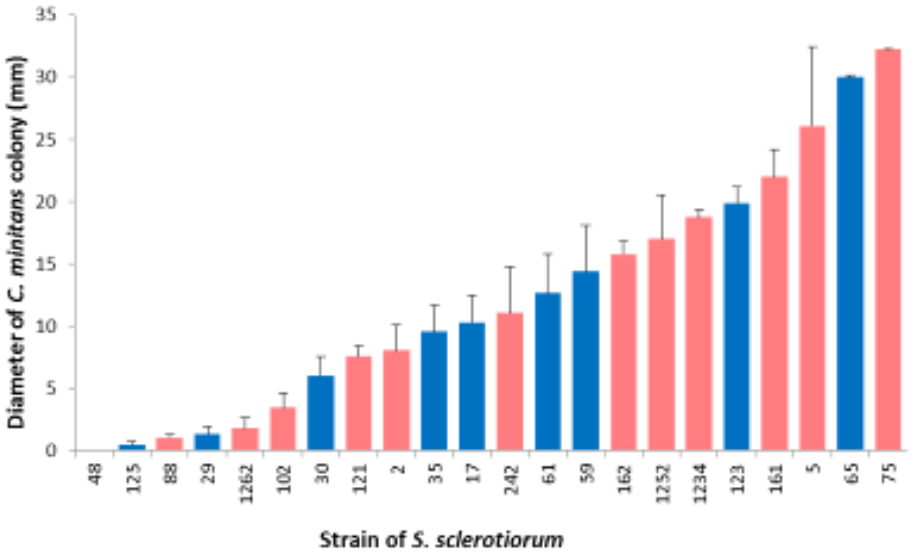

Figure 1. Development of *Coniothyrium minitans* from half-sclerotia of 22 strains of *Sclerotinia sclerotiorum* (from the North: ■ and from the south: ■ of France) 7 days after their deposition on PDA medium. Each data point represents the average of 60 observations; error bars are the standard error of the mean.

Germination of* S. sclerotiorum *sclerotia on PDA medium

Development of *S. sclerotiorum* colonies was observed from almost all half-sclerotia plated on the PDA medium, wether they had been inoculated or not with *C. minitans* before their incubation in sand. The diameter of *S. sclerotiorum* colonies after 3 days on PDA medium varied widely wether the sclerotia had been inoculated with *C. minitans* (Figure 2A) or not (data not shown). In both cases, highly significant differences were found among strains ($P < 0.001$). The comparison of colony diameter for inoculated and non-inoculated control sclerotia (using the "inhibition" index described above) showed that for many strains of *S. sclerotiorum*, mycelial growth was reduced in the presence of *C. minitans*, presumably reflecting the desctruction of biomass by the mycoparasite in inoculated sclerotia (Figure 2B). For certain strains of *S. sclerotiorum*, however, inoculation of sclerotia with *C. minitans* did not impact mycelial growth negatively and surprisingly, a strong stimulation (negative values of the "inhibition" index) was even observed in some cases (Figure 2B). The differences among strains were highly significant ($P < 0.001$).

***Regional differences among strains of* S. sclerotiorum**

According to our initial hypothesis, strains from the South of France would be less susceptible to *C. minitans*, lowering the efficacy of biocontrol compared to the situation in Northern France. However, both groups of strains showed wide differences in their susceptibility to *C. minitans* (Figures 1 and 2). Furthermore, strains for the North were among those for which

the recovery of *C. minitans* from inoculated sclerotia was the lowest (for example N° 48, 125 and 29) and that of *S. sclerotiorum* the least reduced. On average, no significant differences were found between the groups of North and South strains for either susceptibility criteria ($P > 0.05$).

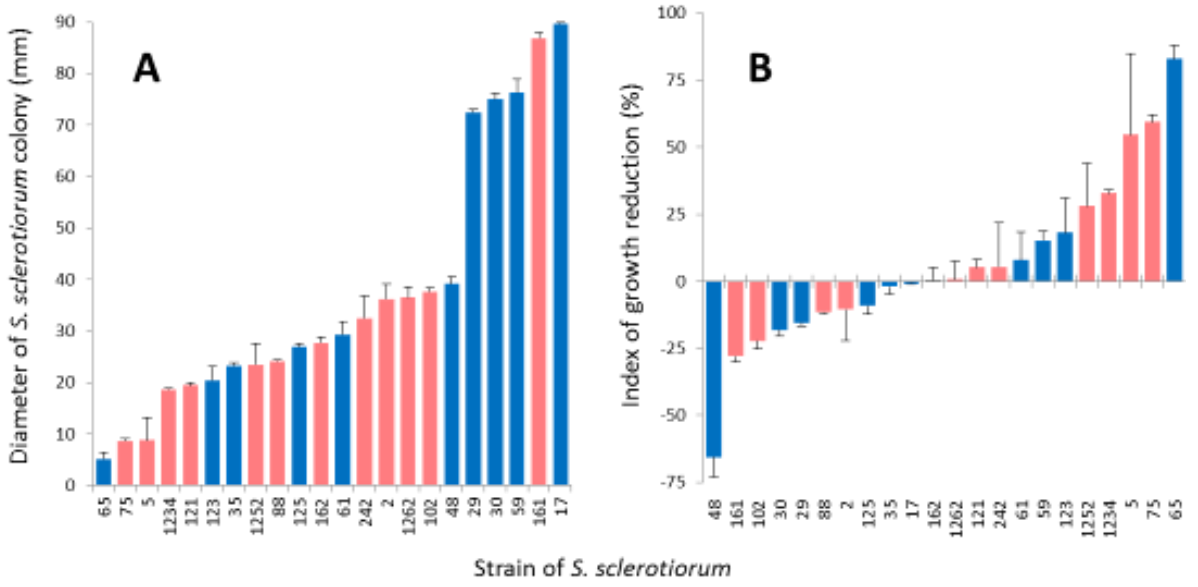

Figure 2. Development of 22 strains of *S. sclerotiorum* (from the North: ■ and from the south: ■ of France) from half-sclerotia 3 days after their deposition on PDA medium. A. Sclerotia inoculated with *C. minitans*; B. Comparison of growth from inoculated and non-inoculated sclerotia. Each data point represents the average for 60 half-sclerotia; error bars are the standard error of the mean.

Conclusions and perspectives

In the present study, the impact of *C. minitans* on sclerotia of *S. sclerotiorum* was assessed through (i) the extent of mycelial growth of *C. minitans* from inoculated sclerotia of *S. sclerotiorum* and (ii) the comparison of mycelial growth of *S. sclerotiorum* from inoculated and non-inoculated sclerotia. Both of these criteria revealed wide and highly significant differences in susceptibility to *C. minitans* among 22 strains of *S. sclerotiorum* examined so far. This finding suggests that the efficacy of this biocontrol method might vary locally depending on the frequency of susceptible *vs* less susceptible strains of *S. sclerotiorum*. However, differences in susceptibility observed so far for strains from the North and the South of France cannot explain reported regional differences in the efficacy of Contans®.

The present data will be complemented shortly with the assessment of ca 50 additional strains. If this consolidates the present results, more focus will need to be put on other hypotheses evaluated in the ScleroLeg project to explain North-South differences in biocontrol efficacy.

Acknowledgements

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Integration of biocontrol agents and thermotherapy to control *Fusarium fujikuroi* on rice seeds

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Abstract: *Fusarium fujikuroi*, the causal agent of bakanae disease, is the most important seedborne fungal pathogen of rice. Chemical alternatives to seed treatments against bakanae disease consist in biological and physical control methods. Four yeast isolates were selected among all potential antagonists obtained from various rice seeds on the basis of *in vitro* and *in vivo* assays. Two of them were identified as *Metschnikowia pulcherrima*, one as *Pichia guilliermondii* and one as *Sporidiobolus pararoseus*. Biocontrol efficacy of *P. guilliermondii* and *M. pulcherrima* on *F. fujikuroi* was higher in comparison with some applied commercial biofungicides. The seed dressing with antagonistic yeasts diminished the bakanae disease index to 70% compared to control seeds. Biocontrol yeasts resulted even more efficient when combined with thermotherapy, decreasing the disease index less than 5%, and ameliorating the seed germination as well. Selected biocontrol agents could be efficient alternative method to control *F. fujikuroi* on rice seeds, particularly when combined with thermotherapy.

Key words: *Fusarium fujikuroi*, antagonistic yeasts, thermotherapy

Introduction

Fusarium fujikuroi Nirenberg [teleomorph *Gibberella fujikuroi* (Sawada) Ito in Ito & K. Kimura], the causal agent of bakanae seedborne disease, represents a seriuos threat to rice production (Desjardins *et al.*, 1997). Beside its spread throughout Asia, it has also been diffused recently in California and Italy (Carter *et al.*, 2008; Amatulli *et al.*, 2010). A continuous increase of the disease incidence has been observed as a consequence of the partial efficacy of the available fungicides and the underestimation of the phytosanitary phenomena importance.

Biological control with the application of antagonistic microorganisms represents an alternative sustainable approach in control of plant diseases (Adams, 1990). It can be applied successfully agains seedborne pathogens as a seed dressing method on different cereal and vegetable crops (Tinivella *et al*., 2009; Debnath *et al*., 2012).

Material and methods

Selection of microorganisms from rice and a dual culture assay
Epiphytic microorganisms were isolated from rice seeds of the cultivar Galileo naturally infected with *F. fujikuroi*. Ninety-six microorganisms were identified initially on potato

dextrose agar (PDA) medium previously loaded with the serial supensions of Ringer solution containing the seeds. A single colony per each microorganism was further grown on NYDA medium. Mycelial growth inhibition of *F. fujikuroi* by isolated microorganisms was measured as previously described (Zhang *et al*., 2010).

Antagonistic efficacy of selected microorganisms in vitro and in vivo

Four microorganisms were selected on the basis of the dual-culture assay results, and they were characterized by morphological characteristcis as yeasts: *Metschnikowia pulcherrima* (isolates R23 and R26), *Pichia guilliermondii* (isolate R9), and *Sporidiobolus pararoseus* (isolate SB1). Antagonistic efficacy of selected yeasts were evaluated on a large number of seeds *in vitro* in Petri dishes on Komada medium (infection rate) and on a 2-layer filter paper (for seed germination). For *in vivo* testing the seed germination and the disease index were evaluated by sowing of treated seeds in greenhouse and growing of rice plants under controlled conditions.

Combined biocontrol and thermotherapy in vivo

Efficacy of combined application of each selected yeast with thermotherapy (60 °C for 5 or 10 min) was also evaluated by treating of seeds and growing of plants in greenhouse conditions.

Results and discussion

Antagonistic efficacy in vitro

Among ninety-six isolated microorganisms, four isolates belonging to the three yeast species *M. pulcherrima* (isolates R23 and R26), *P. guilliermondii* (isolate R9), and *S. pararoseus* (isolate SB1) were most efficient. Four selected isolates were tested for *in vitro* antagonistic activity on rice seeds at concentration of 10^8 cells/ml. The infection rate by *F. fujikuroi* was decreased below 18% by application of *P. guilliermondii* (R9), and *M. pulcherrima* (R23 and R26) in contrast of commercial biofungicides which showed the higher infection (above 28%) (Figure 1). *S. pararoseus* (SB1) – seed dressing was less efficient in infection supression compared to the other selceted antagonistic yeasts. Antagonist seed dressing resulted also in increased seed germination comparing to the control seeds.

Antagonistic efficacy in vivo

The disease index was importantly reduced by application of selected antagonists *in vivo*. Thus, *P. guilliermondii* (R9) and *M. pulcherrima* (R23) showed the highest reduction of the bakanae disease (Figure 2). These isolates were also more efficient in comparison with applied commercial biofungicides. Seed germination was also positively stimulated by antagonist seed dressing.

Combination of antagonists and thermotherapy in controlled trials

Combined effect of each antagonist and thermotherapy was observed against bakanae disease within 28 days after sowing. The higher efficacy of the combined treatment was found when *P. guilliermondii* (R9) and *M. pulcherrima* (R23) were applied together with 60 °C thermotherapy for 10 min in comparison to single tretaments allowing the suppression of the bakanae index below 5% and improving the seed germination (data not shown).

The present work indicates that biocontrol agents may be used as alternative to chemical control for suppression of *F. fujikuroi* infection on rice. Additionally, where possible they can be combined with thermal treatments increasing further their disease-control potential.

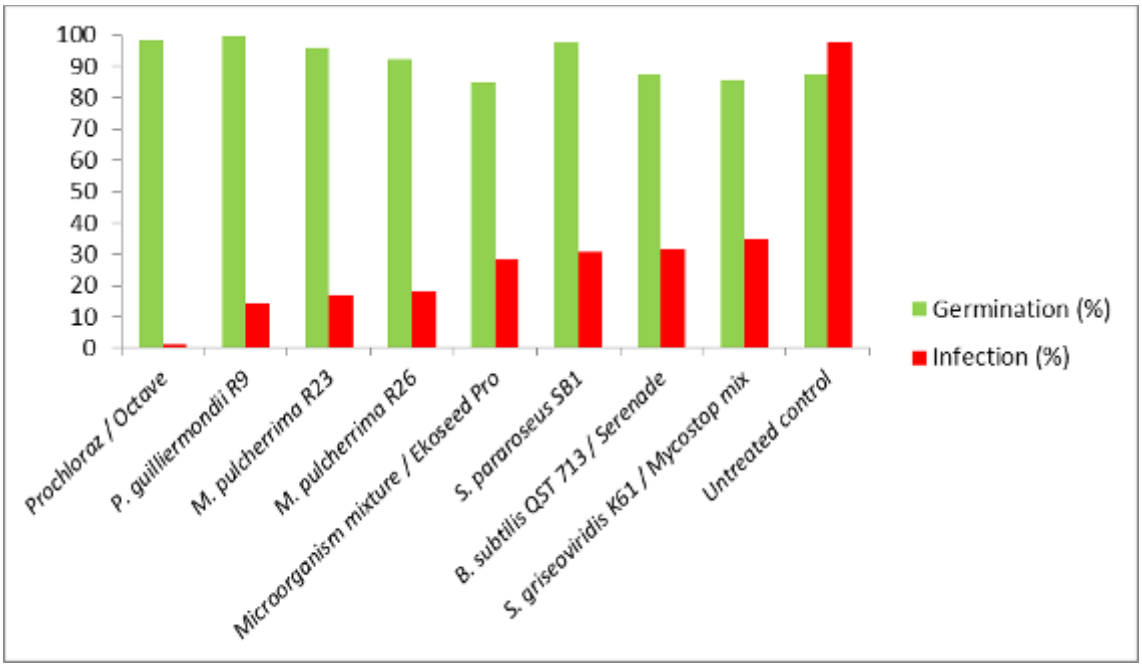

Figure 1. Influence of antagonists on *F. fujikuroi* infection and rice seed germination *in vitro.*

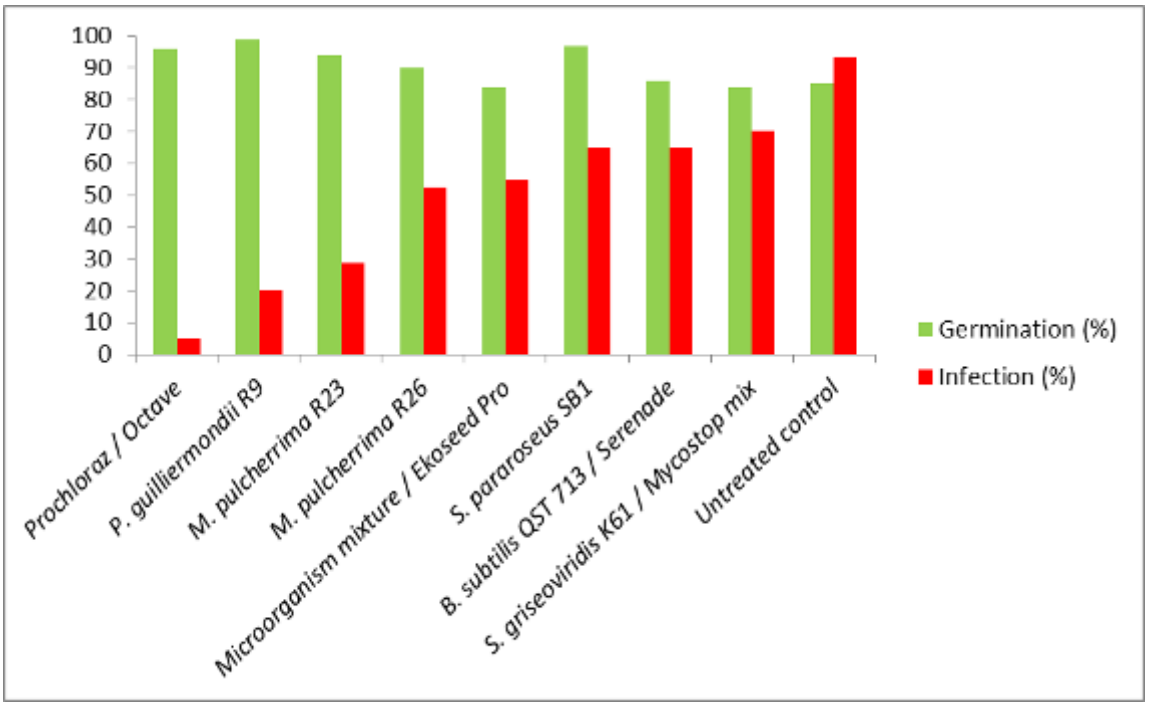

Figure 2. Influence of antagonists on bakanae disease index and rice seed germination *in vivo.*

Acknowledgements

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Biocontrol of olive anthracnose by *Aureobasidium pullulans*

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Abstract: Anthracnose is one of the most severe and widespread disease of olive (*Olea europaea* L.), caused by fungi belonging to the genus *Colletotrichum*. Among the species associated to the disease, some in the complexes *C. acutatum* and *C. gloeosporioides* are the most frequent (Schena *et al.*, 2014). *Colletotrichum* species can use diverse host colonization strategies, ranging from fruit infection and rapid symptom development to long latent infections (Agosteo *et al.*, 2005). Typical anthracnose symptoms appear on mature fruits under wet autumn conditions, as dark sunken lesions with abundant production of orange masses of conidia. Symptoms can also be found on branches and leaves, leading to necroses, severe defoliation, dieback of branches and reduced tree vigour (Talhinhas *et al*., 2011). During the spring, conidia of the pathogen are dispersed by rain splash from mummified fruit to flower and/or developing fruit, causing latent infections (Moral & Trapero, 2012). In autumn, when the drupes start to ripe, the pathogen becomes active, causes fruit rot, and produces large amount of conidia that initiate secondary disease cycles. If the temperature rises and humidity decreases, the affected drupes fall prematurely to the ground and only a few of them remain mummified on the tree canopy. It has therefore been assumed that mummified fruits act as sources of inoculum for infection of developing fruit during the following spring (Moral *et al.*, 2012). Moreover, disease can also rapidly develop on harvested drupes if they are stocked in the oil mill before the extraction process. A very poor olive oil quality is obtained from anthracnose-affected drupes, because of the increase of free acidity, color alterations (red), and deterioration of the organoleptic properties.

Traditional control methods rely on protectant and systemic fungicides, which remain the main tools to reduce anthracnose to date (Kefialew & Ayalew, 2008). However, increasing public concern over food safety and regulatory restrictions over pesticide residues on foods, as well as the development of fungal resistance, highlights the need for replacing synthetic compounds with alternative, non-chemical methods. Although still permitted at present, the European authorities would like to see banning of copper fungicides in conventional and organic farming across Europe, thus having a major impact on disease management in olive production. Under EU Directive 2009/128, the Sustainable Use Directive, National Action Plans for reductions in pesticide use and the implementation of Integrated Pest Management on all farms in member states has been reviewed by the EC at the end of 2015 and became mandatory throughout the member states.

Biological control, alone or as part of integrated disease management, has been suggested as a promising and sustainable long-term solution (Pantelides *et al.*, 2015) to control plant pathogens and increase product quality. One of the most effective strategies to optimize the efficacy of BCAs and control latent infections is the application of antagonists in pre-harvest stage, i.e. at blooming stage, or just prior the harvest (Ippolito & Nigro, 2000). Endophytic microorganisms, which colonize symptomless the living plant tissue, produce bioactive compounds having antifungal and antibacterial activity (Dutta *et al*., 2014). Compared to

epiphytic microorganisms, internal colonisers provide additional benefit as biocontrol agents. As the plant provides shelter and nutrients, microorganisms can develop under less competitive conditions and shield the plant interior against pathogens.

The yeast-like fungus *Aureobasidium pullulans* (de Bary) Arnaud is a member of the black yeast family, and it is one of the most widespread and well-adapted saprophytes in the phyllosphere, as demonstrated by its presence in washing water of leaves and fruits (Gaur *et al*., 2010), and its frequent occurrence as endophyte (Schena *et al*., 2003). It has been frequently reported as an effective biocontrol agent against both pre-harvest and post-harvest diseases (Di Francesco *et al*., 2015; Parafati *et al*., 2015). Therefore, the aims of this work were i) to determine the antagonistic activity of *A. pullulans* strains against *Colletotrichum* spp. causing olive anthracnose; ii) to ascertain the biocontrol activity of *A. pullulans* strains applied at the blooming stage and before the harvest, in controlling latent and active anthracnose infections, and iii) to determine the surviving rate on the field-treated olive trees of the strains L47, by using strain-specific markers. Results indicated that some isolates were as effective as the chemical fungicides, providing high protection levels against drupes anthracnose. Among the tested strains some resulted very effective in controlling *C. acutatum sensu stricto* and *C. gloesporioides sensu lato*, both in laboratory assays and in field trials. Applications at the pre-blooming and veraison stages determined significant reduction of latent and active anthracnose infections, as compared to the untreated control and copper-treated fruits. Among the most effective strains, *A. pullulans* L47 showed also high population levels on fruit and leaves surface. Tests on the survival rate, as determined by the use of specific markers, indicated that strain L47 can be detected up to one year after the first application, although its incidence on the total epiphytic population of *A. pullulans* was no greater than 5%. These results confirm that *A. pullulans* is an effective biocontrol agent against both the active and latent infections by *Colletotrichum* spp. if applied at the flowering and veraison stages, respectively.

Key words: *Colletotrichum* spp., latent infections, endophytes

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Biological control of chestnut blight: an interplay between chestnut, fungus *Cryphonectria parasitica* and *Cryphonectria hypovirus* 1

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Abstract: Chestnut blight is a disease caused by the fungus *Cryphonectria parasitica* Murrill Barr. This aggressive ascomycete infects bark and cambium of the sweet chestnut trees through wounds, and induces bark cankers which can lead to dieback of the distal parts of the plant, after girdling branches or the entire tree trunk (Heiniger & Rigling, 1994). *C. parasitica* had been accidentally introduced from Asia to North America and Europe and caused devastation of *Castanea dentata* and *C. sativa* populations, respectively. However, this severe disease can be controlled by naturally-occurring hypovirus *Cryphonectria hypovirus* 1 (CHV-1) which significantly reduces fungal virulence, sexual reproduction, pigmentation and sporulation (Chen & Nuss, 1999). Hypovirulent (CHV-1 infected) *C. parasitica* strains do not induce lethal cankers on chestnut trees. Moreover, they cause healing of cankers which were previously induced by virulent fungal strains (Heiniger & Rigling, 1994). The hypovirus can be transmitted from infected to non-infected *C. parasitica* strains via hyphal anastomosis, but the transmission is limited by the vegetative incompatibility *vic* system (Cortesi & Milgroom, 1998; Cortesi *et al*., 2001). CHV-1 is therefore transmitted between fungal strains belonging to the same vegetative incompatibility (vc) type, but between strains that belong to different vc types transmission rate is considerably lower or transmission does not happen at all (Cortesi *et al.,* 2001). The hypovirus can also be transmitted via asexual conidia, but not via sexual ascospores (Peever *et al*., 2000; Prospero *et al*., 2006). In Europe CHV-1 occurs as several subtypes: I, F1, F2 and E/D (Prospero & Rigling, 2013) that differ in their virulence towards *C. parasitica* (Bryner & Rigling, 2011). French subtypes F1 and F2 of CHV-1 are believed to be more virulent towards *C. parasitica* and inhibit its growth and sporulation more than Italian subtype I (Robin *et al*., 2010). The importance of the use of specific virus isolates belonging to certain CHV-1 subtype in biological control had been studied before, but three lateral interaction of sweet chestnut, pathogen *C. parasitica* and biocontrol agent – CHV-1 has been only recently studied by our team (Ježić *et al*., 2014; Krstin *et al*., submitted). It was found that chestnut genotype contributes a lot to this three-lateral interaction. Both, susceptibility (Krstin *et al*., submitted) as well as recovery (Ježić *et al*., 2014) are considerably influenced by chestnut genotype, and therefore not only the genotypes of the fungus and virus, but also of the chestnut are important for successful biocontrol. We found out that marrons, sweet chestnut cultivars obtained through selection and propagated by grafting, that are grown primarily for the production of large quality fruits, are especially vulnerable to chestnut blight. In the presence of naturally-occurring hypovirulence, these trees recover with much lower frequency than trees from naturally growing chestnut population in

the same area. However, there is a difference is susceptibility of naturally growing trees as well, and certain hypovirulent fungal strains harboring specific virus isolates are more or less efficient against chestnut blight depending on chestnut genotype. Therefore, generally efficient hypovirulent *C. parasitica* strain does not exist, only strains which are efficient on majority of chestnut genotypes. Furthermore, as opposed to previously published results that imply higher virulence of French than Italian CHV-1 subtypes towards chestnut blight fungus, our results reveal that some virus isolates that belong to subtype I, which is widespread in Croatia and neighboring countries (Krstin *et al*., 2008; 2011) have equal effect on fungal host as the strong isolate CHV-1/EP713 that belongs to F subtype.

Key words: hypovirulence, chestnut genotype, chestnut susceptibility and recovery, virus efficiency

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Use of a plant oil extract biostimulant to control grapevine fungal diseases

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Abstract: Grapevine is a perennial crop which is sensitive to many fungal pathogens requiring numerous pesticide treatments, potentially leading to environmental problems and fungicide resistances. Today, it is necessary to develop new more sustainable pest management strategies, while maintaining wine quality. The use of biostimulants appears to be an interesting alternative or a complementary method to conventional pest management. However, until now, excellent results were obtained in controlled conditions, but were often disappointing in vineyard.

We identified a plant oil extract able to stimulate grapevine defenses taking the salicylic acid way, without any direct action on pathogens. The aim of this study was to test its indirect effect on the major grapevine fungal diseases: Powdery mildew, downy mildew, *Botrytis* bunch rot and grapevine trunk diseases. An important decrease of symptoms of each disease was observed in controlled conditions and in the vineyard after treatments with the plant oil extract. Severity of the first three diseases was reduced by 45% to 62% on different vineyards in the Bordeaux area without modification in the harvested grape quality. In greenhouse, the wood necrosis of cuttings infected with grapevine trunk disease fungi was halved 4 months after treatment with the biostimulant. In addition, the expression of several genes involved in grapevine defenses and metabolism were followed to support our result and demonstrated the biostimulant nature of this plant oil extract.

Key words: grapevine fungal diseases, biostimulation, field experiment

Potential contribution of biological control to integrated management of plant diseases in UK gardens

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Abstract: The gradual loss of conventional chemical fungicides for amateur use makes integrated disease management in domestic gardens, ideally with biocontrol options, ever more important. In the UK, the Horticulture Innovation Partnership (HIP) has identified that research is required for the 'development of new practical bio-control and integrated pest management approaches, designed with industry input for UK gardens, nurseries and landscapes. However, while there is a huge potential for the use of biological control of plant diseases in gardens, there are also a number of constraints to their uptake.

Several fungal and bacterial biofungicides are already available in commercial horticulture, with more likely to come on the market in the near future. None are currently registered for amateur use, the key constraint being that 'Micro-organisms may have the potential to provoke sensitizing reactions'. Development of 'safe' formulations or evidence of their safe use by amateurs could enable such registration, but size of the market will also be a factor.

The use patterns of biofungicides in gardens will differ from those in commercial horticulture for several reasons. The domestic market is less cost-sensitive and the degree of disease control may be less than in commercial horticulture. Plant diversity is greater in a typical domestic garden and species are more likely to be grown in small groups or in mixtures. These differences mean that integrated disease management strategies will need to be developed for domestic gardens.

Biological control could be applied in ways that do not require amateur-application. For instance, those that provide a long-lasting effect could be applied in nurseries (or even to seed) before sale to home gardeners. Several root endophytes or rhizosphere colonizers are available. Such products have been reported to increase nutrient uptake, photosynthesis and vigour in the absence of disease, protect against root diseases and also induce plant resistance to aerial diseases.

The FAO state that 'Healthy soils maintain a diverse community of soil organisms that help to control plant disease'. Various microbial products are marketed to home gardeners with the promise to improve soil biodiversity and reduce disease problems. The encouragement of beneficial microbes and development of suppressive soil has potential in disease management, but further work is required to develop systems for domestic gardens.

In this paper we give examples of the potential use of biological control to manage diseases in two areas of the domestic garden – the fruit and vegetable garden and a mixed ornamental border.

Key words: gardens, biocontrol, IPM

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Impact of use of *Trichoderma* spp. on greenhouse tomato crop and the bio control of late blight caused by *Phytophthora infestans* (Mont.) de Bary

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Abstract: As a part of the search for alternative methods that respect the environment, to reduce the increased use of fertilizers and fungicides, the present work focused on the study of bio-stimulant and eliciting effects on tomato of *T. asperellum* and *T. atroviride*, issued from the rhizosphere of tomato plants grown in producing areas of Algeria. Indeed, conidial suspensions of 2 x 10^7 conidia/ml were prepared and applied on tomato cultivar "Saint Pierre" growing in pots and in greenhouse as follows: incorporation into the soil, foliar spraying and mycorhization. The impact of the use of these two isolates on the vegetative growth of tomato plants, on fruit yield, on total polyphenol contents and on foliar pigments content (including chlorophyll A and B, the total chlorophyll and carotenoids) was evaluated. Moreover, the biocontrol of downy mildew caused by *Phytophthora infestans* was assessed by *in vivo* antagonistic activity on detached leaves of tomato.

The results showed that the two isolates of *Trichoderma* spp., particularly *T. atroviride*, have a biostimulant effect on vegetative growth parameters such as plant height, leaf number, bunch of flowers, number of flowers and fresh weight of roots, whatever their modes of application. These isolates also improved the fruit yield (number of fruits per bunch, weight and caliber). Levels of total polyphenols and leaf pigments were significantly higher on treated leaves than on untreated leaves.

In addition, the mycorhization obtained by dipping the plant roots in the conidial suspension turns out to be the best mode of application to increase fruit weight and caliber. Moreover, the two isolates induced tomato resistance against *Phytophthora infestans* with a disease reduction of 60.47% and an inhibition of sporulation of 96.32% for the three application modes. Foliar spraying gives the best inhibition rate. This study confirmed the biostimulant and biofungicides potential of *T. aroviride* isolate on tomato plants applied either by incorporation into the soil, mycorhization or foliar spraying.

Key words: *Trichoderma atroviride*, *Trichoderma asperellum*, biostimulant and elicitor effects, *Solanum lycopersicum*, *Phytophthora infestans*

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Management of groundnut stem and root rot complex by using *Trichoderma harzianum* Th3 at field level

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Abstract: Stem and root rot complex disease of groundnut is a devastating disease caused by *Sclerotium rolfsii* and *Macrophomina phaseolina*. To avoid extensive use of pesticides, biological formulation developed from *Trichoderma harzianum* (Th3) was used as an environment friendly option. In the dual culture method, *T. harzianum* Th3 showed around 62% inhibition against *S. rolfsii* and 71.9% inhibition of *M. phaseolina.* Seed treatment, soil application and drenching with *T. harzianum* Th3 showed minimum disease incidence of 21.6% in Banswara and 12.6% in Jhabua districts. Thus it reduces the disease incidence by 66.3 to 78.1% as compared with control. In the same way, there was significant response of application of *T. harzianum* Th3 was observed on plant growth promoting parameters. at field level to increase number of pods per plant and shelling % and thereby produces maximum yield of 3.37 and 3.6 t/ha in Banswara and Jhabua district, respectively.

Key words: *Trichoderma*, groundnut root rot, seed and soil application

Introduction

Groundnut (*Arachis hypogaea* L.) is an important food and oil seed crop because of its high protein and oil content. In India, area under groundnut crop is 0.557 million ha, with an annual production of 9.14 million tons and productivity of 1007 kg/ha in the year 2013-14 (Annual Report, Agricoop, 2013-14). Groundnut stem and root rot disease complex also known as 'kalijad' (in hindi), affects the production and quality of groundnut crop, it is a seed-borne disease caused by a pathogen complex of *Aspergillus niger* Van Tieghem and *Aspergillus flavus* J. H. F. Link of the family Trichocomaceae, *Thievaliopsis basicola* (Berk. & Broome) Ferraris of the family Ceratocystidaceae, *Rhizoctonia solani* J. G. Kuhn of the family Ceratobasidiaceae and *Pythium aphanidermatum* (Edson) Fitzp. of the family Pythiaceae. The disease progresses in different forms like collar rot (*A. niger*), stem-rot (*Sclerotium rolfsii*), root-rot (*Macrophomina phaseolina = R. bataticola*), along with aflatoxin contamination (*Aspergillus flavus* and *A. parasiticus*). Awareness about the pesticides hazards to human health and environment is increasing. Use of biocontrol agent is considered a safe option to control the disease.

Trichoderma harzianum Th3 isolate was identified and developed as bioformulation at Biocontrol lab, Division of Plant Pathology, Indian Agricultural Research laboratory, New Delhi. Rigorous lab and field trials of *T. harzianum* Th3 has already been conducted against several soil borne diseases to validate the performance of this isolate against soil borne diseases of various crops (Sharma *et al*., 2012; Sharma *et al*., 2014; Jambhulkar *et al*., 2015). To carry forward our evaluation to different agro-ecological conditions, we conducted a field

trial of *T. harzianum* Th3 against stem and root rot of groundnut for two years at Agricultural Research Station Banswara, a southern most district of Rajasthan and Krishi Vigyan Kendra, Jhabua in Madhya Pradesh.

Material and methods

In vitro antagonistic activity and bioassay of T. harzianum Th3 *with* S. rolfsii *and* M. phaseolina

Dual culture method was employed to analyze whether *T.harzianum* Th3 inhibits the growth of fungus *S. rolfsii* and *M. phaseolina* as described by Sharma *et al*. (2004). The test pathogens *S. rolfsii* and *M. phaseolina* were grown in Sand maize medium. The efficacy of the biocontrol agent was tested by both seed treatment and soil application. The germination percentage, seedling vigour and root colonisation were recorded.

Determination of rhizosphere colonization

The rhizosphere region of the randomly selected plants tested above have been collected. To this end, the soil adhering to the root surface was removed by gently tapping the roots. Serial dilution technique was adopted for quantification of *Trichoderma* colony on TSM plates.

Field experimental site selection

The experiment was conducted during 2013 and 2014 rainy seasons. The field experiment was conducted at Banswara and Jhabua districts. The susceptible variety used for experiment was RSB 103-87 in Banswara and JGN-23 in Jhabua. The treatments of the experiment were T1-seed treatment with *T. harzianum* Th3 at 8 g/kg seeds; T2-soil application with *T. harzianum* Th3 enriched FYM (10: 200) + seed treatment with *T. harzianum* Th3; T3-T2 + drenching with *T. harzianum* Th3 at 4 ml/l water at 40 days after sowing; T4-seed treatment with Carbendazim 12% + Mancozeb 63% WP at 2g/kg seeds + drenching with Carbendazim 12% + Mancozeb 63% WP Saaf™ at 3g/l water at 40 days after sowing; T5-control. For soil application, formulation of *T. harzianum* Th3 was mixed with thoroughly decomposed farm yard manure (FYM) in 1:200 ratio.

Results and discussion

In the dual culture method, *T. harzianum* Th3 showed around 62% inhibition against *S. rolfsii* and 71.9% inhibition against *M. phaseolina* (Table 1).

Table 1. Antagonistic activity of *T. harzianum* Th3 against *Sclerotium rolfsii* and *M. phaseolina.*

Treatments	Radial growth of *S. rolfsii* (mm)*	% of inhibition	Radial growth of *M. phaseolina* (mm)*	% of inhibition
T. harzianum Th3	30.3 ± 0.44	62.1	23.3 ± 0.35	71.9
Carbendazim	28.4 ± 0.31	64.5	28.5 ± 0.24	65.7
Control	80.0 ± 0.10	-	83.2 ± 0.53	-

*Each value is mean of four replications ± Standard deviation

In the rhizosphere, *T. harzianum* Th3 grow profusely up to 20 days after sowing and then declined gradually (Table 2). Challenged inoculation of pathogens along with Th3 showed 87.2 x 10^6 cfu/g of soil at 20 days after sowing while in pathogen free soil the Th3 maintained maximum population of 102.1 x 10^6 cfu/g of soil at 20 days after sowing. Similarly, Jash and Pan (2007), observed a rapid increase in the antagonist population up to 20 days and in few cases up to 30 days in the rhizosphere soil. Higher growth rate with strong rhizosphere competence of the selected strains are clear indications of the better antagonistic potential.

Table 2. Root colonization of different isolates of *Trichoderma harzianum* Th3 in infected and uninfected soil.

Treatment	**Population (1x 10^6 cfu/g) at different period of time**			
	10 DAS	20 DAS	30 DAS	40 DAS
Th3+ *S. rolfsii* + *M. phaseolina*	57.3 (1.76)*	87.2 (1.94)	71.2 (1.85)	42.5 (1.63)
Th3	67.4 (1.83)	102.1 (2.00)	88.5 (1.95)	48.7 (1.69)
CD (0.01)	0.113	0.051	0.0014	0.036

*****Figure in parenthesis is log-transformed value

The seed treatment, soil application and drenching of Th3 bioformulation against stem and root rot complex of groundnut reduced significantly ($P \leq 0.05$) disease severity but also increased pod yield in both locations. Soil application of *Trichoderma* enriched FYM followed by seed treatment with *T. harzianum* Th3 reduced sclerotium rot by 54.7% in Banswara and 67% in Jhabua districts (Table 3). Minimum disease incidence of 21.6% was observed when soil application, seed treatment along with drenching of *T. harzianum* Th3 was done. This treatment reduced stem and root rot complex to the maximum extent (66.3% in Banswara and 72.9% in Jhabua) and thereby produced maximum yield of 3.37 to 3.6 t/ha.

Trichoderma sp. controls the pathogen but also improves the overall health of the host. There is significant increase in plant vigour with application of *T. harzianum* Th3 bioformulation. Treatment with *T. harzianum* Th3 as soil application, seed treatment and drenching as well as treatment with fungicide produce maximum number of pods per plant (~ 39). Thereby, maximum pod yield is also recorded by these treatments as discussed above. Maximum shelling of 72.1% is given by soil application of *T. harzianum* Th3 enriched FYM, seed treatment with *T. harzianum* Th3 + drenching with *T. harzianum* Th3 at 40 days after sowing. This is equivalent with the results obtained with the fungicide treatment (Table 4).

Table 3. Effect of application of *T. harzianum* as seed treatment, soil application and drenching for management of stem and root rot complex of groundnut.

Treatments	**Banswara**			**Jhabua**		
	% Disease Incidence Pooled	**ROC %**	**Pooled Yield (t/ha)**	**% Disease Incidence Pooled**	**ROC %**	**Pooled Yield (t/ha)**
T1- Seed treatment with *T. harzianum* Th3 at 8 g/kg seeds	32.4 (34.7)	49.4	2.79	22.2 (28.1)	61.4	2.68
T2- Soil application of Th3 with *T. harzianum* enriched FYM + seed treatment with *T. harzianum* Th3	29.0 (32.6)	54.7	3.00	19 (25.8)	67.0	3.29
T3- Soil application of Th3 with *T. harzianum* enriched FYM + seed treatment with T. *harzianum* Th3 + drenching with *T.harzianum* Th3 at 40 days after sowing	21.6 (27.7)	66.3	3.37	12.6 (20.8)	78.1	3.60
T4-Seed treatment with Carbendazim 12% + Mancozeb 63% WP at 2g/kg seeds + drenching at 40 days after sowing at 3g/l water	25.9 (30.6)	59.5	3.22	15.6 (23.3)	72.9	3.61
T5-Control	64.1 (53.2)	-	2.08	57.6 (49.4)	-	2.35
CV SEm ± CD (0.05)	13.4 0.89 2.81		14.7 0.12 0.37	11.5 1.01 3.05	-	11.4 0.14 0.41

Table 4. Effect of application of *T. harzianum* as seed treatment, soil application and drenching on pods per plant and shelling % at Banswara and Jhabua agroclimatic conditions during 2013 and 2014 kharif seasons.

Treatments	**Pooled Pods per plant**	**Pooled Shelling %**
T1- Seed treatment with *T. harzianum* Th3 at 8 g/kg seeds	32.65	67.3 (55.1)
T2- Soil application of Th3 with *T. harzianum* enriched FYM + seed treatment with *T. harzianum* Th3	36.45	68.6 (55.9)
T3- Soil application of Th3 with *T. harzianum* enriched FYM + seed treatment with T. *harzianum* Th3 + drenching with *T.harzianum* Th3 at 40 days after sowing	39.25	72.1 (58.1)
T4-Seed treatment with Carbendazim 12% + Mancozeb 63% WP at 2g/kg seeds + drenching at 40 days after sowing at 3g/l water	39.95	72.0 (58.1)
T5-Control	28.70	65.7 (54.2)
CV SEm ± CD (0.05)	13.4 0.60 1.77	11.8 0.36 1.13

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Biological and integrated control of plant pathogens
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Over 30 years of research, more than 15 years of using *Bacillus subtilis* and production of other beneficial microbes by ABiTEP – a vision of a sustainable agriculture becomes reality

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Abstract: A collaboration of the Humboldt University Berlin, Department of Phytomedicine, the Research Center for Biotechnology (FZB) and the company 'Berliner Stadtgüter' was launched in 1984 to select *Bacillus* strains from soils with the aim of reducing soilborne plant pathogens such as *Rhizoctonia, Fusarium* and *Verticillium*.

Initial successes in horticulture was found in cooperation with Schering AG and since 1994 with Bayer Crop Science using the product FZB24® – marketed as a plant strengthener in Germany, Austria and Switzerland since 1999.

In the meantime FZB24®, RhizoVital®42 and other products were renamed as *Bacillus amyloliquefaciens* ssp. *plantarum* and their application in many cultures as coating or drenching agent where successfully proved, especially in potatoes, vegetable crops and ornamental plants.

Extensive scientific investigations of the mechanism of action in *Bacillus* took place within the framework of various research projects funded by German ministries. It has been shown that yield increases and improvement in the quality of the harvested products are possible without using or in combination with chemical fungicides. That is due to the microbial competition for nutrients and settlement area at the root.

No residue loads and the use of sustainable, environmentally friendly manufactured products distinguish this modern trend in agricultural production.

The company ABiTEP GmbH in Berlin – certified by GMP + B2 and working conform to DIN ISO 9001:2015 – performs intensive research in order to optimize the use of *Bacillus amyloliquefaciens ssp. plantarum*, ensures high quality of products and advises farmers and gardeners for over 10 years now in the targeted application of microbial products to protect the environment and to conserve the biodiversity.

Key words: *Bacillus*, FZB24, agriculture

Biological and integrated control of plant pathogens
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In vitro and *in vivo* co-inoculation of soil biocontrol microbial agents: methods for the evaluation of their persistence and performance

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Abstract: Two species of fungi, *Beauveria bassiana* (Bals.-Criv.) Vuill. and *Beauveria brongniartii* (Saccardo) Petch were jointly applied in the field as biological agents in strawberry plantations in Poland to control *Melolontha melolontha* L. (European cockchafer). We adapted a genotyping approach based on SSR (Simple Sequence Repeat) marker to a discriminating tracing of the two inoculants *in vitro* and in soil. We analysed the behaviour of the two fungal species *in vitro* in the presence of different carbon sources, to assess possible antagonistic and/or synergistic effects of the consortium. Further, we used SSR markers in the assessment of inoculants persistence and traceability in soil after their field application.

Key words: *Beauveria bassiana*, *Beauveria brongniartii*, carbon sources, integrated control, *Melolontha melolontha*, traceability

Introduction

Entomopathogenic fungi act as natural regulators of insects' populations and in many cases have some very species-specific actions that can be exploited to control agricultural pests. Naturally occurring biocontrol phenomena often result from complex assemblages of antagonistic species rather than from a single antagonist or parasitic organism (Mishra *et al.*, 2013). The combination of different biocontrol organisms applied to control pests can help to increase their efficacy (Jaronski, 2010). However, the action of consortia can lead to either increased activity of some enzymatic activities related to biocontrol mechanisms (Hua *et al.*, 2011) or inhibition between the microorganisms (Mishra *et al.*, 2013). Thus it is worthwhile to test the degree of compatibility between different organisms or strains before the formulation of combined biocontrol mixture for plant protection. Moreover, when applying biocontrol fungi to soil, the assessment of their persistence in the environment is fundamental to fine tune the method and dose of application. Protocols are therefore needed to better understand the effects of mixed inocula on agricultural systems and the survival or dominance of the strains, in order to optimize their blending and improve their efficacy. In this study a culture independent method based on qPCR with SSR (Simple Sequence Repeat) markers was used for the detection and quantification of two fungal inoculants *in vitro* and in soil, after field application. The proposed method, even though set up for tracing entomopathogenic fungi, could also be adapted to study the behaviour and persistence of fungi used as control

agents against plants' pathogens. Specificity of SSR markers was verified using: i) mixed inocula of the fungi on different carbon sources; ii) soil samples from field trials treated with mixed inocula.

Material and methods

In vitro experiment

The isolates *B. brongniartii* (ID KT932309) and *B. bassiana* (ID KT932307) described in Canfora *et al.* (2016) were singly and jointly inoculated in microarrays containing different carbon sources. We utilized a method based on FF MicroPlate™ (Biolog, Inc., Hayward, California, USA) which is a commercial multiwell plate that contains low-molecular weight carbon sources (Bochner, 2003). The inoculation procedure was based on the protocol used by Tanzer *et al.* (2003). The joint inoculum consisted in a mixture of equal volumes of the single strains spores' suspensions, which had a final optical transmission of 80-75% at 590 nm. Measurements of optical density of the plates with a spectrophotometer at 750 nm were used to quantify fungal biomass development in the different wells (Tanzer *et al.*, 2003).

Three biological replicas of the fungal biomass were sampled after 230 h incubation from the wells of the microplate containing N-Acetyl-L-Glutamic Acid, and D-Mannose as substrate. DNA was extracted from the mycelium of these samples using the PowerSoil DNA Isolation Kit (Mo Bio Laboratories, Carlsbad, CA) according to the manufacturer's protocol. The concentration of DNA crude extracts was checked by Qubit® 2.0 Fluorometer kit, following manufacturer's instructions, and stored at -20 °C for further PCR analysis.

Field experiment

The field experiment was established near Brzostówka (Lublin, Poland) in fields (about 1 ha) naturally highly infested by *M. melolontha* larvae. The trial was carried out on an established strawberry plantation. The inocula of *B. bassiana* or *B. brongniartii* were applied as an aqueous suspension. Each inoculum was applied singly at a dose of 15 kg/ha to the soil, splitted into two applications with three weeks interval. When the two species were applied together, 7.5 kg/ha of each inoculum was distributed with the same application schedule. The applications were carried out in June, near the strawberry plants row. Soil samples were collected three weeks after the last application. Ten sub-samples of about 50 g each were gathered from each plot and then they were mixed to obtain a composite sample used for the DNA analyses. They were collected either in the vicinity of the plant roots or in the inter-row.

Fungal quantification from in vitro and field experiments

A culture-independent method based on Real Time qPCR was used for the quantitative discrimination of fungal growth and persistence in the in vitro joint inoculum trials, and in the field trials. Simple sequence repeat (SSR or microsatellites) markers, species specific, were used in a qPCR protocol developed for the discriminant and simultaneous detection and quantification of *Beauveria bassiana* and *B. brongniartii* (Canfora *et al*., 2016). The genetic marker used for quantifying *B. brongniartii* with this method was the SSR amplified by the primers pair of the locus Bb4H9, while that used to quantify the biomass of *B. bassiana* was the SSR amplified by the primers pair of the locus Ba01. The protocol was applied to fungal DNA extracted from both the soil samples and from the microplate wells containing the fungal co-inoculums. Real Time results were analysed using linear ANOVA ($\alpha = 0.05$).

Results and discussion

The results obtained from soil samples inoculated with *B. bassiana* or *B. brongniartii* and collected from the strawberry plants' roots (R) and from the inter rows (IR) are compared in Figure 1.1. The persistence of *B. bassiana* was detected only in soil samples gathered close to plants' roots (R) while the samples collected from inter rows (IR) did not result in a statistically significant amount of PCR product, even after a significant number of PCR cycles. *B. brongniartii* was absent, or present in undetectable amounts, in both inter rows (IR) and strawberries' roots (R) samples.

The different carbon sources in the microplates differentially affected the fungal development: N-Acetyl-L Glutamic Acid depressed the growth of co-inoculated fungi in comparison to the single strains, while D-Mannose triggered the fungal biomass development of the co-inoculum (Figure 1.2). Moreover, the growth of the two fungi was also differentiated when co-inoculated, as shown by the different gene copy number measured by RT qPCR of *B. bassiana* and *B. brongniartii* growing jointly on the two substrates (Figures 1.3 and 1.4). The mycelium of *B. brongniartii* was dominant in the joint inoculum grown on N-Acetyl-L Glutamic Acid, while *B. bassiana* was dominant when the consortium was grown on D-Mannose. The role of these compounds in triggering or depressing the co-cultivation of the two fungi could be related to the different ecological behaviour of the two species, purely entomoparasitic in case of *B. brongniartii*, also saprophytic for *B. bassiana* (Cory & Ericsson, 2010). A deeper study on the relationship between nutrition, conidia production and co-occurrence of the two species is the object of a larger study currently underway.

Cultivation-independent multilocus genotyping, combined with the qPCR (qPCR-SSR analysis), allowed detecting with high specificity and sensitivity the fungal inoculants in soil, even when present in a very low concentration. The lack of detection of *B. brongniartii* after the application under field conditions, unlike *B. bassiana*, which could be related to the ecology of the species or an insufficient dose applied, showed the potentiality of the method to evaluate the efficacy of biocontrol treatments to soil. The possibility of using the qPCR-based tracing method here described for studying *in vitro* the compatibility, and degree of metabolic overlap, between species of entomopathogenic fungi discloses wide fields of investigation useful to support the formulation and/or application method of inocula for pest control.

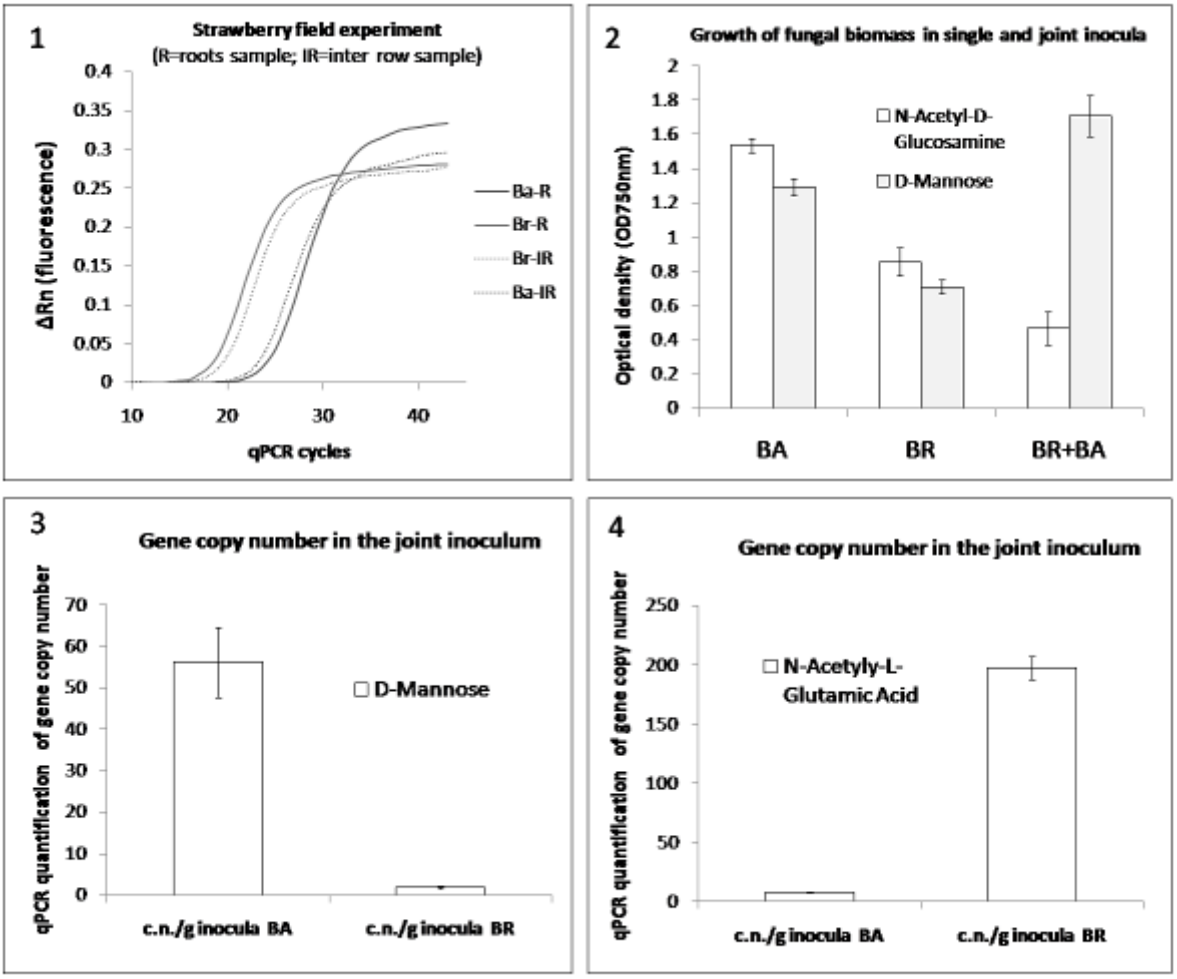

Figure 1. (1) Relationship between PCR cycle number and PCR product amount; comparison of results obtained from soil samples inoculated with *B. bassiana* or *B. brongniartii* and gathered from the strawberry plants' roots (R) and from the inter rows (IR).
(2) Fungal biomass (OD at 750 nm) developed by single inoculum of *B. bassiana* (BA) and *B. brongniartii* (BR), and by their joint inoculum (BA + BR), in the wells of microplates containing N-Acetyl-L-Glutamic Acid or D-Mannose.
(3) Fungal biomass of *B. bassiana* (BA) and *B. brongniartii* (BR), measured by qPCR, when growing in a joint inoculum on N-Acetyl-L-Glutamic acid. Units expressed as gene copy number for gram of inoculum (c.n./g inoculum).
(4) Fungal biomass of *B. bassiana* (BA) and *B. brongniartii* (BR), measured by qPCR, when growing in a joint inoculum on D-Mannose. Units expressed as gene copy number for gram of inoculum (c.n./g inoculum).

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In vitro screening of *Trichoderma* species isolates for potential bio-control of black foot disease causing pathogens in grapevine nurseries

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Abstract: Black foot disease (BFD) of grapevines is a decline and dieback disease caused by a soilborne pathogen complex including *Ilyonectria*, *Dactylonectria* and *Campylocarpon* species. Grapevine nursery plants get infected by BFD pathogens present in the soil. Apart from hot water treatment of dormant nursery plants, no treatment is available to prevent nursery plants from becoming infected with BFD pathogens after planting. *Trichoderma* species are well known biocontrol agents and have shown good efficacy to prevent grapevine trunk disease infections of pruning wounds. However, the efficacy of *Trichoderma* species against BFD pathogens has not been determined *in vitro*. The following *Trichoderma* species *Trichoderma atroviride*, *T. fertile*, *T. harzianum* and *T. virens* were evaluated *in vitro* against four of the most commonly occurring BFD pathogens, *Ilyonectria liriodendri*, *Dactylonectria macrodidyma*, *Campylocarpon fasciculare* and *Campylocarpon pseudofasciculare*. The effects of volatile and non-volatile antibiotics produced as well as the direct antagonistic effects were determined in Petri dish assays. The percentage growth inhibition was determined for both the volatile and non-volatile assays. Microscopic observations were made of the interaction zone for the dual plate assay. Preliminary assays with *T. atroviride* and *T. harzianum* showed very good inhibition of *Ilyonectria* and *Campylocarpon* species for both volatile and non-volatile antibiotic tests. The assays with the fuller array of isolates are currently underway.

Key words: *Trichoderma*, black foot, grapevines

Protection of apple and pear flowers against fire blight infections using biocontrol organisms applied via bumble bees

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Abstract: During bloom, apple and pear are susceptible to fire blight infection which occurs when *Erwinia amylovora* bacteria colonize the stigma and subsequently reach, due to rain or dew droplets, the flower hypanthium where they penetrate the openings of the nectary or nectarthodes. First the flowers necrotize then the peduncles, the shoots and finally the complete stem. While the disease gradually spreads through the tissues, ooze droplets are formed that contain high concentrations of bacteria. In Belgium the infection risk increases with increasing temperature making the secondary bloom later in the season more vulnerable than the primary bloom in early spring when night temperatures are still low and frost occurs. Similarly, trees that are planted late in the season and flower in summer as well as young fruits and shoots damaged by hail in the summer can become rapidly infected by *E. amylovora.* This infection is worsened if ooze droplets are present in the orchard. The current strategies for control focus on preventive treatments such as sprayings with plant defense enhancer molecules like fosethyl aluminium (Aliette®) and laminarin (Vacciplant®) as well as with heavy metals including copper and manganese that help to reduce the inoculum in the orchard during the season. To avoid phytotoxicity, Aliette® treatments are done before and/or after bloom making the use of biocontrol organisms (BCOs) during bloom an attractive approach to protect the flowers.

Although the antagonistic yeast *Aureobasidium pullulans* (Blossom Protect™) that can block flower colonization by *E. amylovora* is registered in Belgium, its use is limited today. As flowers gradually open, the BCO should be sprayed at least two to three times during bloom to protect all flowers, which is time consuming and expensive. In the research project that is presented here, we aim at a continuous application of the BCO at the sites of flower infection (*i.e.* stigma and hypanthium) by bumble bee (*Bombus terrestris*) vectoring. Besides *A. pullulans*, also existing BCO strains of *Bacillus subtilis* and *Pantoea agglomerans* as well as a new strain of *Paenibacillus polymyxa* are tested. A search for new candidate BCOs naturally present in apple and pear flower microbiomes is also ongoing. Initial results show that (i) under optimal infection conditions there is a risk of *E. amylovora* spread by bumble bees after visiting infected flowers (ii) BCOs are acquired to a variable degree by bumble bees, (iii) in 2015 primary bloom flower visitation in the orchard was absent in pear and limited in apple, whereas bumble bees have better visited secondary flowers and (iv) tested BCOs show potential for fire blight control in the greenhouse.

Key words: fire blight, entomovectoring, pipfruit

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Biological control methods for soil-borne pathogens in organic onion production – the on-farm study

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Abstract: *Fusarium* basal rot (FBR), caused by *Fusarium oxysporum* f. sp. *cepae* and *F. proliferatum*, is the most important soil-borne disease of onions in Finland. *Fusarium proliferatum* was identified for the first time in Finland in 2015. This species has proven to be highly pathogenic on onion. The pathogen causes economic losses by reducing onion quality and yield both in the field and during storage. Although this disease is soil borne, the significant sources of infection are contaminated onion sets, which are almost completely of foreign origin. During the last few years *Fusarium* infections have increased in Finland. This paper describes the effect of biological pesticides and microbial fertilizers against *Fusarium* species in farm trials.

The effect of onion sets treatment with biological pesticides (Prestop®, Mycostop®) and microbial fertilizers (FZB24®, Rhizo Vital 42®, Rhizocell) were tested in pot and field trials located in South-Karelia, South-Savo and North-Savo regions in Finland. The bulbs were treated by submerging into the treatment solution or by spraying with the treatment solution. The part of the bulbs was planted immediately after treatment and another part was dried 2-3 days or 9-10 days. After harvesting onion bulbs were visually graded and stored for 4-5 months. The occurrence of the fungi and the losses in weight of the onions during storage were evaluated.

Biological control methods of onion bulbs had no clear positive effect on the quality of organic onions in the trials. No differences were found in either of the biological pesticides or microbial fertilizers. The results indicated that the dipping treatment without any drying period before planting is not an effective method because the moisture remains in bulbs which may activate *Fusarium* fungi. Instead, the effect of spraying seemed to be more beneficial. Effectiveness of biological control was modest probably due to the utilization of highly infected onion sets in the farms.

Based on this study, different disease control methods still require further research. Solving the disease problems of the onion cultivation will require the development of alternative production and biological control methods.

Key words: organic onion, biological control, *Fusarium* basal rot

Biological control of potato diseases: control of *Phytophthora infestans* and *Alternaria solani* by indigenous Belgian bacteria

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Abstract: Potato is one of the most important crops in Belgium. The management of fungal diseases, especially potato late blight (*Phytophthora infestans*) and early blight (*Alternaria solani*), is a major concern for farmers. In order to reduce the use of chemical pesticides, the present study aims at taking benefit from bacteria isolated in Belgium for the biological control of these diseases. More than 2600 bacteria belonging to the *Bacillus* and *Pseudomonas* genera were isolated from fields, manure, composts, vegetable gardens and potato plants. The antagonistic activities of bacteria against *P. infestans* and *A. solani* were evaluated *in vitro* using high throughput screening assays. Several strains with promising potential to control both diseases were tested by direct confrontation tests on solid medium. The best candidates were then used for *in vivo* tests on whole plants in greenhouses. These bacteria were evaluated for their ability to protect potato plants from subsequent challenge infection by either *P. infestans* or *A. solani* under controlled conditions. Phytotoxicity was also evaluated on plants. The spraying of chosen bacterial strains reduced significantly the fungal infection of plants and protected the whole plants from secondary infections. Moreover, none of them caused damage on plants. These results encourage the use of indigenous Belgian bacteria for the biological control of fungal diseases in the field. Tests in fields are under progress in order to evaluate the potential use of some antagonistic bacteria in an "integrated pest management" approach. Performances of our strains will be compared with conventional and biological approaches for potato diseases management.

Key words: antagonistic activity, *Bacillus* spp., biocontrol, field trials, *Pseudomonas* spp.

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Influence of mode of application and strain effect on the antagonist efficacy of bacterial strains to control the fungal pathogen *Neofusicoccum parvum* in grapevine

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Abstract: Grapevine trunk diseases (GTDs), such as Esca and *Botryosphaeria* dieback, markedly impact the worldwide wine grape and table grape industry. Detection and development of antagonistic microorganisms, particularly bacteria, to achieve biological control of GTDs would be of prime importance as a future innovative alternative practice in viticulture. Our aim was to select bacterial strains, among 46 strains issued from Bordeaux vineyards, showing significant antagonism activity against *Neofusicoccum parvum*, one of the major pathogenic fungi involved in GTDs. Different bioassays performed under greenhouse conditions with foliar grapevine stem cuttings have shown that the inhibition of the two types of the pathogen symptoms, *i.e.* necrosis and canker, depends on both the bacterial strain effect and the application method to the plant. The effectiveness of different strains of *Pantoea agglomerans* and *Enterobacter* sp., as potential biological control agents against *N. parvum*, was demonstrated (see also Haidar *et al.*, 2016). By comparing three methods of bacterial application, our results concerning inhibition of the fungal canker showed that preventive inoculation of the bacterial strain into the cutting trunk wood (at the inoculation hole) was the most efficient method. The two other methods tested were co-inoculation with the pathogen at the inoculation hole, and soil inoculation by drenching.

Key words: *Vitis vinifera*, grapevine trunk disease, Esca, bacteria, biological control

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Opportunistic endophytism of *Trichoderma* spp. and its biocontrol activity against *Rhizoctonia solani* causing sheath blight in rice

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Abstract: In this study we explored opportunistic endophytism of *Trichoderma* spp. in rice [Pusa basmati-1 (PB-1)]. Pusa basmati-1 was subjected to soil and seed treatment with five isolates. After 28 days, the root samples were assessed by microscopic analysis (light and SEM) and isolation of endophytic fungi from root samples revealed that the isolated endophytic fungus were identical to the inoculated ones. These results were further confirmed by PCR amplification of the rDNA region and *tef* 1 with the newly isolated *Trichoderma asperellum* and *T. asperelloides.* Consequently, the *in vitro* (dual culture assay) and *in vivo* (plant infection) studies of the antagonistic activity of the five isolates against *Rhizoctonia solani* showed that all isolates significantly inhibited the growth of the pathogen. In both treatments, TaR3 proved to be the best isolate. The mean horizontal spread range and relative lesion height were studied in both seed and soil treatment. In this study, it has been reported that *T. asperellum and T. asperelloides* is endophytic in rice upon inoculation through seed and soil treatment. This may indicate the role of *T. asperellum and T. asperelloides* as endophytes protecting the host against biotic stress.

Key words: endophytism, *Trichoderma* spp., biological control

Introduction

Endophytic fungi have some important roles in nutrient cycling, biodegradation and bioremediation. Endophytes are involved in nutrient pedaling where dead biomasses gets degraded and is returned back to the environment. Endophytes can also be used to remove contaminants and wastes from the atmosphere. Apart from the clear fact that they help in uptake of essential nutrients necessary for plant growth it is also known that they help in anchorage of plants in soil, absorption of water and ions, and nutrient storage. Furthermore endophytes can be anti-cancerous. Even though few species of *Trichoderma* have been isolated as endophytes, it might be that other species can be facultative endophytes. *Trichoderma hamatum* for instance is an endophyte as well as common inhabitant of soil and rhizosphere. It is not known whether obligate endophytic *Trichoderma* spp. can be present in roots. The relationship between endophytic *Trichoderma* spp. and the plant is yet being investigated to understand whether there is some potential fungal prey and plant root-derived nutrients that encourage the internal colonizing of plant roots or whether presence of certain proteases, chitinases and secondary metabolites is playing a role in endophytism (Druzhinina *et al.*, 2011). In depth studies of *Trichoderma* as an endophytes and its importance in antagonistic nature against *R. solani* in rice plants has not been investigated so far.

Material and methods

Isolation of endophytic fungi in rice fields

To study endophytism, we collected 28 days old healthy Pusa basmati-1 rice plants from different plots belonging to the Division of Genetics, ICAR-IARI, New Delhi. Plant pieces were washed five times in sterile double distilled water. Sterility checks of the root samples were done by plating out 0.1 ml from the fifth rinse onto Potato Dextrose Agar and *Trichoderma* specific media in Petri plates and incubating plates for 6 days. The fungi were identified by their microscopic and macroscopic characters.

Trichoderma inoculum preparation and treatment

Trichoderma isolates (TaR1,TaR2, TaR3, TaR4 and TaR5) were applied to soil and seeds. Soil was treated with *T. asperellum* and *T. asperelloides* spore suspensions (2×10^6 spores/ml) 5 days before sowing of PB-1. For seed treatment, 5ml of conidial suspension (2×10^6 spores/ml) was used per 10 gram of seed and were thoroughly mixed in a small plastic container with constant shaking to obtain uniform coating. Seeds were kept overnight and were sown on the next day at the rate of 20 seeds/pot. The pots were prepared by using sterilized soil (pH: 6.5-7.5 and 100% moisture) along with FYM (4:1) to avoid microbial contaminations. Control pots were prepared with untreated seeds and untreated soil.

Isolation and identification of opportunistic endophytic fungi from treated roots

Endophytic fungi were isolated from roots to confirm endophytism in rice. Freshly uprooted roots were washed under tap water to remove soil debris, dried and washed with SDW. The roots were allowed to dry on blotter paper followed by cutting them into small pieces (~1 cm). Subsequently, pieces were immersed in 70% ethanol. The surface sterilization process continued by dipping the root pieces in 2.5% sodium hypochlorite for 2 minutes. The roots were then rinsed five times in SDW. Sterility checks for the root samples were done by plating 0.1 ml from the fifth rinse on PDA and TSM followed by an incubation at 28 ± 2 °C for 5-6 days. Surface sterilized roots were dried on sterilized blotter papers, crushed under aseptic conditions and finally plated on PDA. The plates were incubated for 5 days at 28 ± 2 °C. After the appearance of fungal growth, all the isolates were subcultured and further characterized. The identification was based on conidia shape, colony appearance, phialides characteristics and branching patterns of conidiophores (light microscopy and SEM). Molecular identification was based on DNA sequencing of the ribosomal ITS region (28s rDNA, 18s rDNA, 5.8s rDNA, ITS1 and ITS2*)* and *TEF1* region.

Evaluation of antagonistic activity of Trichoderma* spp. *against R. solani

Mycelial discs from 7 days old cultures of *Trichoderma* spp. and *R. solani* were placed at equal distance from the periphery on PDA plates and kept at 28 ± 1 °C. Pathogen alone served as a control. Standard procedure was used to measure the percentage of inhibition. Inoculum of the sheath blight pathogen was prepared on shoots of water sedge (*Typha angustata*) (Bhaktavatsalam *et al.*, 1978), and inoculated at maximum tillering stage (45 DAS) by placing the colonized *Typha* pieces in between rice tillers. Standard scale was used for disease scoring.

Results and discussion

Endophytism is one of the important tools in the use of biocontrol agents to control plant diseases as well as to boost up the plant growth (Sharma *et al*., 2014). In India, *Trichoderma harzianum* and *T. viride* have been fully investigated and exploited as potential antagonists. Up to date *T. harzianum* and *T. viride* has been commercialized successfully. *T. asperellum and T. asperelloides* are also important biocontrol agents which are close to exploitation and commercialization. In this study different segments of rice tissues (stem, leaves and roots) were used to explore endophytes found in rice Pusa basmati-1 (PB-1) under natural conditions. But we found the four genera of fungi *Penicillium* sp., *Alternaria* sp., *Fusarium* sp. and *Aspergillus* sp. There was no occurrence of *Trichoderma* species in natural conditions. Three isolates of *T. asperellum* (TaR1, TaR2 and TaR3) and two isolates of *T. asperelloides* (TaR4 and TaR5) collected from different agro-climatic zones Rajasthan, India, (Table 1), were used and morphological characters reveled conidiophores which were loosely tufted and regularly branched with side branches at right angles to the branches. The conidiophores were crowded. The conidiophores terminated with flask shaped phialides which were swollen at the middle. The phialides were either single or formed a whorl of 2-4 divergent phialides.

Table 1. Details of the *Trichoderma* cultures used in this study.

SL. No.	Isolate Name	Isolate Code	Source & Place of collection	ITCC No.	NCBI No.	
					ITS	*Tef1*
1	*Trichoderma asperellum*	TaR1	Soil & Vegetable field, Jaipur, Rajasthan	7845	KT001076	KT722733
2	*Trichoderma asperellum*	TaR2	Soil & Vegetable field, Jaipur, Rajasthan	7846	KT001077	KT722734
3	*Trichoderma asperellum*	TaR3	Soil & Vegetable field, Jaipur, Rajasthan	7847	KT001078	KT722735
4	*Trichoderma asperelloides*	TaR4	Soil & Vegetable field, Jaipur, Rajasthan	7848	KT722738	KT722736
5	*Trichoderma asperelloides*	TaR5	Soil & Vegetable field, Jaipur, Rajasthan	7849	KT722739	KT722737

Treatment with *T. asperellum* isolates and *T. asperelloides* isolates was done by two methods *viz* soil inoculation and seed treatment. For soil inoculation the soil was treated with *T. asperellum* and *T. asperelloides* spore suspension with 2×10^6 spores/ml. At 28 days after sowing of seed and soil treatments, rice plants were assessed by light microscopy. *T. asperellum* and *T. asperelloides* hyphae were observed inside the roots, indicating that the inoculated isolates were growing inside the root zone. Roots samples were subjected to SEM analysis. *Trichoderma* mycelium was observed in all root samples (Figure 1) for both soil and seed treatment.

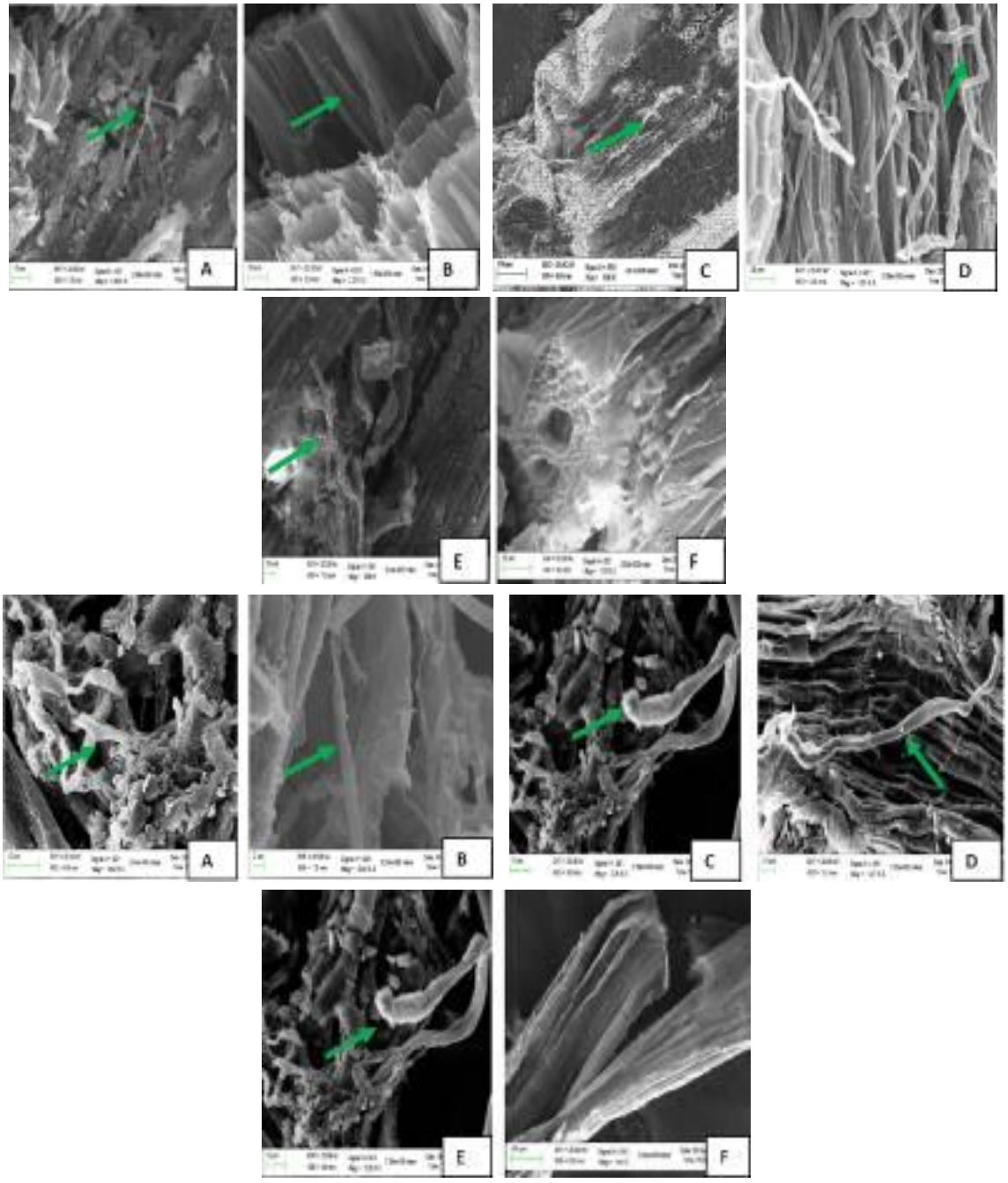

Figure 1. SEM photos showing internal colonization of Pusa basmati-1 (PB-1) rice roots treated (seed at the top and soil at the bottom) with *Trichoderma asperellum*: (A) TaR1 (B) TaR2 (C) TaR3 and *Trichoderma asperelloides* isolates: (D) TaR4 (E) TaR5 and (F) Control image showing no mycelial growth isolated from *Trichoderma* untreated Pusa basmati-1 rice roots.

Re-isolated fungi from the root samples were further subjected to molecular analysis by amplification of ITS and *Tef1* gene and the results confirmed the identification (Figure 2). Phylogenic tree results for the ITS and *Tef1* gene also confirmed that both the inoculated and re-isolated *T. asperellum* (TaR1, TaR2 and TaR3) and *T. asperelloides* (TaR4 and TaR5) isolates were within the same cluster. It clearly infers that all the five isolates are endophytic

in rice roots. This observation is matching with a recent study, where *T. asperellum* isolates were re-inoculated in cacao seedlings through the roots and they were recovered from the roots and stems after one month (Rosmana *et al.*, 2015). In dual culture tests, TaR3 mean percentage inhibition of *R. solani* was 86.84% while its relative lesion height at twenty days in soil and seed treatment was the lowest at 38.9% and 40.2%, respectively. The mean horizontal spread ranged from of 72.87 to 92.93% in the soil treatment and 78.4% to 93.33% in the seed treatment. *TaR3* showed the lowest mean horizontal spread of 72.87% in the soil treatment and 78.4% in the seed treatment. This is an example of opportunistic endophytism since the *Trichoderma* species is not endophytic but introduced through seed and soil entering the root system and surviving in the roots.

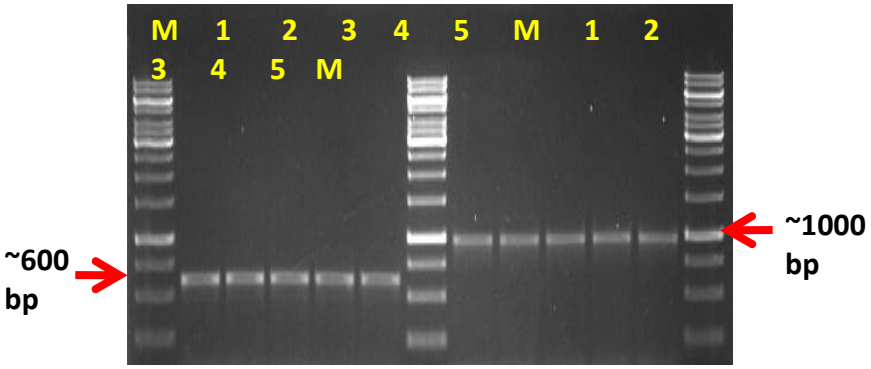

Figure 2. ITS and *Tef1α* gene amplification of *Trichoderma* isolates using primer pair ITS1/ITS4 and EF1F/TEF1R respectively. M: 1Kb DNA ladder; Lane 1: TaR1; Lane 2: TaR2; Lane 3: TaR3; Lane 4: TaR4 and Lane 5: TaR5.

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Pythium oligandrum root colonization of grafted vines and protection against the Grapevine Trunk Disease pathogen, *Phaeomoniella chlamydospora*

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Abstract: Esca is the most destructive Grapevine Trunk Disase (GTD). It causes substantial losses in vineyards, for instance 13% of the French vineyard is unproductive because of Esca. No efficient chemical product is registered to control this disease but a biocontrol strategy is presently developed. *Pythium oligandrum*, a biological control agent, frequently isolated from the roots of grapevines planted in the Bordeaux region (France), displays interesting properties to control grapevine diseases, including Esca. *P. oligandrum* root colonization of cv. Cabernet Sauvignon cuttings (not grafted) reduced by half the necroses caused by *Phaeomoniella chlamydospora*, a pathogen involved in Esca, In order to apply *P. oligandrum* in nurseries and vineyards that mainly used grafted plants, plant protection induced by the oomycete on grafted plants has to be verified. A greenhouse assay showed that *P. oligandrum* colonizes rootstock (SO4) roots over the whole experiment period. Moreover, necroses in the trunk and *P. chlamydospora* DNA quantities in the rootstock were reduced in *P. oligandrum* inoculated plants.

Key words: Esca, biocontrol, grafted vines, *Pythium oligandrum*, *Phaeomoniella chlamydospora*

Introduction

Esca is the most destructive disease that affects the woody tissues of grapevine (*Vitis vinifera* L.) in Europe. This disease has increased over the last 20 years. This increase caused considerable losses of productivity and vine death. In France, it was estimated that around 13% of vineyards are unproductive due to trunk diseases, mainly Esca (Grosman & Doublet, 2012). To control this disease, no efficient chemical molecules are registered, since the ban of sodium arsenite in the early 2000's in many grape-producing countries (Bertsch *et al*., 2013). Therefore, the use of alternative control methods, such as biocontrol, has to be developed. This plant protection strategy has been employed since many microorganisms are able to protect the vine against pathogenic attacks, including Esca. Recently, a study showed that the necrosis on the grapevine cultivar Cabernet Sauvignon (not-grafted cuttings) caused by *Phaeomoniella chlamydospora*, were significantly reduced (40-50%) when the oomycete *Pythium oligandrum* colonized the root system of these young vines (Yacoub *et al*., 2016). However, since 1870, all traditional European grape varieties are grafted on resistant American vines (rootstocks), to control the phylloxera (*Daktulosphaira vitifoliae*) attacks. So,

it seems useful to assess the ability of *P. oligandrum* to colonize and protect young grafted grapevine (made of a rootstock: the SO4 and a scion: the Cabernet Sauvignon) against *P. chlamydopsora* attacks.

Material and methods

Plant culture and experimental layout

The experiment was conducted in 2014 on one-year-old cv Cabernet Sauvignon (*V. vinifera* L.) vines grafted to SO4 rootstock. Plants were provided by the grapevine nursery company, Mercier Frères (France). Young grafted vines were grown in a greenhouse in which the temperature was maintained between 22 °C and 28 °C. A total of 90 plants were used in this experiment.

Treatments consisted of plants (i) inoculated at the root level with *P. oligandrum*, (ii) infected only by the pathogen fungus *P. chlamydospora* at the trunk level, (iii) inoculated on roots with *P. oligandrum* and then one week later with *P. chlamydospora* at the trunk level, (iv) control plants not inoculated with microorganisms, (v) mock inoculated plants to mimic the first step of the procedure infection of *P. chlamydospora*. Treatments, in which plants were inoculated with the pathogen, included 30 plants per treatment. For the other treatments, 10 plants were used.

Oomycete and fungal inoculations

Inoculum of *P. oligandrum*, strain Po37 was prepared by Biovitis SA (Saint Etienne Chomeil, France). At the 5-6 leaf stage, plants were inoculated twice with *P. oligandrum* inoculum at the collar level of each plant: one time with 50 ml of inoculum and then again with 50 ml, three days later.

The inoculum concentration was 2×10^4 oospores per ml. Seven days after the first root inoculation with *P. oligandrum*, infection with *P. chlamydospora* strain SO37 (INRA-UMR SAVE collection, Bordeaux, France) was carried out according to Laveau *et al*. (2009). Briefly, the stem of each plant was surface-sterilised and artificially wounded by drilling a hole (2 mm diameter, 5mm deep) at the scion level. Then, the inoculation site was immediately covered with paraffin wax, in order to prevent contamination with other fungi.

Plant tissue samplings

Roots samples were collected at 3 time points: 60 dpi (60 days post infection with *P. chlamydospora*), 90 dpi and 120 dpi. For each time, 9 plants per condition (3 plants * 3 replications) were sampled. The sampled roots were used to assess *P. oligandrum* root colonization. *P. oligandrum* assessment was made by using 2 methods: plate-counting and quantitative PCR (qPCR).

Wood samples were collected at the end of experiment (120 dpi). Depending of the treatment, 30 or 10 plants were collected, the stem of each plant was cut longitudinally and the wood necrosis lengths were measured. Then, wood samples were stored at -20 °C in order to quantify *P. chlamydospora* DNA by using qPCR. For each plant, wood samples from rootstock and scion were analysed separately. For each treatment, 9 plants were randomly grouped to obtain 3 biological repetitions.

Assessment of grapevine root colonization by P. oligandrum

Root colonization by *P. oligandrum* was assessed using two different approaches: plate counting method and qPCR. For the first one, the presence of *P. oligandrum*, at the root level was determined according to the protocol described by Gerbore *et al*. (2014). For qPCR analyses, DNA extraction from root samples was carried out using the Invisorb Spin Mini Kit (Invitek), according to the manufacturer's instructions, and *P. oligandrum* root colonization was evaluated by qPCR as described by Vallance *et al.* (2009).

For all analyses, the differences between treatments were evaluated by Kruskall-Wallis test ($p < 0.05$) using software R.3.1.2.

Assessment of plant protection induced by P. oligandrum against P. chlamydospora attack

The rate of necrosis was obtained by calculating the ratio between the length of necrosis and the total length of the stem. Analysis of variance (ANOVA) was carried out to assess differences between treatments, using the software R.3.1.2. For qPCR, DNA extraction from wood samples was carried out using a commercial kit: the DNeasy Plant Mini Kit (Qiagen) with some modifications (Pouzoulet *et al*., 2013). After DNA extraction, qPCR reactions were achieved according to Pouzoulet *et al*. (2013). As regards *P. chlamydospora* DNA extraction, the protocol was the same as the one used for *P. oligandrum*.

Results and discussion

Assessment of root colonisation by P. oligandrum

In order to assess the colonisation and persistence of *P. oligandrum* on the roots, the percentage of root pieces from which *P. oligandrum* echinulated-oospores were recovered, was calculated. Table 1 shows that root colonization by *P. oligandrum* remained relatively steady over the whole experimental period. The highest colonization levels were observed at 60 dpi with 52.59% and 55.56% of colonized roots, when plants were inoculated, respectively, with *P. oligandrum* and the 2 microorganisms. These percentages decreased at 90 dpi but, at the end of experiment (120 dpi), the root colonization rates by *P. oligandrum* increased without reaching those measured during the first sampling point. The highest amounts of *P. oligandrum* DNA quantities evaluated by qPCR were detected at 60 dpi: 9.28×10^{-2} pg/µl for plants inoculated with *P. oligandrum* and 3.78×10^{-2} pg/µl for those inoculated with 2 microorganisms (Table 1). At 90 dpi and 120 dpi, DNA detected quantities decreased for the two treatments. However, whatever the method used, the differences were not statistically significant. *P. oligandrum* was detected until the end of the experiment and showed the ability to colonized the SO4 rootstock. These results are consistent with those obtained by Gerbore *etal.* (2014), who showed that *P. oligandrum* colonized the root system of the rootstock SO4 associated with Cabernet Sauvignon in vineyards.

Table 1. Assessement of *P. oligandrum* root colonization (± standard error) by plate counting method and qPCR in young grafted vine transplant plants. Values with the same letter are not significantly different at $p < 0.05$ by Kruskall-Wallis. dpi: days after inoculation with the pathogen.

	Treatment	*P. oligandrum* root colonisation (%)	DNA quantities (pg/µl)
60 dpi	*P .oligandrum*	52.59 ± 12.14 a	$9.28\ 10^{-2}$ ± $7.24\ 10^{-2}$ a
	P. oligandrum + *P. chlamydospora*	55.56 ± 10.42 a	$3.78\ 10^{-2}$ ± $1.24\ 10^{-2}$ a
90 dpi	*P .oligandrum*	17.37 ± 9.13 a	$3.14\ 10^{-2}$ ± $2.18\ 10^{-2}$ a
	P. oligandrum + *P. chlamydospora*	30.00 ± 12.47 a	$1.14\ 10^{-2}$ ± 6.6010^{-3} a
120 dpi	*P .oligandrum*	30.49 ± 12.42 a	3.8210^{-2} ± 3.1010^{-2} a
	P. oligandrum + *P. chlamydospora*	44.44 ± 17.57 a	$1.21\ 10^{-2}$ ± $6.34\ 10^{-3}$ a

Protection of young grafted vines against P. chlamydospora attacks

Necrosis was naturally presents in control plants (17%), mock inoculated plants (32%) and plants inoculated with *P. oligandrum* (17%) (Figure 1). However, for the plants inoculated with *P. oligandrum* at the root level, a decrease of necrosis length was observed. Differences between treatments were not statistically significant.

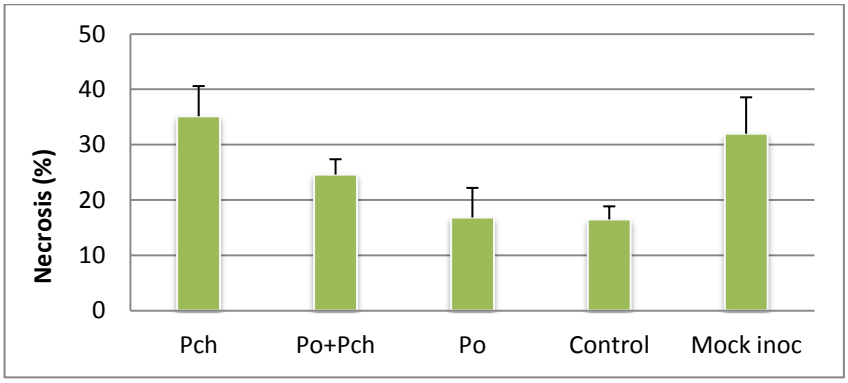

Figure 1. Wood necrosis (± SE standard) measured in trunk of young grafted vines, 120 dpi. Po = plants treated with *P. oligandrum*; Pch = plants infected with *P. chlamydospora*; Po + Pch = plants treated with the two microorganisms and Mock inoc = mock inoculated plants. No significant differences between the different treatments were observed (Kruskall Wallis, $p < 0.05$).

Frequently, necrosis length is used to measure the level of attack by *P. chlamydospora* on grapevine cuttings (Pouzoulet *et al*., 2013). In our study, this visual evaluation of pathogen attack was not fully relevant. Thus quantification of *P. chlamydopsora* DNA in the wood may be useful. *P. chlamydospora* DNA quantities detected at the scion level were more important than those measured at the rootstock level (data not shown). At the scion level, *P. chlamydospora* DNA was detected in all treatments. However, the amount of DNA recovered was significantly higher in plants where the pathogen was inoculated (*P. chlamydospora* and *P. oligandrum* + *P. chlamydospora* tratments) than in plants of the

other treatments, except for mock inoculated plants. The presence of *P. oligandrum* on the roots was not associated with a decrease of the *P. chlamydospora* DNA amount detected in the trunk. However, at the rootstock level, pathogen DNA quantities in plants inoculated with *P. oligandrum* + *P. chlamydospora* were lower than those observed in plants inoculated solely with the pathogenic fungus. However, no significant differences between the different treatments were observed (Kruskall Wallis test $p < 0.05$).

In the literature, several experiments on biological control of grapevine trunk diseases were performed by using the simplified model with cuttings (Bruez *et al.*, 2015; Haidar *et al.*, 2016; Yacoub *et al.*, 2016). To our knowledge, our study is the first one that evaluated the ability of a biocontrol agent, *P. oligandrum*, to protect young grafted grapevine against *P. chlamydospora* attacks. It was demonstrated that *P. oligandrum* colonized the roots of the rootstock SO4 over the whole experimental period. Then, when *P. oligandrum* was present at the root level, a reduction of *P. chlamydospora* DNA quantities was observed in the wood of the rootstock.

Acknowledgements

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Effects of selected sebacinoid endophytic fungi on tomato plant health

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Abstract: Fungal endophytes such as the sebacinoid fungus *Piriformospora indica* can alleviate abiotic and biotic stresses and thus represent interesting candidates for biological control strategies against soil-borne diseases.

In this work the effects of the sebacinoid fungi *Piriformospora williamsii*, *Serendipita herbamans*, *Sebacina vermifera* and *Piriformospora indica* on the soil-borne pathogen *Fusarium oxysporum* f. sp. *lycopersici* (Fol) in tomato were investigated in greenhouse experiments. Furthermore, effects on tomato root development and morphology were assessed in *in-vitro* assays by using the software WinRhizo™.

The preliminary results showed a reduction of Fol disease incidence by the application of *P. williamsii* and *P. indica* depending on the inoculation method. The *in-vitro* assays on early plant development revealed positive effects on root growth and branching triggered by all tested fungi compared to the control treatment.

These preliminary data suggest a great potential of other sebacinoid fungi than *P. indica* in disease control and plant growth promotion which should be further investigated.

Key words: Sebacinales, fungal endophytes, *Fusarium oxysporum*

Efficacy of biopesticides on root diseases and pests in hydroponic production of vegetables

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Abstract: Soil-borne pathogens cause significant losses in hydroponic greenhouse vegetable production, even when fungicides and water treatments are employed. Several microbes and microbe-derived products protect plants from pathogens. Many new products are available but comparative efficacy data is lacking. This research evaluated biopesticide efficacy under commercial conditions to identify implications for IPM. A small scale hydroponic system was constructed to evaluate efficacy of 10 bio-pesticides on commercial greenhouse tomato and cucumber cultivars. The effect of cultivar, grafting, and substrate type on product efficacy was also determined. Products were applied as a drench at transplant to rockwool blocks and at transplant to coco or rockwool slabs. Products were compared to fungicide and water controls. Plants were challenged with *Pythium* spp. 3-5 days after product application and root rot, plant height and weight was evaluated after 3 weeks. Several products reduced root rot equal to or greater than the fungicide Previcur. Disease reduction varied depending on the substrate, time of year and rootstock genetics. The best performing products in the disease trials were tested for potential positive or negative effects on performance of greenhouse pests *Trialeurodes vaporariorum* and *Tetranychus urticae*. Results from this research will allow growers to make informed management decisions and derive maximum benefit from their investment in disease control products.

Key words: *Pythium*, biopesticide, tomato

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EUCLID: Leveraging IPM for sustainable production of fruit and vegetable crops in partnership with China

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Abstract: EUCLID is a project funded by the EU in the context of H2020. The objective of the EUCLID project is to contribute to secure the production of food for the increasing worldwide population while developing sustainable production approaches to be used in the European and Chinese agriculture. EUCLID is coordinated by INRA and it includes 19 partners from public research and private companies (15 representatives from 6 countries of the European Union and four Chinese partners). It started in September 2015 for a period of 4 years. The project will exploit knowledge developed in the last decades and will explore new methods of IPM to provide solutions to pest management for specific problems of European and Chinese farmers for a few important and emblematic crops that represent different production systems. These include leafy vegetables, table and wine grapes and tomatoes. These crops also represent different production systems. As one of the partners of the EUCLID consortium, the MISTRAL team at INRA-Avignon will contribute its experience in the development of biological control agents of diseases of fruit and vegetable crops and in elucidating life history and evolutionary ecological processes that can be leveraged to innovate new means to manage plant health that are compatible with sustainable agriculture and agro-ecological principles. We will present the objectives and work packages of EUCLID and the research that the MISTRAL team will develop pertinent to the management of fungal diseases of the model crops of this project.

Key words: IPM, biological control, leafy vegetables, grapes, tomatoes

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PGPR induces resistance against soft rot on Chinese green mustard

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Abstract: Plant growth promotion rhizobacterium (PGPR), *Bacillus subtilis* strain CaSUT007 was isolated from soil in northeast of Thailand. Application of CaSUT007 reduced the disease severity of soft rot up to 60% when compared to pathogen inoculated control plants. Our results revealed that phenolic compounds were found to accumulate in Chinese green mustard leaf tissues treated with CaSUT007 when plants were 5 days old, with the level of phenolic compounds reaching 2.05 µg/g fresh weight. FT-IR analyses indicated that protein was highly accumulated in response to the strain CaSUT007. Chinese green mustard treated with CaSUT007 showed changes in the beta sheet secondary structure of proteins and appeared to have a higher polysaccharide content, but lipid content was decreased compared with plants treated with distilled water. These results demonstrate that the application of PGPR strain CaSUT007 has potential to control soft rot disease and reduce the risk associated with high use of chemical pesticides in commercial Chinese green mustard production in Thailand.

Key words: *Bacillus subtilis*, induces resistance, biochemical change, PGPR

Introduction

Soft rot disease caused by *Erwinia carotovora* pv. *carotovora* (Ecc.) is one of the most serious bacterial diseases of *Brassica* vegetables such as Chinese green mustard, cauliflower, cabbage, Chinese cabbage, pak-choi, spinach, carrot and Chinese radish in tropical countries (Phokum *et al*., 2006). This disease appears every year and reduces the quality and quantity of vegetables, causing up to 55% crop losses in many countries. The symptoms usually develop in rainy season as high humidity and high temperature result in rapid reproduction and spread of Ecc. The use of PGPR- *B. subtilis* strain CaSUT007, isolated from soil, as biocontrol agent has been reported for protecting various plants from several diseases caused by bacteria and fungi including soft rot of Chinese green mustard. Biocontrol mechanism by this PGPR strain CaSUT007 enhances of plant growth promotion and induces systemic resistance of plants (Prathuangwong & Buensanteai, 2007; Buensanteai *et al*., 2009). The aim of this study was to identify the mode of action of bacterial antagonist *B. subtilis* strain CaSUT007 and to evaluate the efficacy of strain CaSUT007 for enhancing growth and control of soft rot disease caused by Ecc under laboratory and greenhouse conditions. Moreover, the induced resistance mechanism was determined.

Material and methods

Effect of seed treatment and spray with CaSUT007 controlling soft rot disease
Chinese green mustard seeds cv. *max108* were treated by the strain CaSUT007 (1×10^8 cfu/ml) prior to the challenge inoculation with Ecc. Treatments with chemical bactericide (copper hydroxide) and untreated control were included in each experiment. The strain CaSUT007 was seed treatment and then foliar sprayed every 7-day for 7 times within 49 days. Disease severity of each treatment was evaluated from score 1 to 9, slightly modified from Prathuangwong & Buensanteai (2007). The experiments were conducted in a greenhouse with 2 treatment, 4 replications and 10 plants per each replication with completely randomized design (CRD). Data were analyzed and subjected to analysis of variance (ANOVA) (SPSS software, version 16). The significance of treatments was determined by the magnitude of F value (P = 0.05). Treatment means were separated by Duncan's Multiple Range Test (DMRT).

Extraction and quantification of phenolics in Chinese green mustard
Plants from each treatment were investigated for accumulation of phenolics. The level of phenolic content was expressed as mg catechol/100 g of plant fresh weight of chinese green mustard. The method described by Prathuangwong & Buensanteai (2007) was slightly modified.

Chinese green mustard cellular composition measurement using FTIR
Chinese green mustard leaf samples of both CaSUT007-treated and non-treated plants were collected at 7 DAI and dried in a hot air oven at 60^{o}C for 3 days, then ground by sterile crystal pestles and mortars into the fine powder. Each powdered sample was taken at an equal weight and subsequently subjected to FTIR spectroscopy (Baseri & Baker, 2011) at the Synchrotron Light Research Institute, Thailand. The infrared spectra were recorded using FTIR spectroscope (Bruker Optics Ltd., Ettlingen, Germany) which was collected in the 4000-900/cm mid-infrared range at a spectral resolution of 4/cm. The individual spectra from each group were analyzed using Principal Component Analysis (PCA) to distinguish different biochemical components of the samples by the Unscrambler X 10.1 software (CAMO, Norway). The Savitzky-Golay method (3rd polynomial, 9 smoothing points) was employed to perform second derivative spectra, then normalized using the Extended Multiplicative Signal Correction. Unsupervised hierarchical cluster analysis (UHCA) was performed on the second-derivative spectra employing Ward's algorithm. This method utilized a matrix to define inter-spectral distances and calculated spectra distances as D-values.

Results and discussion

Effect of seed treatment and foliar spray with CaSUT007 under greenhouse conditions
Strain CaSUT007 significantly reduced the percentage incidence of soft rot and increased Chinese green mustard yield compared to bactericide spray, although the last spray with either CaSUT007 or bactericide had been conducted on 49-day-old plants. Overall reduction of bacterial soft rot by CaSUT007 was approximately 60%, the disease symptom was firstly found 5 days after last spray (Table 1). Bactericide treatment was slightly less effective in controlling this disease.

Table 1. Effect of seed and foliar treatment of the strain CaSUT007 on severity of bacterial soft rot disease in Chinese green mustard cultivar *Max018*.

Treatment	Disease assessment[1]	
	Disease severity[2] (%)	Disease reduction (%)
CaSUT007	44.4b	55.6b
Copper hydroxide	42.5b	57.5b
Control	100.0a	0.0a
F-test	**	**
CV (%)	31.12	

[1]Chinese green mustard leaves were challenged with strain Ecc at 7 days after final foliar treatment with the strain CaSUT007.
[2]Disease severity was evaluated 7 days after being challenged with strain Ecc. Each value represents a mean of three replicate plants with two leaves per plant. Mean values in the column followed by the same letter are not significantly different according to the LSD test ($\alpha = 0.05$).

Levels of Chinese green mustard phenolic compounds

CaSUT007 was examined for its ability to induce defense related compounds to protect Chinese green mustard from Ecc infection. In Chinese green mustard treated with a combined application of a seed treatment and a foliar spray with CaSUT007, the highest level of phenolic compound accumulation was observed, reaching 2.05 µg/g fresh weights at 4 days after Ecc pathogen inoculation (Figure 1). In contrast, phenolic accumulation was considerably lower in non-treated plant and challenge inoculation with Ecc pathogen. We found that pathogen inoculated negative control plants, accumulated the lowest level of phenolics after 5 days, reaching 0.91 µg/g fresh weight. The phenolic accumulation response was more pronounced in plants inoculated with bacterial pathogen, suggesting pathogen recognition and a subsequent plant defense response**.**

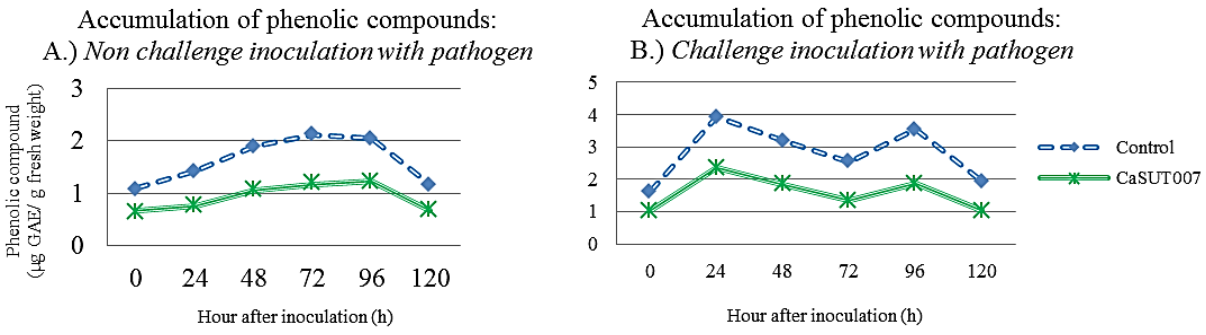

Figure 1. Accumulation of phenolic compounds in leaves of Chinese green mustard cv. Max018 with or without seed and foliar treatment with strain CaSUT007 and with challenge inoculation with Ecc. A) Non-challenge inoculation with pathogen and B) Challenge inoculation with Ecc pathogen.

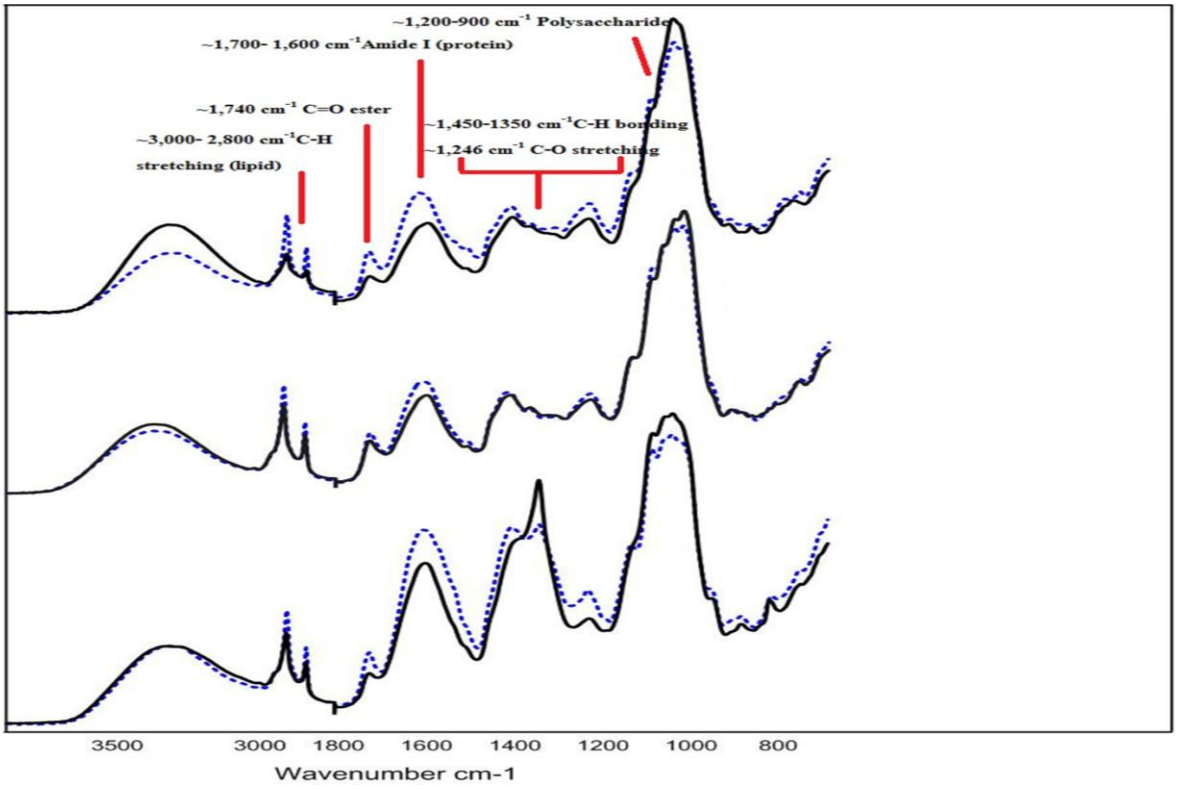

Figure 2. Infrared spectrum absorbance of biomolecules in Chinese green mustard treated with the strain CaSUT007 compare to negative control at 49, 56 and 63 day after planting.

Changes in planta cellular components in response to CaSUT007 using FTIR analysis

FT-IR spectroscopy was performed in order to explore the cellular and biochemical changes of Chinese green mustard plants after treatment with the strain CaSUT007. The IR spectra of Chinese green mustard plants reflect cellular components of the cell wall and membrane such as polysaccharides, protein secondary structure and lipid content. The conformational change of protein amide noted between 1700-1600/cm which give the spectra of protein secondary structure such as alpha-helix (entered at 1653/cm), beta-sheet (centred at 1635/cm) and beta-turn (centred at 1685/cm) (Figure 2). Chinese green mustard treated with CaSUT007 shows higher content of polyshaccharides associated with cell membrane structure when compared with those of plants treated with distilled water. The spectra shown in Figure 2 indicate that there are variations in polysacharide components. Clearly, the higher content of polysaccharide in the spectral region of C-O-C stretching (1150-900/cm) was seen under distilled water negative control when compared with plants treated with CaSUT007. We found that shifts in the absorbance intensities at different frequencies between control and treated leaves can be related to the concentrations of different molecules such as cellulose, protein, pectin, and lignin. Pectin plays an important role within plant cell walls. Pectin and pectin methyl-esterification are involved in plant defence. Chemical compounds with beta-sheet secondary structure play an important role in cell wall metabolism (Yu, 2004). These biochemical changes may also involve enzymes implicated in degradation processes such as pectinases that catalyze de-esterification of pectin by the action of pectin esterferase and other pectin enzymes (Manrique & Lajolo, 2004). Interestingly, peaks in the carbohydrate fingerprint region (1200-1000/cm) show an increase in absorbance, particularly in the bands at 1076/cm and 1153/cm. This might be related to pectin polysaccharides, such as

homogalactruonan, and beta-glucan (callose), known to be found in cell walls of plant leaves (Yu, 2004), increasing in response to strain CaSUT007 to build up plant defence mechanism against pathogen infection. In conclusion, the results of this experiment suggest that CaSUT007 is a valuable biopesticide for crop production in Thailand with simultaneous meachanisms of PGPR and induced plant defence.

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Session 4:
Microbial environments – suppressive soils

Biological and integrated control of plant pathogens
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Suppressive soils: back on the radar screen

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Abstract: Suppressive soils are those in which a pathogen does not establish or persist, establishes but causes little or no damage, or establishes and causes disease for a while but thereafter the disease is less important, although the pathogen may persist in the soil (Weller, 2002). 'General suppression,' the ability of essentially all soils to suppress the growth or activity of soilborne pathogens to a limited extent, is due to the activity of the total soil microbiome competing with the pathogen and is not transferable between soils. 'Specific suppression' is superimposed over the background of general suppression, is highly effective, is transferable between soils, and is due to the effects of individual or select groups of microorganisms. Suppressive soils owe their activity to both general and specific suppression, with the latter the dominant force and the focus of most studies. Suppressive soils occur worldwide and are known for many pathogens (Cha *et al*., 2016; Mendes *et al*., 2011; Weller *et al.*, 2002; 2007; Yin *et al.*, 2013). Take-all decline (TAD), the spontaneous reduction in take-all and increase in yield that occurs with monoculture of wheat or barley following a severe attack of the disease (Weller, 2015), is the classic example of a suppressive soil. Growers in the Pacific Northwest (PNW) of the USA rely on TAD, with about 0.8 million ha of wheat suffering little damage from the disease even though the pathogen is still present (Weller, 2015). This TAD suppressiveness results from the enrichment of certain fluorescent *Pseudomonas* spp., now classified as *P. brassicacearum* (Loper *et al.*, 2012), that produce the antibiotic 2,4-diacetylphloroglucinol (DAPG). Biotic and abiotic factors as well as management practices influence the robustness of take-all suppression, with irrigation and wheat cultivar impacting the abundance and effectiveness, respectively, of DAPG producers (Mavrodi *et al*., 2012b; M.-M. Yang & D. M. Weller, unpublished data). Populations of phenazine-1-carboxylic acid producers likewise are influenced by soil moisture in the PNW, where they are localized to arid soils throughout the Columbia Plateau (Parejko *et al*., 2013) and comprise up to 10% of the total culturable heterotrophic aerobic bacteria on the roots of dryland spring wheat (Mavrodi *et al*., 2012). While these bacteria are suppressive of *Rhizoctonia* root rot (Parejko *et al.*, 2013), recent evidence also has implicated microbial communities of copiotrophic bacteria including members of the Oxalobacteriaceae and Sphingobacteria (*Flavobacterium* and *Chryseobacterium*) in the decline of the disease (Yin *et al.*, 2013). One bacterium, *Chryseobacterium soldanellicola*, was isolated, tested in greenhouse bioassays, and shown to reduce symptoms caused by *Rhizoctonia* on wheat, completing the biocontrol version of Koch's postulates and demonstrating causation for *Rhizoctonia* suppression. In another case, the application of DNA-based technologies revealed not only a key role for Actinobacteria in the suppression of strawberry wilt caused by *Fusarium oxysporum*, but also genes responsible for the ribosomal synthesis of a novel thiopeptide inhibitory to the pathogen and the antibiotic's mode of action against fungal cell wall biosynthesis (Cha *et al*., 2016). Since the pioneering work of Mendes *et al.* (2011), the use of DNA-based tools has opened the door to unprecedented new insights into the complex interactions among members of rhizosphere microbial communities and their plant hosts.

These tools, in combination with classical approaches, are essential not only to unravel the network of molecular and biochemical mechanisms that underpin soil suppressiveness, but also to extend the lessons learned from these special soils to develop microbial community management strategies integral to plant health.

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Microbiome studies give new insights in *Rhizoctonia*-suppressive microbial communities

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Abstract: Disease suppressive soils are soils in which plant pathogens cause little or no plant infections due to microbial activities in the soil or rhizosphere. This worldwide phenomenon has been described for many plant species and a range of plant pathogens, including fungi, oomycetes, bacteria and nematodes. For most disease suppressive soils, however, the responsible microbial communities and the underlying mechanisms of disease suppression still remain unknown.

Rhizoctonia suppression was demonstrated in several arable soils, after amendment of specific organic compounds, as well as after continuous cropping of a host in the presence of the pathogen. Characterization of the soil microflora by classical plating techniques and quantitative PCR showed some correlation between disease suppression and the presence of *Streptomyces* and antagonistic *Lysobacter* species. The role of these bacterial groups as a key factor in *Rhizoctonia*-disease suppression, however, could not be confirmed.

In a recent study, the rhizosphere microbiomes in conducive and suppressive soil samples were compared to unravel the mode of action. Disease suppressiveness in soils against the soil-borne fungus *Rhizoctonia solani* was induced by growing the host plant (sugar beet) successively in presence of the pathogen. The induced suppressiveness could be transferred to a conducive soil, suggesting that it has a (micro)biological basis. To study the dynamics and *in situ* activities of the microbial communities during the transition from a disease conducive to a disease suppressive state, total DNA and RNA were isolated from the rhizosphere of sugar beet and subjected to metataxonomic and metatranscriptomic analyses. 16S amplicon analyses revealed only minor changes in the rhizobacterial community composition during transition from a disease conducive to a suppressive state, with most OTU changes observed in the Bacteroidetes phylum. Results of metatranscriptomic analyses highlight which functions in the rhizobacterial community are induced during rebiosis, i.e. the transition from a diseased to a healthy soil.

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Microbial profiling of a suppressiveness-induced agricultural soil amended with composted almond shells lead to isolation of new biocontrol agents

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Abstract: This study focused on the microbial profile present in an agricultural soil that becomes suppressive after the application of composted almond shells (AS) as organic amendments. The role of microbes in the suppression of *Rosellinia necatrix*, the causative agent of avocado white root rot, was determined after heat-treatment and complementation experiments with different types of soil. Bacterial and fungal profiles based on the 16S rRNA gene and ITS sequencing showed that the soil under the influence of composted almond shells revealed an increase in Proteobacteria and Ascomycota groups, as well as a reduction in Acidobacteria and Xylariales (where *R. necatrix* is allocated). Complementary to these findings, functional analysis by GeoChip 4.6 confirmed the improvement of a group of specific probes included in the "soil benefit" category was present only in AS-amended soils, corresponding to specific microorganisms previously described as potential biocontrol agents, such as *Pseudomonas* spp., *Burkholderia* spp. or Actinobacteria. Based in such data, a model for the microbial-based suppressiveness is proposed and further isolation of representative microorganisms was performed.

Key words: *Rosellinia necatrix*, organic amendment, biocontrol, Gammaproteobacteria

Introduction

The enhancement of soil suppressiveness using organic amendments has been widely described, especially for soil-borne diseases (Bailey & Lazarovits, 2003). The soils that become suppressive soils provide an environment in which plant disease development is reduced, even in the presence of a virulent pathogen and a susceptible host. This phenomenon could be induced as a direct result of the activity of microorganism consortia that are naturally established on soil after application of the amendment (Weller *et al*., 2002). For this reason, understanding the diversity, composition, structure, function and interactions of microbial communities is crucial to gain insight into the basis for suppressiveness mediated by this organic amendment (Janvier *et al*., 2007).

Our research interest is focused on the avocado (*Persea americana* Mill.), for which southern Spain is one of the most relevant zones in the Mediterranean area. In this part of the world, one of the most limiting soilborne diseases affecting avocado trees is white root rot, caused by the fungus *R. necatrix*. Several approaches have been implemented, among them, the use of organic amendments or mulches, such as composted almond shells, which have been previously shown that they can affect soil physicochemical properties and microbial communities (Bonilla *et al*., 2015; López *et al*., 2014).

Material and methods

Soil sampling

Natural field soil samples allocated underneath of unamended avocado trees (CT) or amended with composted almond shells (AS) were taken to perform the different experiments, as previously described by Vida *et al*. (2016) from an experiment located at the Experimental Station 'La Mayora' (IHSM-UMA-CSIC, Málaga, Spain) on the coast of the Malaga Province (SE Spain).

Samples processing

To test the potential role of soil microorganisms in suppressiveness, we prepared three types of processed soils using different treatments: Field soils (raw soils), heat-treated soils and complemented soils (Vida *et al*., 2016). To isolate Gammaproteobacteria strains from rhizosphere and bulk soil, we plated the samples on selective medium (King's B [KB] agar supplemented with antibiotics (Larkin & Honeycutt, 2006). The most abundant colonies with different morphology (n = 267) were isolated and stored at -80 °C.

Suppressiveness assays

Suppressiveness assays against white root rot caused by the virulent strain *Rosellinia necatrix* CH53 were conducted using two different susceptible pathosystems, avocado and wheat (*Triticum aestivum*) using raw, heat-treated soils and combinations (Cazorla *et al*., 2006; Vida *et al*., 2016).

Microbial profile analysis

Composite DNA samples from each soil type (AS and CT) were sent for sequencing to obtain the microbial DNA sequences of the 16S rRNA gene and ITS hypervariable regions. Sequences were analysed using QIIME and CLcommunity^{TM} software (ChunLab, Korea). Moreover, additional composite samples of DNA from the different soil samples studied were sent to Glomics Inc (Norman, Oklahoma, USA) for the functional analysis (Tu *et al*., 2014).

Isolates characterization

Focused in Gammaproteobacteria strains (n = 267), antagonistic activity assays (Cazorla *et al*., 2006), detection by colony blot of biosynthetic genes of antimicrobial compounds and production of exoenzymatic activities were performed. Partial sequencing of 16S rDNA gene, plant growth promotion and biocontrol assays were performed only with some representative selected isolated.

Results and discussion

White root rot suppressiveness assay

AS field soil samples displayed better suppressive ability than CT field soil samples (data not shown). The disease suppressiveness activity was reduced when AS soil samples were heat-treated (ASt). Moreover, suppressiveness was complemented in conductive soils when incorporating AS soil samples. Thus, the microbiota evolved in the composted almond shells was crucial for suppressiveness because the reduction of the bacterial population after a heat treatment in the organic amendment resulted in a more conducive phenotype. Moreover, total or partial suppressiveness was recovered when these heat-treated soil samples were complemented with a portion of soil influenced by AS.

Microbial profile analysis

Taking together the results obtained in this work and in previous works related, a theoretical model about the role of the microorganisms in enhancing suppressiveness after amendment with composted almond shells can be proposed (Figure 1).

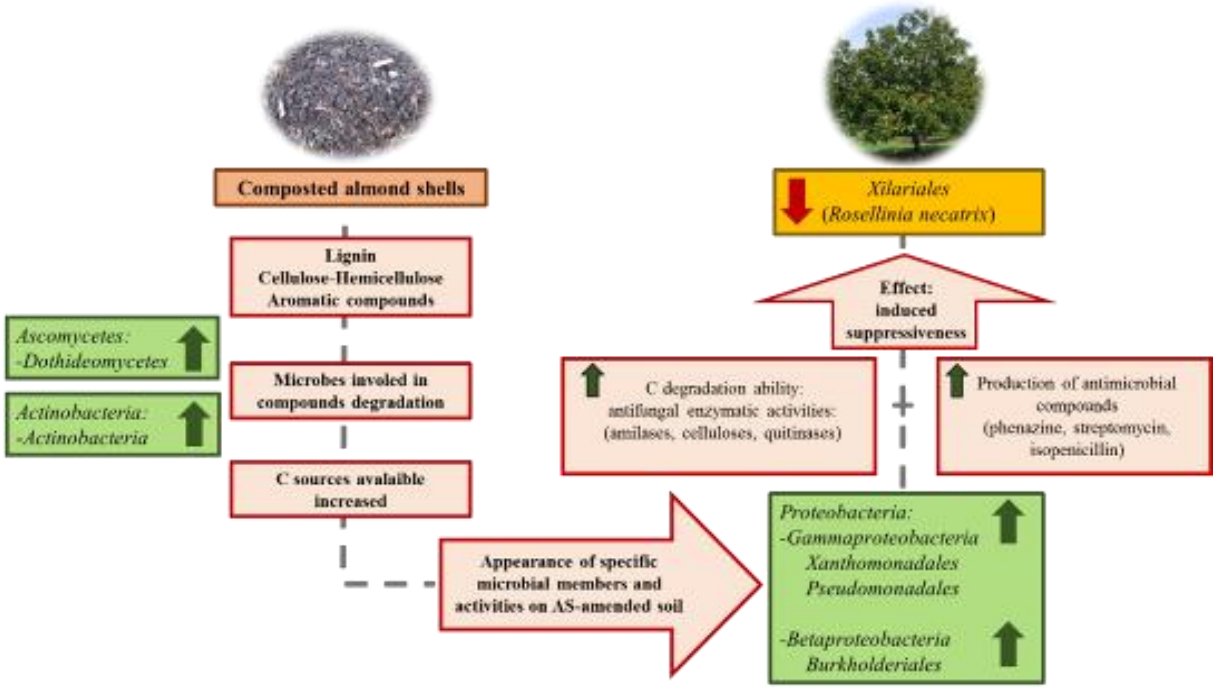

Figure 1. Hypothetical mode of action of almond shells amendment**.**

Briefly, soil amendment with composted almond shells resulted in an extra input of organic matter rich in lignin that could be initially degraded by fungal members of the community (such as *Dothideomycetes*) and Actinobacteria. Lignin degradation would produce a progressive release to the soil of more simple compounds. Those released compounds lead to an increase in available carbon sources, such as cellulose, hemicellulose and aromatic compounds. At this point, some Proteobacteria already present in the soil (mainly Gammaproteobacteria and Betaproteobacteria) could take advantage metabolizing that available organic matter, thus slightly enhancing their population. A selected group of enhanced microorganisms harbor, among other, genes involved in antifungal enzymatic activities and production of antimicrobial compounds that could have an effect on the interaction with other microbes. This resulting modified microbiota after addition of composted almond shells could be more active against some groups of phytopathogenic fungi (as Xilariales, where *Rosellinia necatrix* is included) finally showing a phenotype of induced suppressiveness effect.

Selection of new biocontrol agents

Finally, the isolation of Gammaproteobacteria strains from a suppressive soil could represent a strategy for selecting microorganisms with biocontrol abilities. Thus, some isolates have been obtained, and displayed combined plant growth promoting activities or antagonistic interactions with the biocontrol activity. The main genera selected correspond to *Serratia* spp., *Pseudomonas* spp. and *Stenotrophomonas* spp.

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Abundance of plant beneficial pseudomonads in the rhizosphere of winter wheat grown in different agricultural management systems

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Abstract: Sustainable soil management systems, such as organic fertilization and reduced tillage, are increasingly adopted by farmers to protect soils and to decrease the application of mineral fertilizers. However, it is still not well known how these practices influence the presence and abundance of key groups of soil microorganisms, such as fluorescent pseudomonads. This group of bacteria can improve plant health by protecting roots against the attack of soil borne fungal pathogens through the production of antifungal metabolites and by activating plant defence mechanisms.

In this study, the abundance of fluorescent pseudomonads producing the antifungals 2,4-diacetylphloroglucinol (DAPG), phenazines and pyrrolnitrin was measured in soil and rhizosphere of wheat with a qPCR based approach in two long term trials that compare conventional to organic cultivation, reduced tillage to conventional tillage and monoculture to crop rotation.

DAPG and phenazine producers were significantly less abundant in unfertilized plots compared to plots under conventional or organic cultivation. Phenazine and pyrrolnitrin producers were less abundant in organic plots compared to conventional plots. Monoculture, which had been found to favour the build-up of pseudomonads populations in past studies, had no significant effect on the abundance of any of the three quantified pseudomonads groups. Our results indicate that the quantity of fertilization, rather than the form of fertilization, influences the abundance of plant beneficial pseudomonads in wheat cultivation systems. Further research is needed to identify soil management systems favouring the growth of plant beneficial pseudomonads populations in different cropping systems.

Key words: *Pseudomonas* spp., organic agriculture, monoculture, antifungal compounds

Introduction

Increasing awareness of the importance of soil protection for agricultural production is leading to the adoption of cultivation practices designed to decrease erosion and compaction, such as organic fertilization and reduced tillage. However, it is not known how these soil management practices affect the abundance of key groups of plant beneficial soil

microorganisms, for example fluorescent pseudomonads. Fluorescent pseudomonads, a group of soil bacteria belonging mainly to the species *Pseudomonas protegens*, *Pseudomonas corrugata*, *Pseudomonas fluorescens* and *Pseudomonas chlororaphis*, have many plant beneficial effects. They can directly promote plant growth or they can improve plant health either by activating plant defence mechanisms or by protecting roots against the attack of soil borne fungal pathogens through the production of secondary metabolites with antifungal effects (reviewed by Haas & Defago (2005). Fluorescent pseudomonads were found to be abundant in suppressive soils, where pathogens were present but plants showed reduced or no symptoms (Weller *et al*., 2002). Monoculture can lead to an increase in the abundance of certain groups of plant beneficial pseudomonads, therefore leading to a shift from conducive to suppressive soil (Weller *et al*., 2002, Berendsen *et al*., 2012). While the effect of tillage and fertilization on the soil microbiome have been studied recently (Hartmann *et al*., 2015; Degrune *et al*., 2016), their effect on fluorescent pseudomonads remains largely unknown. Rotenberg *et al*. (2007) quantified pseudomonads producing the antifungal 2,4-diacetylpholoroglucinol (DAPG) in maize fields under different tillage and crop rotation managements, but no clear effects were found.

Therefore, the aim of our study was to compare the abundance of plant beneficial pseudomonads that produce antifungal metabolites in two long-term field trials. The management systems compared in the trials are organic versus conventional fertilization, monoculture versus crop rotation, and conventional tillage versus reduced tillage. Knowledge about management systems that favour plant beneficial pseudomonads could help to limit the damage caused by soilborne fungal pathogens with a conservation biocontrol approach.

Material and methods

Long term experiments

Rhizosphere samples were collected in 2013 and 2014 in two long-term experiments in Switzerland. Samples were collected from plots cropped with winter wheat in the year of sampling. Trial DOK, started in 1978 and located in Therwil, Switzerland, compares different fertilization regimes (Mäder *et al*., 2002). For this study, samples were collected in plots fertilized with mineral fertilizer only (treatment "Mineral"), plots fertilized with organic and mineral fertilizers (treatment "Conventional"), plots fertilized with organic fertilizers only (treatment "Organic", cultivation according to Swiss regulations for organic agriculture), and plots that have not been fertilized since the start of the experiment (treatment "Unfertilized"). Trial Plot 20, started in 1967 in Nyon, Switzerland, compares reduced tillage (15 cm depth) to conventional tillage (25 cm depth) in a wheat monoculture and in a 4 year-crop rotation system (Charles *et al*., 2011).

Real-time quantitative PCR

Key groups of biocontrol pseudomonads were quantified in the rhizosphere of winter wheat. Abundance was measured with qPCR targeting biosynthesis genes of metabolites known to have a biocontrol effect against fungal pathogens. Assays for the following genes were developed and tested for specificity: *phzF* (biosynthesis pathway of phenazines) and *phlD* (biosynthesis pathway of 2,4-diacetylphloroglucinol). A previously published method (Garbeva *et al*., 2004) was used to quantify *prnD* (biosynthesis pathway of pyrrolnitrin). One pooled sample (five plants) per replicate (four replicates per treatment) was analyzed.

Results and discussion

Plant beneficial pseudomonads producing the antifungal metabolites DAPG, phenazines and pyrrolnitrin could be detected in all treatments of the DOK trial and in all treatments of the Plot 20 trial. In the DOK trial (Figure 1), the abundance of DAPG producers and phenazine producers was significantly higher in all fertilized treatments compared to the unfertilized treatment. There was no significant difference between samples from conventional and organic plots for the DAPG producers, but phenazine producers were significantly less abundant in the organic treatment. Furthermore no significant difference could be found between conventional (where a mixture of organic and mineral fertilizer was amended) and mineral treatments for any of the three quantified groups of plant beneficial pseudomonads. According to (Mäder *et al*., 2002), the amended amount of soluble nitrogen was approximately 70% lower in the organic treatment compared to the conventional and mineral treatments, while the amended amount of total nitrogen was in a similar range. This result indicates that the final quantity of nutrients in the soil and not the form in which they are applied impacts on the abundance of DAPG and phenazine producing pseudomonads in the wheat rhizosphere. In a third long term trial (FAST trial) performed at Agroscope, Zürich Switzerland, where the organic treatment received less nitrogen than the conventional treatment, the abundances of phenazine and DAPG producers were consistently lower in the organic treatment (unpublished data). Pyrrolnitrin producing pseudomonads were apparently less influenced by the quantity of nutrients in the soil, since they were not significantly less abundant in the unfertilized treatment compared to the conventional and mineral treatments. Interestingly, lower numbers of pyrrolnitrin producing bacteria were detected in the organic treatments compared to the other three treatments. The qPCR assay used in this study detects also pyrrolnitrin producers belonging to the genera *Burkholderia* and *Serratia* (Garbeva *et al*., 2004), which might respond differently to the nutrient level than fluorescent pseudomonads.

In field trial on plot 20, 47 years of wheat monoculture had no significant effect on any of the three quantified groups of plant beneficial pseudomonads (data not shown), despite the many reports indicating that monoculture can lead to take-all suppressive soils and a higher abundance of plant beneficial pseudomonads (Weller *et al*., 2002). Reduced tillage also did not lead to any significant differences in the abundance of plant beneficial pseudomonads. It is possible that the close proximity of plots belonging to different treatments led to the transfer of a certain quantity of soil between plots, for example through rain, thus minimizing the differences between treatments.

Our results indicate that soil management systems, in particular fertilization, indeed can have a strong impact on the abundance of plant beneficial pseudomonads in the wheat rhizosphere. However, collection of data over more cropping seasons, at more field sites and with different plant species are needed to better understand which soil management type is best to favour the growth of plant beneficial pseudomonads for a certain crop or environment.

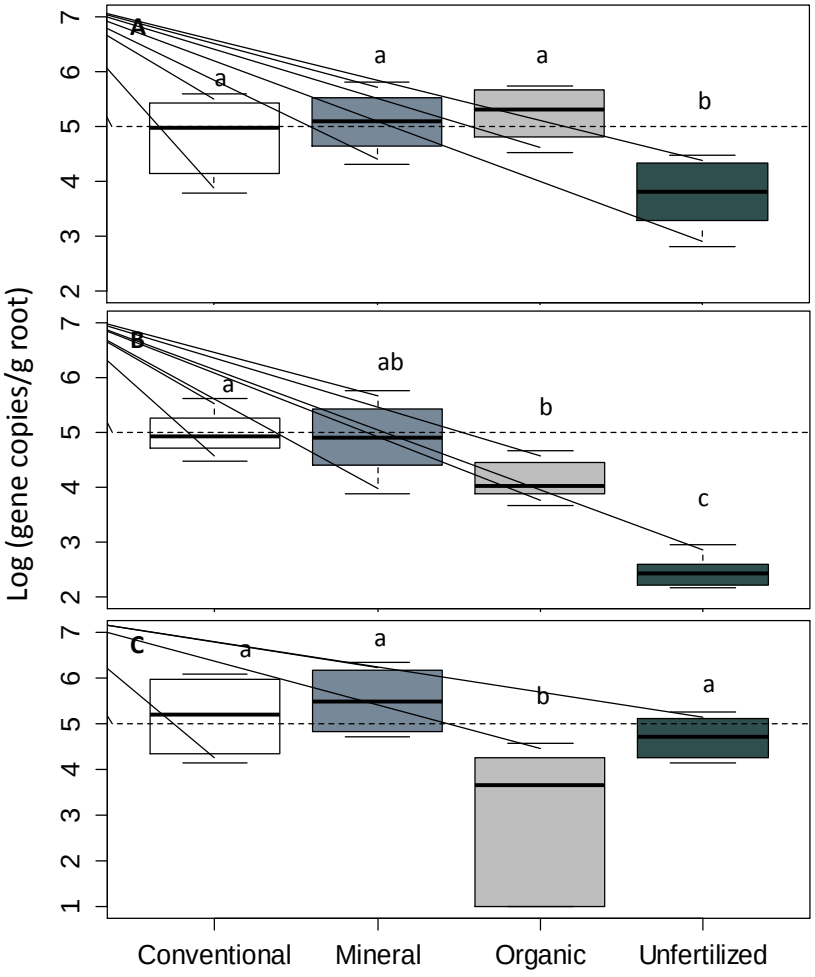

Figure 1. Abundance of plant beneficial pseudomonads producing DAPG (A), phenazines (B), and pyrrolnitrin (C) in the rhizosphere of wheat harvested from the DOK trial. The dotted line indicates the threshold of 10^5 gene copies/g root required for biocontrol effects. Letters indicate significant differences between treatments (linear mixed effect model and Tukey's HSD, $p < 0.05$).

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Effect of biochar on pre-emergence damping-off in nursery growth media and its influence on microbial community structure

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Abstract: Biochar (the solid co-product of biomass pyrolysis), has several agronomic benefits in soil and has a great potential as a supplemental amendment in soilless and nursery media. As biochar has a significant influence on the severity of foliar and soilborne diseases in various crops, its influence on young seedlings health and the potential mechanisms involved in the process has to be studied. This study tested i) the impact of biochar on pre-emergence damping-off caused by *Pythium aphanidermatum* and ii) biochar's influence on soil bacterial community composition and diversity. We found that biochar suppressed pre-emergence damping-off by up to 76%. PCR-DGGE analyses of the 16S rRNA gene showed substantial differences in bacterial composition between biochar amended and control soils. Illumina sequencing revealed a significant enrichment of bacterial abundance, potentially beneficial microorganisms and shift in microbial community structure. These changes may play an important role in the overall effects of biochar on disease suppression either through direct antagonist effect towards pathogen or indirectly via induction of systemic resistance in the plant.

Key words: damping-off, nurseries, disease control, Illumina sequencing, microbiome, soil amendments

Introduction

Biochar, the solid co-product of biomass pyrolysis, has excited robust scientific and commercial interest in the last decade, particularly in terms of understanding and quantifying agronomic benefits it may provide when added to soil and soilless substrates (Graber *et al.*, 2014). Yet, researches show that the influence of biochar on soilborne pathogens is quite complex and the effective dose of biochar for disease suppression was lower than that needed for plant growth promotion (Jaiswal *et al.*, 2015). One of the most promising directions for use of biochars might be the nursery stage where biochar (as a supplemental amendment in the growth media) has the potential to improved disease control of pathogen such as seedling damping-off caused by the fungi *Rhizoctonia solani* and the oomycetes *Pythium* spp. (Dhingra *et al.*, 2004). However, the influence of biochar on initial seedlings growth period during the nursery stage was not yet studied. One of the most intriguing mechanisms that might be involved in disease suppression is the alternation in diversity and composition of the bacterial community and promotion of beneficial microorganisms that are antagonistic to pathogens or induce plant resistance.

The goals of this research were i) to assess the impact of biochar amendment on pre-emergence damping-off caused by *Pythium aphanidermatum* and ii) to study the biochar influence on soil bacterial community composition and diversity by using molecular culture-independent techniques (PCR-DGGE and Illumina sequencing).

Material and methods

Biochar and plant growing condition

Two contrasting types of biochar, EUC-600 and GHW-350, produced from eucalyptus wood chips at 600 °C pyrolysis temperature and from greenhouse pepper plant wastes at 350 °C HTT, respectively, were used throughout the research. Biochar at 0, 0.5, 1, or 3% (wt:wt) was mixed with the potting mixture (peat:tuff 7:3, vol:vol).

Cucumber seeds (*Cucumis sativus*, cv. Muhasan) were used as the host plant for pre-emergence damping-off tests. Seedling raising nursery trays were used to conduct pre-emergence damping-off experiments. PDA agar disks containing *P. aphanidermatum* mycelium were used as inoculum. Similarly, agar disks without pathogen serve as non-inoculated treatments. Cells were filled with potting mixture with or without biochar and single cucumber seed was sown in each cell. There were five replicates and each replicate consisted of 8 cells containing one seed per cell. Replicate cells were used to calculate percentage of damping-off. The experiment was arranged in a randomized block and maintained at 26 ± 1 °C in greenhouse. Damping-off was determined 7 days after seed sowing.

Soil sample collection and DNA extraction

Soil samples amended with either 0% (control) or 3% biochar were collected, each treatment with 3 biological repeats. DNA was extracted from 0.3 g soil with 3 technical repeats using a commercial soil DNA extraction kit (Exgene Soil SV, Geneall).

PCR-DGGE (denaturing gradient gel electrophoresis) and Cluster analysis

DGGE was performed on control and biochar-amended soil DNA samples as previously described (Jaiswal *et al.*, 2016). Clustering was determined by the UPGMA method.

Illumina high-throughput sequencing and data analysis

Illumina sequencing was performed on 16sRNA gene amplicons using the 515F and 806R primer sets (Caporaso *et al.*, 2012). Sequences quality processing and data was analyzed using QIIME (version1.9.1) pipeline (Caporaso *et al.*, 2010).

Experimental design and statistical analysis

All the experiments were conducted twice and the repeats were analyzed together with experimental repeat as a block factor. Data was analyzed by ANOVA (analysis of variance) after arcsine square-root transformation, using JMP 12 software. Multiple comparisons of the means was done with the Tukey-Kramer (HSD) test ($\alpha = 0.05$).

Results and discussion

Effect of biochar on pre-emergence damping-off caused by Pythium

The effect of biochar on cucumber pre-emergence damping-off caused by *Pythium* was evaluated 7 days after seed sowing. EUC-600 biochar at 1 and 3% concentrations significantly reduced the damping-off as compared with the control by up to 71% (P = 0.0015). Similarly, GHW-350 also significantly reduced damping-off at 1 and 3% concentrations by up to 76% (P = 0.0004). This result suggests that biochar has ability to suppress the disease at young seedlings under nursery condition.

Effects of biochar amendment on bacterial community structure

DGGE UPGMA based on Pearson correlation indices showed that soil bacterial communities formed three distinct clusters depending on biochar application rates and types of biochar (Figure 1). Derived cluster analysis of the bacterial community compositions well separated biochar amended soil from the control soil with an intergroup similarity of ~ 80% and intragroup similarity of ~ 90%. Collectively this technique demonstrated that the biochar treatment alters the soil bacterial community structure.

Illumina sequencing of 16sRNA gene amplicons reveals that the relative abundance of the Proteobacteria, Verrucomicrobia, Planctomycetes, Gemmatimonadetes and Firmicutes phylum was significantly increased in the biochar-amended soil but no changes in abundance of Bacteroidetes. In contrast, the relative abundance of the Acidobacteria, Actinobacteria, and Chloroflexi phylum was significantly reduced in the biochar-amended soil (Figure 2). Classification of sequences characterized to the genus level, indicated that biochar amendments significantly increased the abundance of genera generally associated with plant growth promotion, soilborne pathogens suppression and inducing resistance in plants against diseases. For instances, biochar addition significantly increased the abundance of genera: *Pseudomonas, Flavobacterium, Devosia, Haliangium, Streptomyces, Bacillus* and others as compared to unamended soil. Nevertheless, biochar amendment caused a significant increase in Shannon's diversity as compared to unamended soil (9.93 ± 0.061 vs. 9.43 ± 0.084, respectively). These changes in microbial composition and diversity may play a part in the overall effects of biochar on disease suppression either through direct antagonist effect towards pathogen or indirectly via induction of systemic resistance in plant. Some of these florescent *Pseudomonas, Bacillus,* and *Flavobacterium* were isolated from the biochar amended treatments and are currently tested for their antagonistic effect and their potential to induce resistance in plants against soilborne pathogens.

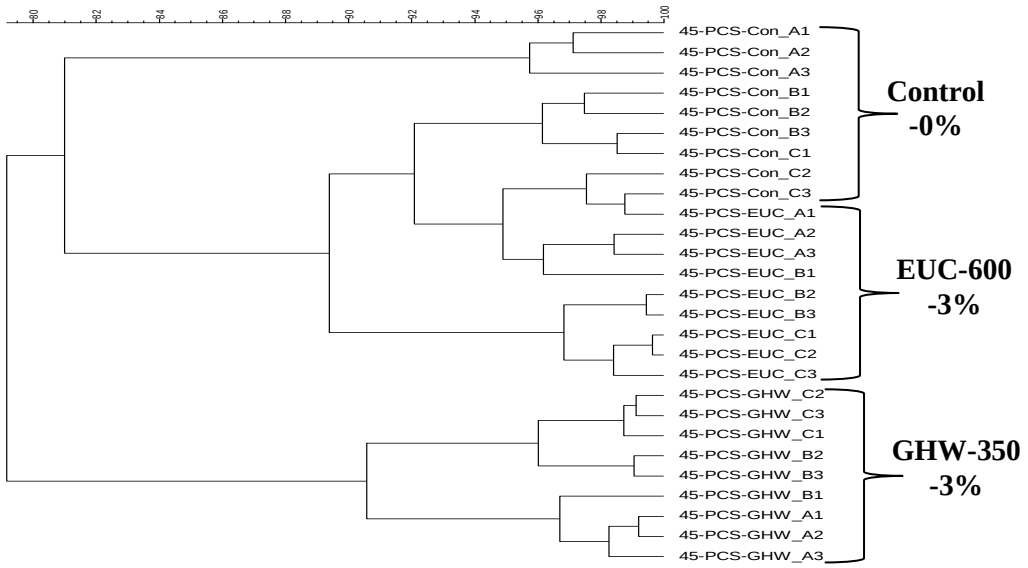

Figure 1. Effect of EUC-600 and GHW-350 biochar on the soil bacterial community structure. Cluster analysis of soil bacterial 16S rRNA gene DGGE ribotypes analyzed and clustered using the Fingerprint II software. The UPGMA tree is based on Pearson correlation UPGMA matrix between the different DGGE patterns of different samples.

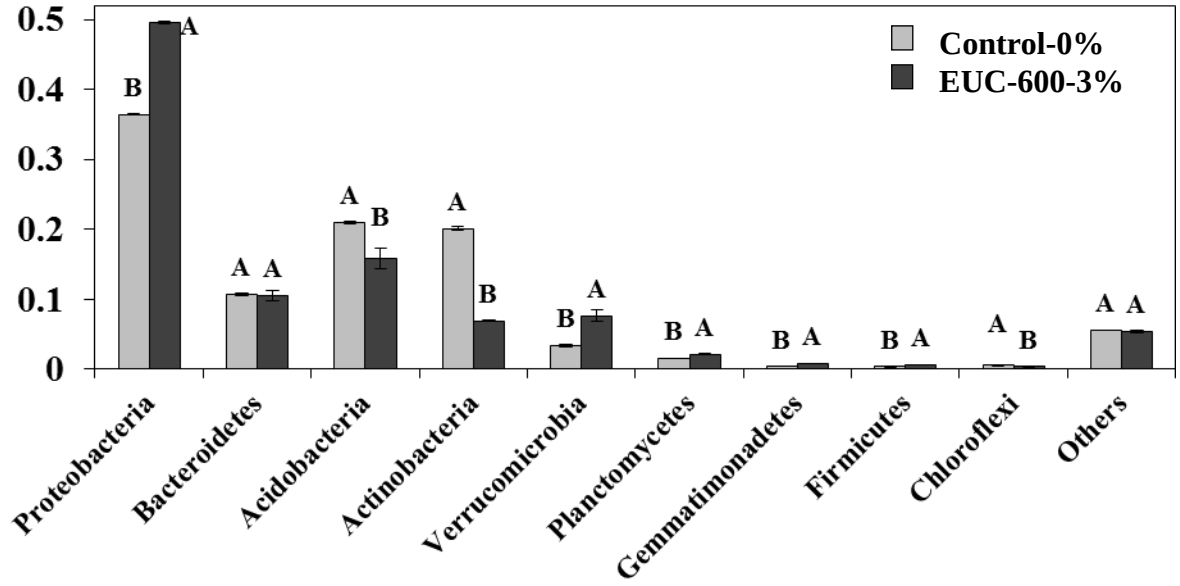

Figure 2. Relative abundance of bacterial phyla in the control and biochar-amended soils identified using the Illumina sequencing of 16S rRNA gene amplicons. Columns labeled by a common letter are not significant different at $P < 0.05$ according to Tukey-Kramer HSD test within a phylum.

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Effect of agricultural management on the soil and rhizosphere microbiome and its implications for soil health

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Abstract: The growing world population poses increasing demands on agriculture. The intensification of agricultural production brought about severe impacts on soils, for instance loss of fertility, pollution by agro-chemicals, soil erosion and enrichment of soil-borne plant pathogens. Hence, there is a growing need for the application of more sustainable farming strategies since soil is an important resource whose health must be maintained and improved. The microbe-mediated natural ability of soils to suppress plant pathogens (suppressiveness) might be a good indicator for soil health and quality. We hypothesized that long-term farming strategies influence the soil and plant microbiome and consequently soil suppressiveness and plant performance. Soils from long-term field trials differing in fertilization and use of pesticides (intensive vs. extensive), soil management (tillage vs. conserved), and the previous crop (wheat vs. rapeseed) were selected for a growth chamber experiment. The different soils were planted with lettuce (*Lactuca sativa*) as model plant and incubated under identical conditions for six weeks. Significant differences in lettuce shoot fresh mass were observed among soils under different long-term farming strategies. The microbial community composition of soils under root influence and from the lettuce rhizosphere was compared to that of unplanted soils by cultivation-independent techniques based on total community DNA. 16S rRNA amplicon fingerprinting by denaturing gradient gel electrophoresis (DGGE) showed that the bacterial community composition of the rhizosphere as well as in root-influenced soils differed among field trial sites but was also influenced by the farming strategy. Using group-specific PCR-DGGE, bacterial responders to fertilization, soil management and crop rotation were found among different taxonomic groups (e.g. Acidobacteria, Actinobacteria). Currently, we are studying functional genes involved in biological control of plant pathogens in the lettuce rhizosphere by quantitative (qPCR) and semi-quantitative analysis (Southern blot hybridization). 16S rRNA Illumina amplicon sequencing will provide further insights into taxa potentially indicative for agricultural management and soil health. In a future experiment, the suppressiveness of these soils will be tested using the pathosystem lettuce/*Rhizoctonia solani* in order to unravel the interaction between farming strategy, soil microbiome and soil health.

Key words: farming strategy, suppressiveness, *Lactuca sativa*

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Survival of *Stenocarpella* spp. in maize debris and soil suppressiveness to maize ear rot pathogens

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Abstract: *Stenocarpella* species (*S. maydis* and *S. macrospora*) overwinter saprophytically in maize stubble but little is known about the factors that contribute to its survival and to the induction of suppressiveness of pathogen colonization. We aimed at determining the role of crop rotation on the survival of the pathogen and induction of specific or broad spectrum disease suppressivity. Maize fields cultivated with soybean crop rotation or maize monoculture were randomly sampled for *Stenocarpella* sp. detection. Stalks were sampled, DNA extracted and the pathogen quantified through qPCR. Soil from the same sampled sites was tested for suppressivity to *F. graminearum*, *F. verticillioides* and *S. maydis.* The crop rotation consistently contributed to the lowest *Stenocarpella* sp. quantification in maize stalks and also to the highest number of soils with suppressiveness to *F. graminearum* and *F. verticillioides* compared to the maize monoculture. The obtained data not only endorsed the importance of soybean crop rotation for broad spectrum control of stalk and ear rot causing pathogens but also pointed out the most promising fields to look for biocontrol agents once the suppressiveness is of biological nature.

Key words: *Stenocarpella maydis*, stalk rot-causing fungi, white ear rot, no-tillage

Introduction

Ear rot diseases of maize result in significant economic losses after harvest and reduce quality and quantity of grains (Matiello *et al*., 2015). Multiple fungi are related to both stalk and ear rot such as *Stenocarpella maydis*, *S. macrospora*, *Fusarium graminearum* and *F. verticillioides* (Ahsan *et al*., 2010). There is a growing market demand for grains with a restricted percentage of maximum values of rot grains: 2% for exportation and 6% for the Brazilian market. In this regard, it is urgent to understand the factors that contribute to the disease development and its management.

Although there is considerable knowledge on the epidemiology of the ear and stalk rot-causing pathogens *F. graminearum* and *F. verticillioides* (Dragich & Nelson, 2014), little is known about *Stenocarpella* sp.-causing diseases, particularly in tropical agriculture, where overwintering is likely to play a major role on the observed epidemics, particularly under no-till cropping system.

Noteworthy, the crop rotation plays an important role on the reduction of the survival of soil-borne pathogens and buildup of antagonist microbiota (Gil *et al*., 2008). On the other hand, successive monoculture with a susceptible cultivar lead to the buildup of antagonists and induction of suppressiveness which also may lead to the reduction of soil-borne pathogen (Song *et al*., 2016).

Considering the lack of knowledge on the epidemiology of the *Stenocarpella* disease complex, the objectives of our study were to verify the presence of *S. maydis* and *S. macrospora* in maize growing areas of Minas Gerais State, to identify the *Stenocarpella* spp. on cultivated areas, to verify soil suppressiveness to maize ear rot pathogens and to verify the contribution of long-term crop rotation or maize monoculture on the pathogen recovery from maize debris.

Material and methods

A total of 25 maize fields were sampled for *Stenocarpella* spp. quantification and suppression to stalk/ear rot causing pathogens in fields under maize monoculure (10/25) or crop rotation (15/25) both managed under no-tillage over five years. Samples of decaying stalks and soil samples (0-5 cm depth) were collected at each site for pathogen quantification and suppressiveness evaluation, respectively.

For the pathogen quantification, total DNA was extracted from the sampled stalks and Sybr Green PCR assays were performed using 2.0 µl DNA of each sample and 23 µl reaction mix containing 12.5 µl SYBR Green PCR Kit (Qiagen), 0.75 µM of each forward and reverse primers (Xia & Achar, 2001). The cycle initially consisted of 95 °C for 3 minutes, denaturation at 94 °C for 30 seconds, annealing at 60 °C for 1 minute, and extension of 72 °C for 1 minute, with final extension of 72 °C for 10 minutes, for a total of 40 cycles. A 5-fold serial dilution ranging from 20 ng to 2 pg of DNA of *S. maydis* CML698, was included in each run as reference.

The Ct was determined as the number of cycles in which the fluorescence generated within a reaction crossed the threshold. The comparative Ct method was used. Samples showing the lowest expression for each gene were used as calibration samples and relative expression was measured using the relative standard curve method. To quantify gene expression using real-time PCR, the values obtained corresponding to sample mRNA levels were compared to the control mRNA level. To calculate expression levels, the following were considered: Ct (exponential increase in PCR product) of the target gene and endogenous control, ΔCt = Ct (sample) - Ct (endogenous control) and $\Delta\Delta Ct$ = ΔCt (sample) - ΔCt (calibrator). Expression levels were then calculated using the formula: $RQ = 2^{-\Delta\Delta Ct}$ (Livak, 2001).

Soil samples were checked for suppressiveness against *S. maydis*, *F. graminearum* and *F. verticillioides*. The soil samples were individually loaded into 90 mm-diameter petri dishes, watered to 60% field capacity and infested with 5 ml suspension of each fungus at 1×10^5 con/ml per plot. One week after soil infestation, seven autoclaved maize stalks, previously determined as bait with high recovery of all three fungi, were deposited on top of the soil in each plate and checked after 7 days for the presence of the pathogen by visually examining through stereo-and light microscopes.

The data of incidence in each replicate from the suppressiveness assay was submitted to variance analysis (ANOVA), using the Software R.

Results and discussion

Stenocarpella sp. was detected at all sampled sites regardless of the crop rotation, at a smaller extent in soybean rotation compared to maize monoculture (Table 1).

Both crop rotation and tillage systems have proven to impact pathogen survival (Flett & McLaren, 2001) but for the first time, the contribution of those cropping systems have been addressed to *Stenocarpella* sp. survival using DNA-based quantification in crop residues on maize fields. Crop residues can serve as shelter and/or nutritional source to pathogens such as for *Stenocarpella* sp. during their saprophytic phase in the life cycle (Scott *et al*., 1994).

Table 1. Soil suppressiveness to *Fusarium graminearum, F. verticillioides* and *Stenocarpella maydis*.

			Colonized baits (%)		
Field	Culture	*Stenocarpella* sp.*	*Fusarium graminearum*	*Fusarium verticillioides*	*Stenocarpella maydis*
10	Maize-Maize	0.20 A	95 A	86 B	76 B
11	Maize-Maize		100 A	86 B	100 A
14	Maize-Maize		71 B	76 B	86 B
17	Maize-Maize		100 A	100 A	33 C
19	Maize-Maize		100 A	100 A	100 A
21	Maize-Maize		90 A	95 A	86 B
23	Maize-Maize		100 A	95 A	100 A
24	Maize-Maize		100 A	100 A	95 A
26	Maize-Maize		100 A	100 A	100 A
29	Maize-Maize		100 A	100 A	100 A
1	Maize-Soybean	0.46 B	85 B	33 C	95 A
2	Maize-Soybean		100 A	100 A	100 A
4	Maize-Soybean		24 C	5 C	5 C
6	Maize-Soybean		95 A	100 A	95 A
16	Maize-Soybean		100 A	100 A	100 A
18	Maize-Soybean		100 A	95 A	95 A
20	Maize-Soybean		100 A	81 B	76 B
22	Maize-Soybean		95 A	100 A	100 A
25	Maize-Soybean		100 A	100 A	100 A
27	Maize-Soybean		71 B	71 B	81 B
28	Maize-Soybean		100 A	100 A	95 A
30	Maize-Soybean		100 A	100 A	100 A
31	Maize-Soybean		86 B	86 B	81 B
7	Maize-Soybean		100 A	90 B	71 B
8	Maize-Soybean		67 B	29 C	33 C

* Mean relative qPCR quantification of *Stenocarpella* sp. associated to maize stalks. Means followed by the same letter within each column are similar according to Skot Knot's test ($p < 0.05$).

From 25 sampled sites (Table 1), out of the 15 managed under soybean rotation, 5 were suppressive to *F. graminearum*, 7 to *F.* verticillioides and 6 to *S. maydis*, while out the 10 sites managed under maize monoculture, the suppressiveness was less common, with 1, 3 and 4 suppressive soils to each pathogen, respectively. Furthermore, under crop rotation, soils from four (27%) sampled sites were suppressive to all three pathogens, while only one (10%) such soil was found under monoculture. Interestingly, baits where no pathogen was detected were colonized by a hallmark of both bacteria and fungi, which points out the biological nature of the observed suppressiveness.

After a soybean-maize or peanut-maize rotation, Gil *et al*. (2008) observed that the reduction in the *Fusarium solani* and *Sclerotinia minor* populations in the soil were acompanied by the buildup of *Trichoderma* spp., *Clonostachys* spp. and actinomycete communities, which are reported to play a role on the biological control of soil-borne disease. In turn, knowing that a soybean crop rotated field is more prompt to harbour antagonistic microrganisms to stalk and ear rot causing pathogens, those microrganisms can be fished out from the soil using stalk baits for the development of a biocontrol products (Köhl *et al*., 2015) that potentially anticipate disease control obtained from the crop rotation in areas where an implementation of crop rotation is not possible.

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Involvement of rhizobacteria in *Fusarium* wilt suppressiveness of soil induced by *Allium* plants cultivation

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Abstract: Cultivation with *Allium* plants is known to induce soil suppressiveness to *Fusarium* wilt of various crops. Soil pasteurization experiment revealed that gram negative bacteria which accumulate in *Allium*-cultivated soil might be involved in the suppression mechanism of *Fusarium* wilt of cucumber. It has been previously reported that four groups of gram negative bacteria, *Pseudomonas*, *Burkholderia*, *Flavobacterium* and *Chryseobacterium*, predominantly inhabit rhizosphere soils of *Allium* plants. Therefore, in this study, bacteria belonging to these four groups were isolated from rhizosphere soils of Welsh onion and onion and their disease suppressive capacity was evaluated by cucumber seedling assay. Each bacterial group obtained from both Welsh onion and onion tended to be suppressive against cucumber *Fusarium* wilt. These findings indicated that four predominant groups of rhizobacteria of *Allium* plants might play crucial roles in the *Fusarium* wilt suppressiveness of soil conferred by cultivation with *Allium* plants.

Key words: *Allium* plants cultivation, *Fusarium* wilt suppressiveness, rhizobacteria

Influence of temperature and culture media on growth, lipopeptide production and antagonistic activity of *Bacillus amyloliquefaciens* Bs006

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Abstract: Cyclic lipopeptides (cLPs) produced by *B. amyloliquefaciens* subsp. *plantarum* can exert direct antagonism (iturins and fengycins) and plant systemic resistance elicitation (surfactins and fengycins) against fungal phytopathogens. Some previous works have suggested that abiotic factors in the rhizosphere can affect the expression of biocontrol traits or metabolites; however, these aspects have not been extensively studied and need more attention to design strategies to increase biological control effectiveness. The aim of the present work was to determine the effect of temperature (15, 25 and 30 °C) on growth, antagonistic activity against *Fusarium oxysporum* MAP5 (FOX), and the profile of cLPs from *B. amyloliquefaciens* Bs006 in routinely used LB medium, PZN and Landy media which have been used to stimulate the production of antimicrobial peptides and cLPs. Results showed that responses varied significantly with temperature/media combination both in liquid and gelified media. For example, Bs006 did not grow below 15 °C in Landy but well in the other media and population levels were similar in liquid fermentation. Globally it was observed that Bs006 produced higher concentrations of fengycins than iturins and surfactins. The highest concentration of fengycins (267 µg/ml) and iturins (57 µg/ml) occurred at 30 °C in rich media PZN while the highest concentration of surfactins (13 µg/ml) was observed at 25 °C in LB. The profile of cLPs detected on inhibition zone during confrontation dual test in gelified media was different compared to liquid conditions. Here concentration of iturins was greater than fengycins and surfactins both in LB, PZN and Landy and the highest concentrations were observed under 30 °C like in liquid conditions. In terms of productivity of cLPs in liquid culture it was observed the highest amount of surfactins (6 µg/1E8 cells) at 15 °C in LB, fengycins (18 µg/1E8 cells) at 25 °C in LB, while productivity of iturins was similar in all treatments (less than 4 µg/1E8 cells). FOX growth inhibition by Bs006 in gelified culture was higher in PZN (49-55%) under all three temperatures followed by LB at 30 °C (46%) and Landy at 25 °C and 30 °C (31-33%). Accumulation of high amount of iturins in the inhibition zone suggests an important role of this cLP against FOX. Overall, the results suggest that expression of biocontrol traits such as cLPs by *B. amyloliquefaciens* Bs006 are influenced by an interaction of nutritional level and temperature.

Key words: cyclic lipopeptides, abiotic factors, *Bacillus amyloliquefaciens*

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Effects of the biological activity in the antagonistic potential of soil to *Fusarium* Head Blight of wheat

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Abstract: All soils are potentially naturally resistant or receptive to plant pathogens due to their microbial activity. In our study, we focused on the ability of agricultural soils to reduce primary inoculum of *F. graminearum*, the major causal agent of *Fusarium* Head Blight. We selected 31 wheat cultivated fields with maize as previous crop and examined soil physicochemical properties, disease symptoms in fields and mycotoxin content as well as *Fusarium* species present on in harvested grains. In parallel, growth of *F. graminearum* was evaluated in autoclaved soil samples and untreated samples incubated under controlled conditions. A significant difference was observed between the two conditions. Pathogen growth was up to 3 logs higher in autoclaved soils when compared to untreated soils. These results demonstrate a key role of soil biological activity in reducing pathogen growth. Besides, when multivariate analyses were used to correlate soil properties to the previous results, any abiotic factors seemed to have a predominant role. Our study demonstrates the key role of soil biological activity in *F. graminearum* repression. Further experiments are ongoing on plant model and sequencing to complete the study on soil suppressiveness.

Key words: *Fusarium* Head Blight, soil suppressiveness, antagonistic potential of soil

Introduction

Soil suppressiveness to plant pathogens has been recognized and demonstrated for several causal agents (Postma *et al*., 2008; Alabouvette, 1986). Although mechanisms of action of abiotic factors remain unclear, the antagonistic role of the microflora is well admitted and the use of beneficial micro-organisms in biocontrol of plant diseases has received a growing interest. Competition for nutrients and space, parasitism, antibiosis and stimulation of induced systemic resistance of plant are modes of action expected for effective biocontrol agents directly introduce in a specific environment (Alabouvette *et al*., 2006). However, effectivenes of these "biopesticides" remains variable with a low reproductibility depending on several parameters and complex interactions (Amein *et al*., 2008). Hence, adapted agricultural practices such as crop rotation and tillage with an appropriate fungicide treatment remains the current best strategy to manage plant disease as *Fusarium* Head Blight (FHB) (Wegullo *et al*., 2013). FHB is a devastating disease of cereals responsible for economical losses due to reduced yield and mycotoxin production in grain. Deoxinivalenol (DON) is the most common mycotoxin in cereals and is produced by several species, including *Fusarium graminearum*, the most important agent of FHB in wheat (Zhang *et al.*, 2011). Since weather conditions at flowering and cropping history play a major role in the disease incidence (McMullen *et al.*,

1997), we investigate the antagonistic potential of soil to *F. graminearum* by taking into account these factors as well as the chemical properties of soil. Therefore, we followed 31 wheat fields in 2014/2015 with maize as previous crop. We evaluated (i) the antagonistic potential of soil against *F. graminearum* and (ii) the disease incidence and severity in the field as well as (iii) mycotoxin production in grains. Multivariate analysis was performed to investigate the correlations between factors.

Material and methods

Agricultural soils: cropping history, sampling and physicochemical characterization
Thirty one agricultural fields were selected in Brittany, France. All of them were under wheat in 2015 with maize as previous crops. Systematic sampling was performed in spring 2015 at the end of tillering to a depth of 5 cm and samples from a field were mixed together to consitute an homogenized sample. Soil samples were stored at 4 °C until use after removal of residues and homogenization. For each field, information on cropping history going back until 2012 was obtained from the farmers and physicochemical properties (texture, organic matter, organic carbon, total nitrogen, pH, CEC nutrients) were determined by Capinov, Landerneau, France.

Disease monitoring: symptoms in field and mycotoxin content in harvested grains
FHB was assessed at the beginning of the ripening (Feekes stage 11.2). In each field, 400 ears were assessed and rated on a 0-4 scale where 0 = no symptoms, 1 = from 0 to 25% of the ear damaged, 2 = from 25 to 50% of the ear damaged, 3 = from 50 to 75% of the ear damaged and 4 = more than 75% of the ear damaged. Disease severity and incidence were calculated from these observations. Mycotoxins were quantified in harvested grains by LC-MS (Capinov, Landerneau, France). Species belonging to *Microdochium* and *Fusarium* genus were also estimated/counted by cultural approach.

Antagonistic potential of soils
Each of the 31 soil samples was divided in two batches. One batch was autoclaved 3 times for 20 minutes at 121 °C with 24h intervals and the other batch received no treatments (Control). All batches were then inoculated with ground maize kernels infected with *F. graminearum*, randomized in seed plates with 3 replicates per batch, and incubated under controled conditions (day/night cycle, 22 °C/18 °C ± 2 °C, 16 h photoperiod, air humidity = 70%). *F. graminearum* DNA was quantified by a quantitative species-specific Real-Time PCR from 1 g of each replicate at 0, 15 and 30 days.

Results' analysis
Data obtained were examined using analysis of variance (ANOVA), paired t tests and multivariate analysis (Pearson correlation, PCA) in order to investigate correlations between selected variables (soil properties, cropping history), disease responses (disease expression in field, mycotoxin production in wheat kernels) and soil antagonistic properties.

Results and discussion

Disease expression in fields and mycotoxin content in wheat kernels

FHB was observed only in 13 out of 31 fields. The FHB incidence ranged from 0.25% to 7% and less than 1% of the total ears observed were damaged at the rating classes of 1, 2, 3 and 4.

It is known that warm and wet conditions at flowering constitute a critical factor for the contamination of wheat heads (McMullen *et al*., 1997). However, rainfalls in each of the 31 fields were very low, or absent during flowering with temperatures exceding the seasonal average (Bretagne Environnement and MétéoFrance data). Weather conditions at flowering could explain part of the low incidence and severity of the disease in the fields.

Nevertheless, DON was detected in 7 harvested grain samples with a concentration between 662 ± 225 µg/kg and 51 ± 22 µg/kg. *F. graminearum* was also the second most frequent *Fusarium* species observed on wheat grains after *F. poae*.

In their study, Dill-Macky & Jones (2000) observed that DON content of harvested grain was significantly correlated with disease incidence and severity and was also correlated with previous crops. In our work, we observed a lower but positive correlation between the incidence and severity of the disease, the presence of pathogenic species producing mycotoxins and the mycotoxin content in harvested grains (Pearson correlation: $0.41 \leq r^2 \leq 0.69$; $\alpha = 0.05$). However, the previous crop was maize in all cases but varied before 2013/2014, suggesting a potential role of the entire cropping history.

Table 1. Disease indicators for *Fusarium* Head Blight observed in fields with deoxinivalenol (DON) contamination in grains.

Fields	DON µg/kg	% of isolates belonging to *F. graminearum* species	FHB Incidence	FHB Severity
P01	662 ± 225	22.83%	6.00%	14.50%
P03	391 ± 144	26.19%	0	0.00%
P08	86 ± 38	5.71%	0	0.00%
P13	56 ± 25	13.89%	4.75%	4.75%
P15	51 ± 22	16.67%	0	0.00%
P22	72 ± 32	0.00%	0	0.00%
P27	161 ± 68	19.51%	1.75%	1.75%

Antagonistic potential of soils

Soil ability to reduce *F. graminearum* growth was tested by inoculation of the autoclaved and untreated soil samples from each fields. The pathogen DNA was quantified at 0, 15 and 30 days by species-specific qPCR. *F. graminearum* DNA quantity first increased between 0 and 15 days, followed by a dicrease at 30 days. The increase of DNA was associated to growth since Marstorp & Writter (1999) demonstrated the positive correlation between DNA quantity and microbial biomass. Pathogen growth significantly increased in autoclaved soils when compared to the control, suggesting a role of the biological activity of the soils. Furthermore, the behaviour of the pathogen in the untreated condition varied depending on the fields, leading to a possible classification of the fields according to their antagonistic activity.

Multivariate anaysis performed showed minor correlation between *F. graminearum* growth and soil properties. Thus, organic matter might reduce mycotoxin production in wheat ($r^2 = -0.45$) and sand could favor the disease severity and incidence ($r^2 > 0.4$). This results are in agreement with the previous hypothesis suggesting a role of soil properties in soil suppressiveness of disease (Wu *et al.*, 2008 ; de Boer *et al*., 2003; Höper *et al*., 1995).

With our study, we highlight a key role of the biological activity of soil in reducing the primary inoculum of *F. graminearum* resposible for FHB.

Further experiments are currently ongoing on plant model to study the relationship between inoculum concentration and the reduction of disease incidence and severity. Sequencing is also ongoing to identify microbial communities that might play a role in the antagonistic activity of soils. This work takes also in account soil properties and cropping history, improving our understanding on the factors responsible for soil suppressiveness.

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Gaeumannomyces graminis suppression and microorganisms involved in the decline of disease in southern Chile

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Abstract: The central-south Chile produces 85% of the national cereal production. Wheat production has been affected for an important plant disease called "take-all", which is caused by the soil borne pathogen *Gaeumannomyces graminis* var tritici (Ggt). Conductive soils with elevated incidence of take-all disease may develop into "suppressive" soils under wheat monoculture. Suppressive soils occur as natural phenomena that prevent or affect the pathogen establishment or disease incidence.

This study focused on the screening of suppressive soil from 16 sites located in small communities from la Araucania región where wheat monoculture was applied for more than 10 years. The disease suppression was measures under *in vitro* conditions. According to preliminary result we can suggest 10 putative suppressive soils which inhibit Ggt. However, a greenhouse assay and molecular studies are ongoing to confirm these results.

Key words: *Gaeumannomyces graminis*, suppressive soils, take-all

Introduction

The sourthern Chile region produces most of cereal crops from Chile, corresponding 85% of total national production (ODEPA, 2016). Considering cereal crops for human diet, wheat (*Triticum aestivum* L.) is highly consumed by the Chilean population as the second largest bread consumer around the world (100 kg of bread per person per year).

Wheat production has been affected by an important plant disease called "take-all", which is caused by the soil borne pathogen *Gaeumannomyces graminis* var *tritici* (Ggt) (Andrade *et al*., 2011). The elevated Ggt incidence can be attributed to the edaphic and climatic conditions of Andisols (60% of agricultural soils) and the agronomic techniques as monoculture and crop residue incorporation. Under the highly vulnerable conditions, soil borne pathogens as Ggt develop diseases in so-called "conductive" soils (Chng *et al*., 2015). Conductive soil with elevated incidence of take-all disease may turn into "suppressive" soil as a result of agronomic practices such as several years of production in monoculture (Garbeva *et al*., 2004; Andrade *et al*., 2011), or alternations of wheat with 2 or 3 years of pasture (Andrade *et al*., 2011). Suppressive soils occur as natural phenomena that prevent or affect the pathogen establishment or reduce disease incidence (Jara *et al*., 2011). Thus, the take-all disease incidence is low despite the presence of the susceptible host, favorable climatic conditions and the pathogen. Suppressive soils against Ggt have been reported around the world (Bull *et al*., 1991; Bithell *et al*., 2012; Chng *et al*., 2013). In Chile, studies of Andrade *et al*. (2011) showed several sites in the La Araucanía region to be highly suppressive, where approximately 25% of analyzed soils were suppressive against the Ggt (specific suppression).

Different to general suppression, specific suppression is easily transferable to conductive soils and suppressive can be inhibited by moist heat and chemical compounds (Weller *et al*., 2002).

Studies on soil suppressiveness showed that microbial activities in the soil are responsible for soil suppressiveness against Ggt (Weller *et al*., 2002; Cook, 2003) and other soil borne pathogens. The main objective of our study was to screen suppressive soils from from the la Araucania tregión, where small farms still produce wheat in monoculture.

Material and methods

Soil and wheat plants sampling

Sixteen soils and wheat plants growing in la Araucanía region were collected in January 2016, primarily from low-input wheat-cropping farmers with a long history of monoculture or rotation that include natural pastures for long time. Soils were collected from the top (20 cm) and stored in a room at 5 °C until use.

In vitro test 1 for inhibition in petri dishes

A preliminary screening to evaluate the capability of soils to inhibit the *G. graminis* var. *tritici* (Ggt) pathogen under *in vitro* conditions was made. Ggt was grown on PD agar plates at 25 °C for 1 week. Agar disks (4-mm diameter) containing Ggt were aseptically incised and transferred to the center of agar plates containing fresh LB/PD (1:1) media (Durán *et al*., 2014). Then, a hole of 10 mm inside the petri dish was made to put each soil (0.05 g) and to evaluate the effect of soil in the fungal mycelia growth. Fungal growth was measured after incubation for 3, 5 and 7 days at 25 °C in the darkness as described by Liu *et al*. (2011). Sterilize soil of all soil samples was used as control.

In vitro test 2 for inhibition with soil extract

A second *in vitro* test was carried out. The soil samples capable to inhibit fungal growth were used in this assay. Soil samples of 1 g (soils 2, 3, 4, 5, 6, 8, 11, 13, 14, 15 and 16) were suspended in 9 ml of sterile phosphate buffer saline buffer at pH 7.4 (PBS). The supernatant (1 ml) from each soil suspension was spread on an eppendorf and were inoculated with 1% of Ggt inocula. Samples were left at room temperature for 3, 5 and 7 days. Fungal growth was measured by quantification of total biomass by crystal violet (CV) staining. Later, samples were washed and fixed with 200 µl of methanol, which was removed after 15 min. The microtiter plates were allowed to dry at room temperature, and 200 µl of CV (1% v/v) were added to each well and incubated for 5 min. The wells were then gently washed with sterile, ultra-pure water and 200 µl of acetic acid (33% v/v) was added to release and dissolve the stain. The absorbance of the obtained solution was read in triplicate in a multiplate reader.

Greenhouse assay

A highly pathogenic isolate of Ggt was used as inoculum for all inoculations. Oat kernel inoculum was prepared. Colonized oat were blended, sieved to a particle size of 0.5-1.0 mm, and stored at 5 ºC until use. Soils considered as putative suppressive soil were tested in a greenhouse assay for confirmation. Plastic containers containing 300 g of the soil samples in 5 replicates were used. Five disinfected seeds (15% ethanol + 1% sodium hypochlorite for 2 min) of wheat cv. Otto were used. Plants were watered each three days and nutrient Taylor and Foyd solution was applied each 15 days. Plants were collected after 40 days.

Results and discussion

Screening assay 1

According to screening assay 1, the behaviour of 37.5% of the soils remained undetermined because bacterial growth from soils was highly abundant and therefore fungal growth could not be determined. On the other hand, a similar percentage of putative suppressive soils (soils were 2, 3, 11, 13 and 16) showing inhibition of fungal mycelial growth was found. Most soils preselected as putative suppressive soils were coincidently cultivated with wheat monoculture and natural pasture for more than 10 years, similar as proposed by Andrade *et al.* (2011). However, soil 2 was sown with the wheat-triticale and oat for more than 10 years (Table 1).

Table 1. Screening assay 1. Mycelial growth (cm) in Petri plates with different soil samples.

soil/day	day 3	day 5	day 7	putative classification
control	0.83 ± 0.03^{ab}	1.72 ± 0.67^{a}	2.03 ± 0.03^{a}	-
soil 1	0.80 ± 0.02^{ab}	N	N	undetermined
soil 2	0.33 ± 0.06^{g}	0.67 ± 0.10^{d}	0.67 ± 0.10^{b}	suppressive
soil 3	0.60 ± 0.04^{cdef}	0.98 ± 0.19^{cd}	0.62 ± 0.10^{b}	suppressive
soil 4	N	N	N	undetermined
soil 5	0.83 ± 0.03^{ab}	N	N	undetermined
soil 6	0.77 ± 0.02^{abc}	1.08 ± 0.03^{bcd}	N	undetermined
soil 7	0.56 ± 0.02^{def}	1.00 ± 0.05^{cd}	1.61 ± 0.02^{a}	conducive
soil 8	N	N	N	undetermined
soil 9	0.87 ± 0.02^{a}	1.63 ± 0.05^{ab}	2.07 ± 0.08^{a}	conducive
soil 10	0.53 ± 0.02^{ef}	1.07 ± 0.03^{bcd}	2.04 ± 0.09^{a}	conducive
soil 11	0.52 ± 0.01^{efg}	0.83 ± 0.05^{cd}	0.84 ± 0.02^{b}	suppressive
soil 12	0.66 ± 0.05^{bcde}	1.26 ± 0.10^{cd}	1.76 ± 0.19^{a}	conducive
soil 13	0.42 ± 0.04^{fg}	0.97 ± 0.03^{cd}	N	suppressive
soil 14	0.76 ± 0.04^{abc}	1.37 ± 0.10^{abc}	1.09 ± 0.11^{b}	undetermined
soil 15	0.10 ± 0.00^{h}	N	N	suppressive
soil 16	0.73 ± 0.05^{abcd}	0.93 ± 0.1^{cd}	0.93 ± 0.11^{b}	suppressive

Screening assay 2

Undetermined soils and putative suppressive soils found in assay 1 were assayed in screening assay 2. From 6 soils considered as undetermined in assay 1, 4 soils were suppressive, 1 soil was again undetermined (soil 5) and 1 was conducive (soil 8). On the other hand, soils considered suppressive in the assay 1 were also suppressive in assay 2 except soil 11 (Table 2). Soils determined as suppressive were used in the greenhouse assay.

Greenhouse assay

Greenhouse assays and other molecular assays for detection and quantification of Ggt are currently ongoing.

Table 2. Screening assay 2. Mycelial growth (OD_{590}) in microtiter plate with soil extract

soil/day	day 3		day 5		day 7		putative classification
	no inoculated	inoculated	no inoculated	inoculated	no inoculated	inoculated	
Control+	1.84±0.14	1.88±0.19	3.18±0.03	3.61±0.05*			
soil 1	1.52±0.14	1.26±0.09	1.25±0.02	1.14±0.18	1.61±0.14	1.49±0.05	suppressive
soil 2	1.18±0.06	0.79±0.02*	1.35±0.35	1.26±0.21	0.66±0.04	0.61±0.04	suppressive
soil 3	1.49±0.10	0.79±0.01*	0.97±0.04	1.04±0.09	0.69±0.07*	0.36±0.04	suppressive
soil 4	1.90±0.03	1.55±0.16	2.25±0.41	1.40±0.10	0.94±0.07	0.56±0.02	suppressive
soil 5	1.38±0.08	1.00±0.12	0.89±0.08	2.37±0.09*	1.34±0.07	1.19±0.18	undetermined
soil 6	1.67±0.11	1.40±0.15	1.38±0.08	0.88±0.06*	1.31±0.03**	0.68±0.02	suppressive
soil 8	0.79±0.14	1.65±0.11*	1.91±0.19	2.31±0.26*	0.54±0.03	1.14±0.07**	conducive
soil 11	1.71±0.07	1.76±0.23	1.47±0.04	1.63±0.20	1.14±0.08	1.87±0.25*	conducive
soil 13	0.76±0.04	0.69±0.02	0.73±0.01	0.77±0.07	0.63±0.11	0.40±0.01	suppressive
soil 14	0.52±0.04	0.62±0.03	0.62±0.01	0.65±0.05	0.35±0.02	0.31±0.01	suppressive
soil 15	0.59±0.01	0.62±0.03	0.93±0.03	0.71±0.03	0.51±0.05	0.42±0.01	suppressive
soil 16	0.84±0.18	1.11±0.00	0.81±0.04	0.70±0.04	0.97±0.31	0.57±0.01	suppressive

* denotes significant differences for T value for related samples (** $p < 0.01$, * $p < 0.05$).

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Toward dissecting naturally occurring soil suppressiveness to *Ceratocystis paradoxa*

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Abstract: Stem bleeding caused by *Ceratocystis paradoxa* is of major concern to coconut production in tropical areas and no sustainable control measures are available. The exploitation of naturally occurring suppressive soils offers an unique opportunity to develop sustainable management practices. This study aimed at characterizing soil suppressiveness to *C. paradoxa* and determining the biological, chemical, and physical properties responsible for the suppression of the pathogen. Suppressiveness was evaluated by determining the percentage of banana peel baits colonised by the pathogen added to the soil samples. The five most suppressive and the five most conducive soils were contrasted to determine the nature of suppressiveness. Total culturable bacterial populations were the only biological property implicated in suppressiveness. Among the physical and chemical properties, soil pH, calcium content, sum of bases, effective cation exchange capacity, base saturation, and sand content were higher in suppressive soils. Aluminium and iron contents were higher in conducive soils. In conclusion, soil suppressiveness to *C. paradoxa* could not be narrowed down to a single factor, but the results of this study indicate that a combination of biological, physical, and chemical soil properties contribute to the phenomenon.

Key words: bacterial community, *Cocos nucifera*, *Thielaviopsis*, biological control, conducive soils, chemical soil properties

Introduction

Ceratocystis sp. is a polyphagous soil pathogen, infecting various plant species, including coconut (*Cocos nucifera*), causing a disease called stem bleeding. The disease has been of extreme concern because of its rapid dissemination in coconut production and the lack of effective control measures further complicates the situation (Ferreira & Fontes, 2007).

The fungus produces chlamydospores which ensure a high survival rate in soil but different soils but soil-intrinsic properties may be contributing to hamper the pathogen survival since there has been observed the occurrence of healthy plants in production areas with high incidence of the stem bleeding in the states of Sergipe and Pará (E. A. Carvalho & V. Talamini, personal communication). This natural phenomenon, which acts against the establishment, survival of pathogens, and their pathogenic activities is a strong indication of the so-called suppressiveness (Bettiol *et al*., 2009).

Hence, the study of soil properties governing this phenomenon may lead to the development of sustainable disease management practices. In this study, we examined the soil attributes – chemical, physical and biological properties – in an attempt to determine their contribution to the suppressiveness to *C. paradoxa*.

Material and methods

Soil samples were collected in the vicinity of healthy coconut plants from producing areas infested with *C. paradoxa* TCTL003 inoculum suspension (1×10^5 spores/ml) and incubated at 28 ± 2 °C for 7 days. Then, 13 pieces of banana peel with 1 cm^2 each (ripe fruit of cultivar Prata) were evenly distributed on the surface of the infested soil and incubated for additional 7 days, when colonisation of the baits was evaluated under stereomicroscope.

To study the nature of suppressiveness, 10 soils were selected in the analysis described above, five of them classified as suppressive and five as conducive. The ravine soil (TA), classified as conducive was also included in the analyses. The soils were evaluated for selected microbial communities, chemical and physical analyses (Table 1).

To dissect the possible factors involved in soil suppressiveness to *C. paradoxa*, comparisons of means of different variables determined for the five most suppressive and the five most conducive soils were performed. The obtained data were transformed to comply with the normality test of Shapiro-Wilk and means were compared by using the t test for paired data at 5% probability. All statistical analyses were performed with the software "R" (R Development Core Team, 2009).

Results and discussion

In this study, the nature of soil suppressiveness to *C. paradoxa* was investigated through colonization of banana peel baits. In 56 different soil samples, colonized baits ranged from 4.6% to 98.5% and were classified as highly suppressive (< 20% bait colonisation) or highly conducive (> 70% bait colonisation) (data not shown). According to this classification, 10 soil samples, 5 from each group, were used to determine the variables related to suppressiveness (Table 1).

Bacterial and fungal population densities were similar in suppressive and conducive soils, with exception of total bacterial populations that were higher on average in suppressive soils. For only one of the suppressive soil samples, fluorescent *Pseudomonas* spp. were detected at 10^4 cfu/g.

The paired t test analysis performed between the mean values of the conducive and suppressive soils to determine the chemical, physical and biological properties that may be involved in soil suppressiveness pointed out that culturable bacterial populations, pH in water, calcium, sum of bases, effective cation-exchange capacity, base saturation, and sand content were on average 8%, 12.2%, 58.7%, 32.7%, 57.4%, 13.2%, and 17.7% higher in the suppressive soils than in conducive ones.

Biological properties are at least in part responsible for the suppressiveness in most soils included in this study, since seven out of ten soils showed increased bait colonisation after sterilisation. In spite of the lower percentages of colonisation of baits in raw soils, the only biological property included in our study that could be implicated in the natural suppressiveness was the total populations of culturable bacteria (Postma *et al.*, 2003).

Table 1**.** T test for biological, chemical and physical variables of the selected soils and classified as suppressive and conducive.

Variables	Conducive †	Suppressive ††	*p*-value [a]
Colonised baits (%)	77.44	16.68	<0.00001
Total bacterial (CFU)	5.65	6.14	0.03079
Pseudomonas spp. (CFU)	6.03	6.14	0.6049
Bacillus spp. (CFU)	4.93	5.25	0.08255
Total fungi (CFU)	4.26	4.58	0.2107
Trichoderma spp. (CFU)	2.06	3.10	0.1702
pH (H_2O)	5.02	5.72	0.000216
Available phosphorus (P)	83.20	196.40	0.2064
Potassium (K)	170.50	252.10	0.438
Calcium (Ca)	1.41	3.41	0.003762
Magnesium (Mg)	0.55	1.20	0.09355
Aluminium (Al)	0.44	0.16	0.04465
Sum of bases (SB)	3.65	5.42	0.009972
Effective CEC (t) [c]	2.18	5.12	0.009162
CEC pH 7,0 (T)	2.62	5.28	0.3036
Base saturation (V)	7.60	8.76	0.03115
Aluminium saturation (m)	31.24	57.37	0.08214
Zinc (Zn)	25.60	3.86	0.5318
Iron (Fe)	410.21	19.80	0.01676
Manganese (Mn)	13.79	28.48	0.1479
Copper (Cu)	2.02	16.97	0.2637
Clay (C)	21.20	7.20	0.04
Sand (S)	71.40	86.80	0.028
Silt (Si)	7.20	6.40	0.1778
Particle density	2.55	2.56	0.8065

† Arithmetic mean of each variable for the five most conducive or the five most suppressive soils; [a] Significant at the 0.05 probability level; †† Cationic exchange capacity

Among the 15 chemical factors evaluated, seven were different when suppressive and conducive soils were compared. Soil pH, which was higher in suppressive soils, is involved in the availability of several nutrients and may favour bacterial establishment, which in turn suppresses pathogens (Primavesi, 2002). Additionally, pH may have contributed to the higher levels of calcium, sum of bases, effective cation exchange capacity, and base saturation, all of which are properties linked to the higher fertility of the suppressive as compared to conducive soils. This higher availability of nutrients may have favoured establishment of selected groups of microorganisms with antagonistic activity against the pathogen (Bettiol & Ghini, 2005).

All suppressive soils studied had significantly higher sand (86.80% vs. 71.40%) and lower clay (7.20% vs. 21.20%) contents, which was also reported to correlate with the suppressiveness to *Fusarium oxysporum* f. sp. *lini* (Amir & Alabouvette, 1993).

In conclusion, this study could not pinpoint a single factor with unequivocal certainty as the responsible factor for soil suppressiveness to *Ceratocystis paradoxa* pathogenic to coconut, but it indicates that a combination of biotic and abiotic factors contributed to the suppressiveness.

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Metagenomic analyses of soil microbiomes in a long-term organic farming experiment and its ecological implications

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Abstract: Massive application of chemical fertilizers and pesticides caused severe environmental and social problems such as soil degradation, eutrophication of surface water and pesticides contaminations on food, water and soil in China. Organic farming, using environmental friendly and sustainable techniques to enhance ecological services, has the potential to restore ecosystems and eroded soils. In the present study, a combination of metagenomic analyses by high throughput sequencing of total soil community DNA and PCR amplified 16S *rRNA, nifH and nosZ* gene fragments were performed to study the influence of organic farming on soil microbial diversity and functional groups in a twelve-year greenhouse experiment. The experiment consists of organic, intermediate and conventional farming systems. An integrated bioinformatics pipeline for the TC-DNA sequencing dataset was assembled to explore microbial populations known to be involved in nitrogen cycling, phosphate mobilization, plant disease suppression, plant growth promoting, antibiotic resistances and mobile genetic elements, as well as the taxonomic composition. Dynamics of populations involved in nitrogen fixing (*nifH*) and nitrous oxide reduction (*nosZ*) was monthly followed during Sep. 2013 to Aug. 2015. Multiple approaches were employed to compare the taxonomic distribution of both Bacteria and Archaeae in soils from the organic, intermediate and conventional farming systems. In summary, this study provides insights into the effect of long-term organic farming on soil microbial taxonomic and functional diversity and its implications on biogeochemical processes.

Session 5:
Plant microbiome & biocontrol

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A protein derivative stimulates grapevine resistance and the natural phyllosphere microbiota against downy mildew

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Abstract: Protein derivatives can stimulate plant growth, activate plant resistance and act as nutritional substrate for phyllosphere microbial communities. A protein derivative (nutrient broth, NB) reduced downy mildew symptoms and induced the expression of defence-related genes, indicating the activation of grapevine resistance mechanisms. NB increased the number of culturable phyllosphere bacteria and altered the composition of bacterial and fungal populations residing on grapevine leaves. Modifications in the structure of phyllosphere populations caused by NB application could partially contribute to downy mildew control by competition for space or other biocontrol strategies. Modifying phyllosphere populations by increasing natural biocontrol agents with the application of microbial nutrients can open new opportunities in terms of biocontrol strategies.

Key words: phyllosphere microbiota, induced resistance, biological control

Introduction

Grapevine (*Vitis vinifera*) is one of the major fruit crops worldwide and it can be attacked by a large number of pests and pathogens. Among those, the biotrophic oomycete *Plasmopara viticola* causes grapevine downy mildew and requires frequent application of chemical fungicides to avoid yield and quality loss (Gessler *et al.*, 2011). Concerns about the environmental impact of the overuse of pesticides have sparked interest in developing alternative methods to chemical treatments, such as resistance inducers (Delaunois *et al.*, 2014).

Protein hydrolysates could act as biostimulants by influencing growth and resistance processes in plants (Colla *et al.*, 2015). For example, a protein derivative (nutrient broth, NB) reduced powdery mildew symptoms under field conditions by stimulating defence mechanisms in grapevine (Nesler *et al.*, 2015). On the other hand, protein hydrolysates act as nutritional sources for microbial phyllosphere communities (Colla *et al*., 2015) and could alter their functional properties. The phyllosphere is normally colonized by a variety of bacteria, filamentous fungi and yeasts that could have positive effects on plant health thanks to the ability to reinforce natural plant defences and to their antagonism to pathogens (Vorholt, 2012).

The aim of this study was to dissect the mechanisms of action of NB against grapevine downy mildew by understanding its role as resistance inducer and as stimulator of leaf microbial communities.

Material and methods

Grapevine (Pinot noir ENTAV115) plants were grown for two months under greenhouse conditions or *in vitro* as previously described (Perazzolli *et al*., 2014; Lenzi *et al*., 2016). Plants were kept untreated (UNT) or treated with water (H_2O), 3.0 g/l NB (Nesler *et al*., 2015), or with a commercial product based on laminarin (LAM, 0.75 ml/l Vacciplant, Belchim Crop Protection). Grapevine treatments and *P. viticola* inoculation were carried out as previously described (Perazzolli *et al*., 2012). RNA extraction and quantitative real-time PCR reactions were carried out as previously described (Perazzolli *et al*., 2012) using primers for the amplification of pathogenesis-related genes (*PR-1*, *PR-2*, and *PR-4*), osmotins (*OSM-1* and *OSM-2*) and chitinase (*CHIT-3*). Collection of phyllosphere microorganisms, isolation of culturable microorganisms, DNA extraction and amplification of bacterial (V6-V8 of the 16S rRNA) and fungal (ITS3-ITS4 of the internal transcribed spacer; ITS) fragments were carried our as previously described (Perazzolli *et al.*, 2014), as well as the pyrosequencing and bioinformatic analyses. The composition of bacterial and fungal communities was analysed on leaves collected from UNT, H_2O-, NB- and LAM-treated plants before and 24 h after *P. viticola* inoculation of two independent greenhouse experiments.

Results and discussion

Nutrient broth stimulated grapevine resistance under greenhouse conditions

The effect of NB against grapevine downy mildew was analysed under greenhouse conditions, and it was compared with LAM as a reference of resistance inducers (Aziz *et al*., 2003). The NB treatment reduced downy mildew severity as compared with water (H_2O)-treated and untreated (UNT) plants. Specifically, the efficacy was greater in NB-treated plants than in LAM-treated plants (Figure 1A).

The expression levels of *PR-1*, *PR-2*, *PR-4*, *OSM-1*, *OSM-2* and *CHIT-3* genes was upregulated by NB before *P. viticola* inoculation, demonstrating the induction of grapevine resistance. The expression of defence-related genes remained at a high level at 24 h after *P. viticola* inoculation even after pathogen inoculation, indicating that grapevine resistance induced by NB plays a major role during the early stages of *P. viticola* infection. Although the expression levels of *CHIT-3*, *OSM-1*, *OSM-2* and *PR-4* were greater in LAM- as compared with NB-treated plants, LAM showed lower efficacy than NB against downy mildew, suggesting that multiple mechanisms of action are involved in the biocontrol activity of NB.

Nutrient broth changed the structure and composition of leaf microbial communities

The NB treatment increased the number of bacterial CFU per leaf unit as compared to control plants (H_2O-treated and UNT plants) before and 24 h after *P. viticola* inoculation, and leaf bacteria could possibly compete for space against *P. viticola*. Conversely, LAM treatments did not affect bacterial CFU and culturable fungi were not affected by the treatments tested.

Each experiment evolved a different community structure of bacteria and fungi, however treatments had an impact only on the bacterial populations. Particularly, significant differences were found between populations of NB-treated and control plants (UNT and H_2O-

treated), but not between LAM-treated and control plants. Although all plants originated from the same nursery stock and were grown under the same controlled conditions, indigenous communities differed between the two experiments, in agreement to what already observed in field experiments (Perazzolli *et al*., 2014). Therefore, changes occurred in leaf bacterial and fungal populations after NB treatment were affected by the composition of the originally residing microbiota. The NB treatment increased abundances of the Exiguobacteraceae and Pseudomonadaceae family as compared with H_2O treatment before inoculation in the first experiment and in the second experiment, respectively (Figure 1B). At genus level, NB increased the proportion of some genera (*Exiguobacterium*, *Pseudomonas*, *Serratia*, and *Lysobacter*) that potentially include biocontrol strains, suggesting that these changes may contribute to downy mildew control.

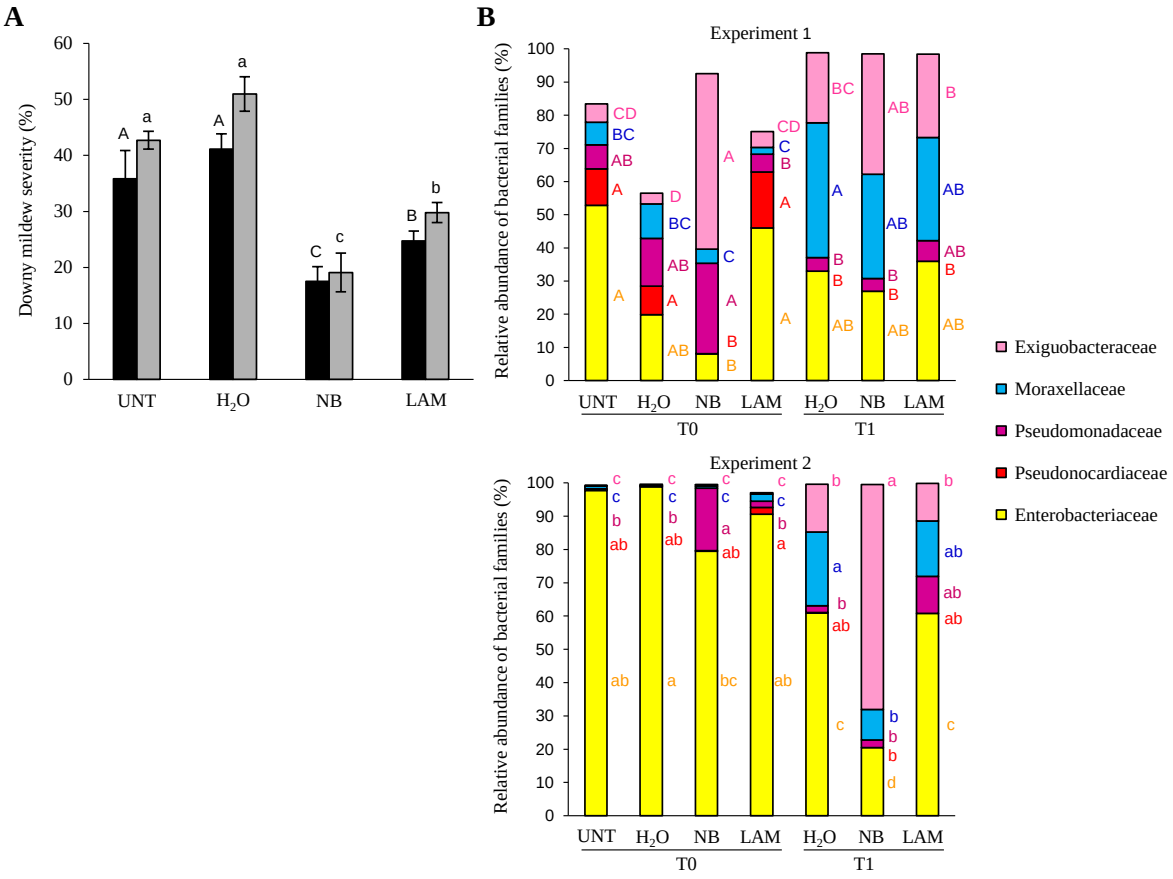

Figure 1. Plants were left untreated (UNT), or treated daily with water (H_2O), nutrient broth (NB) or laminarin (LAM) before *Plasmopara viticola* inoculation. A: Disease severity was assessed as percentage of leaf area covered by *P. viticola* sporulation in experiment 1 (black) and 2 (grey). B: Relative abundance of the dominant bacterial families on grapevine leaves before (T0) and 24 h after *P. viticola* inoculation in the experiment 1 and 2. Mean values of three replicates were analysed for each treatment and time point. Different uppercase and lowercase letters indicate significant differences according to Fisher's test ($\alpha = 0.05$) of experiment 1 and 2, respectively.

Nutrient broth stimulated grapevine resistance under axenic conditions

Since NB was able to change microbial community structure of grapevine leaves, experiments were carried out using *in vitro* grown plants in the absence of phyllosphere microorganisms (axenic conditions). The NB treatment (disease severity: 1.2 ± 0.9%; average ± standard error) reduced downy mildew symptoms on *in vitro*-grown grapevines as compared with H_2O-treated plants (disease severity: 19.1 ± 5.6%). Expression levels of *PR-2*, *PR-4*, *CHIT-3*, *OSM-1* and *OSM-2* were upregulated by NB before *P. viticola* inoculation and they remained at a high level at 24 h after inoculation. The *PR-1* gene, a marker of the salicylate pathway (Perazzolli *et al*., 2012), was induced by NB under greenhouse and not under axenic conditions, suggesting that leaf microorganisms could contribute to the activation of defence signalling pathways.

In conclusion, NB reduced downy mildew symptoms mainly by the induction of defence mechanisms in grapevine. Furthermore, NB stimulated culturable phyllosphere bacteria and changed proportions of some microbial taxa linked to the biocontrol of plant pathogens, possibly providing a partial contribution to the control of downy mildew and to the activation of defence signalling pathways.

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Designing efficient bacterial mixtures from extreme habitats to protect crops in a cultivar-specific manner

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Abstract: Advancements in sequencing technologies and microscopic techniques allow gathering valuable information about the genetics and ecology of plant-beneficial microorganisms. Integrated analysis may result in novel strategies to select and apply potential microbes, and to develop products for biocontrol (BCAs) and stress protection (SPAs) in agriculture. Here, we present a direct enrichment approach to obtain specifically adapted bacteria from the alpine habitats mosses, lichens and primrose, as each is adapted to adverse environmental conditions. Among other crops, sugar beets were used as bait plants and the role of the associated bacteria as potential BCAs and SPAs was evaluated in assays identifying phytopathogen antagonism, plant growth promotion capacities, the ability to allocate nutrients and to cope with abiotic stresses (Zachow *et al*., 2013). By applying a score scheme selecting desired properties, a sugar beet-specific composition of bacteria was designed and applied as seed treatment for field trials. The applied bacterial cocktail increased the number of early emerged seedlings up to 12% in comparison to the untreated control. The genome analysis of one bacterial component of the sugar beet cocktail, *Pseudomonas fluorescens* RM1-1-4, showed genes encoding abiotic and biotic stress protecting factors and other well-known bacterial traits for establishment of beneficial plant-microbe interactions. Additionally, the bacterial community structure of sugar beet cultivars with susceptible and tolerant phenotype towards the phytopathogen *Rhizoctonia solani* was analysed by amplicon sequencing. Principal coordinate analyses (PcoA) showed that tolerant and susceptible cultivars harbour distinct bacterial communities ($P \leq 0.003$) in the rhizosphere. A *Pseudomonas*-specific genotype closely related to the well described BCA *P. poae* RE*1-1-4 was enhanced in *Rhizoctonia*-tolerant cultivars, as example of naturally occurring biocontrol (Müller *et al*., 2013). Summarizing, screening for environmentally-conditioned and host-adapted microorganisms were proved to be a suitable tool for target-oriented exploitation of microbial bioresources for the management of ecofriendly crops facing biotic and abiotic stresses.

Key words: Bacterial mixtures, cultivar-specificity, plant microbiome, biological control, *Pseudomonas* species

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Plant species and soil type independent rhizosphere competence and biocontrol activity of *Pseudomonas jessenii* RU47 under field conditions

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Abstract: Biocontrol inoculants often show inconsistency in their efficacy at field scale and knowledge of the causes is largely lacking. A high rhizosphere competence of inoculant strains is assumed to be a key factor for successful biocontrol effects. We hypothesized that the plant species and the soil types affect the rhizosphere competence of *Pseudomonas jessenii* RU47 and its biocontrol effect against *Rhizoctonia solani* caused diseases. An experimental plot system with three soil types (diluvial sand, alluvial loam, loess loam) kept under similar agricultural management at the same field site enabled us to investigate the rhizosphere competence and biocontrol effect of RU47 in two plant species (lettuce and potato) and three soil types at the same site. A rifampicin resistant mutant of RU47 was used to evaluate the colonization density in the rhizosphere of both crops by plate counts. In addition, we assumed that both plant species and the soil type influenced the bacterial community composition in the rhizosphere. Bacterial community composition was analyzed by denaturing gradient gel electrophoresis (DGGE) of 16S rRNA gene fragments amplified from total community DNA. Unexpectedly, the ability of RU47 to colonize the rhizosphere of potato or lettuce and the biocontrol activity against *R. solani* (AG3/AG1-IB) was not influenced by the soil type. DGGE indicated that RU47 bacterial community composition was more affected by RU47 in the potato rhizosphere than in the lettuce rhizosphere while *R. solani* AG3 had more pronounced effects in the potato rhizosphere compared to the effects of AG1-IB in lettuce. The soil type and plant species independent biocontrol effects of RU47 indicated a promising biocontrol potential of RU47.

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Potential candidates for AHL-interacting proteins in plants

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Abstract: Priming for enhanced resistance of crop plants through biocontrol agents, like for example bacteria, is an efficient disease management strategy. Different studies validated the beneficial effects of bacterial quorum sensing (QS) molecules *e.g. N-acyl* homoserine lactones (AHLs) on the resistance against pathogen in various crop plants including tomato, barley and beans. Similarly, *Arabidopsis* pre-treated with the bacterial QS molecule oxo-C14-HSL was more resistant towards the pathogenic bacterium *Pseudomonas syringae* pv. *tomato* DC3000. Moreover, oxo-C14-HSL-primed plants showed stronger activation of mitogen-activated protein kinases AtMPK3 and AtMPK6, followed by higher expression of defense-associated transcription factors *WRKY22* and *WRKY29* along with the *PR1* gene subsequent to flg22 perception (Schikora *et al*., 2011; Schenk *et al*., 2014). So far, AHLs of different lengths of their lipid moiety ranging from 6 to 14 carbons and substitution with oxo- or hydroxyl groups in the γ position have been identified, and these modifications contribute to the various impacts on plants, such as induced resistance or growth promotion. Transcriptional reprogramming of various defense and growth related genes upon AHL recognition modifies the physiology of primed plants. In bacteria, AHLs are perceived through their cognate receptor(s), often from the LuxR-type family. In animals, Peroxisome Proliferator-Activated Receptors PPARγ and PPARβ, members of the nuclear hormone receptor (NHR) family and ras GTPase-activating-like protein IQGAP1 were proposed as potential candidates for AHLs receptors. Although AHLs induce modifications in development or changes in gene expression of various defense and growth related genes, AHLs interacting proteins in plants are not yet reported. In order to elucidate the role of AHLs in plant defense, it is essential to identify and characterize the AHL perception mechanism(s). In this study, we present a search for the AHL-interacting proteins in plants, and show the expression of defense-related genes in oxo-C14-HSL-primed of wild type plants and two mutants in potential candidates for AHL-interacting proteins in *Arabidopsis thaliana*.

Apart from functioning as an autoinducers for collective behavior among bacterial community, QS molecules are used for inter-kingdom communication with eukaryotic host.

Key words: priming, AHL, quorum sensing

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Ecology of bacterial antagonists and their complex interaction with the pathogen and the host plant rhizosphere microbiome

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Abstract: A better understanding of the environmental factors that influence the fate and activity of inoculants is a prerequisite for their smarter use. In this talk two studies aiming to unravel the complex interaction of bacterial inoculants, pathogens and the indigenous rhizosphere bacterial communities will be presented. Advantages and limitations of the culture-dependent and -independent methods used to study the complex interaction of inoculants with the pathogen and the host plants' microbiome will be discussed.

The influence of the soil type on the rhizocompetence of *Pseudomonas jessenii* RU47 and its biocontrol activity towards the soil-borne fungus *Rhizoctonia solani* AG1/IB (lettuce) was investigated under field conditions in a unique experimental unit where three soil types were stored for more than 10 years under the same weather conditions. Amplicon sequencing of 16S rRNA gene fragments from total community DNA showed a distinct soil type-dependent bacterial community composition from the rhizosphere samples of lettuces. However, independently of the soil type several genera such as *Pseudomonas* were strongly enriched in the rhizosphere compared to bulk soil independent of the soil type. This might also explain our unexpected finding that both the rhizocompetence and biocontrol activity of RU47 were found to be soil type-independent. The inoculant RU47 reduced disease symptoms and severity and caused shifts of the indigenous bacterial communities might have contributed to the biocontrol effects.

Three independent greenhouse experiments with tomato plants were performed to evaluate the rhizocompetence of *in vitro* antagonists and their ability to control *Ralstonia solanacearum* (*Rs* biovar2/race 3). The population densities of *Rs* and antagonists in the rhizosphere of tomato plants were estimated by selective plating and in total community DNA by means of real-time PCR and PCR-Southern blot hybridization targeting the *fliC* gene. Two *in vitro* antagonists, *Bacillus vallismortis* (B63) and *Pseudomonas brassicacearum* (AL2YTEN-142), showed a pronounced delay in or no disease symptoms, and significantly decreased in *Rs* population compared to the uninoculated plants. Amplicon sequencing of 16S rRNA gene fragments amplified from TC-DNA revealed pronounced treatment-dependent shifts in bacterial communities of the tomato rhizosphere. Most important, the strong reduction of *Rs* in the presence of the antagonists was confirmed and dynamic taxa in response to *Rs* or the inoculants were identified. Confocal laser scanning microscopy uncovered colonization patterns of the AL2YTEN-142. Gfp-positive cells were detected in lateral roots, root hairs and epidermal cells and within xylem vessels. Both inoculants hold a lot of promise to control *Rs* under field conditions.

In conclusion, both case studies showed that the combination of culture-dependent and independent methods allowed to gain insights into the rhizosphere competence of *in vitro* antagonists and their effects on the pathogen abundance, disease severity and most importantly, the bacterial community analysis by amplicon sequencing indicated that rhizosphere microbiome shifts in response to the inoculants might be a mode of action of biocontrol strains so far overlooked.

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Menaces from the plant microbiome

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Abstract: Micro-organisms potentially threatening human health are commonly found in plant microbiomes (Berg & Smalla, 2009). In spite of the fact that some species in plant microbiomes have close taxonomical relatedness to human pathogens, their roles in infectious diseases in humans remain inconclusive. Several identified bacteria in our endophyte and rhizosphere soil collections from different plant species and collected over the past twenty years showed close relatedness to potentially risk full organisms, such as *Bacillus cereus*, *Escherichia coli*, *Klebsiella varicola*, *Serratia marcescens*, *Legionella lytica*, *Staphylococcus epidermis*, and *Stenotrophomonas maltophilia*. One of these species, *S. marcescens* strain A2, isolated as an endophyte from *Arabidosis* plants, showed strong suppressive activities to mites (*Tetranychus urticae*) and thrips (*Frankliniella occidentalis*). Strain A2 was, for over a long time, considered as a promising agent for biological control of herbivore arthropods in vegetable and flower production systems in glasshouses. However, because of its close relatedness to potential human pathogens, it was decided not to continue with registration of this strain for commercial applications. Typically, these so called 'suspected human pathogens' occasionally can offer good control properties against plant devastating organisms and thus can be regarded as plant beneficial species.

The key question that will be addressed in this paper is 'what potentially riskful organisms are doing in plant microbiomes?' Do we have sufficient understanding on the ecological behaviour of microbes infectious to humans under agricultural and natural circumstances? Three distinguishable hypotheses on the occurrence of human pathogens are commonly postulated within this context. The first hypothesis is that all human pathogens occurring outside the clinics must be regarded as invasive species and circumstances prevailing in agriculture and nature are hostile to these species. The second hypothesis is that some plant-associated species and human pathogens evolved from a same common ancestor and both groups thus still have many cellular and molecular features in common. The third hypothesis is that human pathogens can become integral parts of plant-associated communities and that their prevalence in these communities occasionally is context dependent (Van Overbeek & Saikkonen, 2016). These three hypotheses may appear to contradict, but do not necessarily have to exclude each other, when human pathogens are regarded as continuously adapting and specializing groups of species.

As an example, we isolated shiga-toxin producing *E. coli* (STEC) strains from grass plants (shoots and rhizosphere soil). Their prevalence in meadow land could be explained by presence of grazing cows, but not by application of stable manure to land. Namely, STEC bacteria were detected in one year, but remained untraceable in the year after on the same land without grazing cows, whereas manure was applied to the soil in both years. We demonstrated that STEC bacteria persisted for weeks and possibly longer in grass land and also it was demonstrated that a STEC isolate was able to colonize pea (*Pisum sativum*) roots in an additional experiment. In fact, two other *E. coli* strains, both from different sources (a plant and a stool sample from a diseased human individual), showed equal persistence near plant roots over a longer period in time (more than six weeks). The three strains were inoculated via

seeds to growing plants and thus were allowed to 'choose' for different plant tissue types, but all unequivocally opted for roots as their preferred sites for persistence. In spite of the fact that *E. coli* (an organism typically dwelling in gut systems of warm-blooded animals) was introduced via cow faeces to grass plants (an environment considered to be hostile for *E. coli*), it was able to select for its preferred site where it remained alive, metabolically active and where it possibly was able to multiply. Plant roots can be considered as an alternative environment for *E. coli* and from an evolutionary perspective this makes sense as root colonization would warrant introduction into gut systems of other grazing animals (Van Overbeek *et al.*, 2014). Circulation across environments could be a strategy for human/ animal pathogens to disseminate and to explore new opportunities for growth. Many plant-associated bacteria belong to the same taxonomical class as *E. coli* (Enterobacteriaceae) and some common rudimentary features remained intact enabling *E. coli* to persist in the plant microbiome.

Crossing ecosystem borders is an important evolutionary aspect for many microbes and not only for human pathogens (Toth *et al*., 2006). Presence of human and animal pathogens in microbiomes can be a natural phenomenon, not exclusively evoked by human activities. However, agricultural management practices may stimulate human pathogens to invade plant microbiomes, so called context dependent events that may obscure generalizing effects on the presence of human pathogens in plant microbiomes. This aspect requires further attention and thanks to current rapid development in high throughput DNA sequencing technologies, other 'omics technologies including bio-informatics pipelines, we are capable to investigate complex microbial communities across different environments and to search for microbial commonalities among soil, aquatic, plant, food, and animal and human gut environments. These efforts are proposed to be combined at a European scale level in the submitted EU COST Action 'HUPLANTcontrol'.

Key words: plant microbiome, human pathogens, biocontrol

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Effect of compost treatments on matched growing media and root microbiome samples in a *Pythium*-cucumber system

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Abstract: The use of compost as a disease suppressive agent against *Pythium* root-rot in cucumber has been demonstrated over the years. While it has also been demonstrated that the microbial activity within composts is responsible for this suppression, the evaluation of microbial communities of compost-based growing media which include a comparison of sterilized and non-sterilized composts, along with the host plant root microbial community, has not been extensively studied. We hypothesized that microbial communities of substrates composed of peat based media would differ between those produced with compost, and that compost which had been gamma irradiated would provide a different fingerprint than either control (peat only) and composts which were not irradiated after 21 days of growth. We conducted a study evaluating the effect of peat based substrates in a cucumber system at three levels of pathogen inoculation in a pot bioassay. Two known suppressive composts produced from differing feedstocks were used, both with and without irradiation. The different substrates were planted with pregerminated cucumber (*Cucumis sativa*) seeds and maintained in a temperature controlled chamber for 21 days. Significant differences in mortality rates of plants were observed between substrates revealing the high suppressive properties of the compost-amended non-irradiated substrates. A strong biological impact of the composts was proven independently from the nutrient status of the substrates. The microbial community compositions of substrates prepared with peat alone, with irradiated composts, and with non-irradiated composts were compared based on total community DNA. 16S rRNA and ITS rRNA amplicon fingerprinting by denaturing gradient gel electrophoresis (DGGE) showed that the bacterial and fungal community composition of the root samples as well as the substrates differed between substrate formulation. 16S rRNA and ITS rRNA Illumina amplicon sequencing will provide further insights into taxa potentially indicative for community members suppressive to this *Pythium* root-rot pathogen.

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Bacterial endophytes from seeds of conifers with potential role in biocontrol: microbiome analysis

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Abstract: It is well established that many forest conifers depend on associations with mycorrhizal and foliar endophytic fungi. However, our understanding of the bacterial endophytes of conifers is limited. In this work we used 16S rRNA pyrosequencing to ask whether these conifers host a core of bacterial species that are consistently associated with conifer individuals and therefore potential mutualists. It was found that for seeds of coniferous the total number of established nucleotide sequences belonging to bacteria is low and for different samples does not exceed 100. Overall, it should be noted that at the level of phyla the dominating position was taken by representatives of the Proteobacteria (about 30-35% of the total) and Actinobacteria (30%), whereas the number of such phyla as Firmicutes, Bacteroidetes, Acidobacteria was significantly lower (1 to 5%). The most interesting results of the microbiome analysis of the seeds of conifers can be monitored at the level of families. For seeds it was found that diversity of endophytic bacteria in the composition of microbiome is low and is determined by four main families: Enterobacteriaceae, Pseudomonadaceae, Oxalobacteraceae and Comamonadaceae. As a general trend, similarity is found with other microbiomes of conifers seeds with the same dominant components that are characteristic for pine and spruce seeds.

Key words: bacterial endophytes, seeds, coniferous, microbiome, ultra-deep pyrosequencing, phylogenetic analysis

Introduction

The strategy of symbiotic (cooperative) adaptations is at least as common, if not more common, in the living nature as that of individual (autobiotic) adaptations (Tikhonovich & Provorov, 2009). However, few plants have been studied in respect of their interaction with endophytic microorganisms. Therefore, the search for new endophytic microorganisms with beneficial properties among plants in various ecosystems is a promising research direction (Ryan *et al*., 2008; Chebotar *et al*., 2015). Bacterial endophytes may act as biocontrol agents by colonizing ecological niches occupied by phytopathogens (Berg *et al*., 2006). It is thought that any host organ may be colonized (Schulz & Boyle, 2005).

The diversity of endophytic bacteria inhibiting the roots, stems, and leaf tissues was examined in conifers (O'Neill *et al*., 1992; Chanway, 1997; Izumi *et al*., 2008). The most typical isolates of bacterial endophytes of conifers were closely related to *Methylobacterium*

spp. (Pirttilä *et al*., 2005), *Bacillus* spp., *Paenibacillus* spp., *Acinetobacter calcoaceticus* (Izumi *et al*., 2008), *Bacillus* spp. and *Paenibacillus* spp. (Bal *et al*., 2012), *Paenibacillus polymyxa* (Anand *et al*., 2013) and *Gluconacetobacter* (Carrell & Frank, 2014). It is well established that many forest conifers depend on associations with mycorrhizal and foliar endophytic fungi, but our understanding of the bacterial endophytes of conifers is limited (Pirttilä & Frank, 2011). While studies of specific bacterial endophytes associated with vegetative parts of conifers have been fragmentary reported, the diversity and ecology of endophytic bacteria in generative organs, such as flowers, cones and seeds (Cankar *et al*., 2005) is not well investigated.

In this work we used 16S rRNA ultradeep pyrosequencing to characterize the taxonomic composition of bacteria in inner tissues of surface-sterilized seeds of two populations of Scotch pine (*Pinus sylvestris* L.), two population of European spruce (*Picea abies* L.) and two populations of Siberian cedar *(Pinus sibirica* L.).

Material and methods

Plant material and total DNA isolation

Cones from *Pinus sylvestris* L. and *Picea abies* L. were collected in summer 2014 at 2 native sites in the Middle Volga: forestry Serunskoe (Republic Mari-El) and forestry Zelenodolsk (Republic Tatarstan). Cones from *Pinus sibirica* L. were collected in autumn 2014 at 3 native sites on the Baikal Lake coast (Irkutsk Region).10 cones from five trees on each site were sampled, in total about 50 cones samples were collected. The seeds were removed with sterile tweezers, placed in sterile bags and sent to ARRIAM, Saint-Petersburg, for processing.

Seeds were surface sterilized with 70% ethanol 2 min, washed with sterile water and twice rinsed in 30% sodium hypochlorite for 15 min. The inner tissues from seeds were removed with a sterile scalpel. Total DNA was isolated from 0.5 g of frozen inner tissues after its mechanical destruction using glass beads in extraction buffer. DNA was purified by phenol-chloroform, precipitated by isopropanol, and then purified by electrophoresis in 1% agarose gel and isolated by sorption on silicon dioxide (Andronov *et al*., 2009).

Pyrosequensing and phylogenetic analysis

When constructing and sequencing amplicon libraries, purified DNA preparation (10-15 ng per sample) is used as a matrix of PCR (temperature profile: 95 °C – 30 s, 50 °C – 30 s, 72 °C – 30 s, altogether 30 cycles) with the use of Encyclo polymerase (Evrogen, Russia) and universal primers to the variable region of V4 gene 16S rRNA F515 (GTGCCAGCMGCCGCGGTAA) and R806 (GGACTACVSGGGTATCTAAT) (Bates *et al*., 2010). The primers also contained oligonucleotide identifiers for each sample (20 identifiers) and accessory sequences for pyrosequencing according to the protocol of Roche (Switzerland). Sample preparation and sequencing were performed in GS Junior (Roche, Switzerland) according to the manufacturer's protocol.

Homologous 16SrDNA sequences were searched against bacterial databases from the Ribosomal Database project (http://rdp.cme.msu.edu), using the BLAST-N nucleotide software with the Megablast algorithm (Cole *et al*., 2009). The identification criterion was based on a > 98% sequence identity. The sequences were aligned using the multiple sequence alignment software (Unipro UGENE). To create the heatmap, the heatmap function in QIIME was used. The function visualizes the operational taxonomic unit (OTU) table generated by QIIME (this table tabulates the number of times an OTU is found in each sample).

Results and discussion

Composition of endophytic microbiome in seeds endosphere

We used the method of flow sequencing to analyze the endophytic community of seed coniferous plants that has allowed to assess more deeply the true scale of the natural genetic diversity of endophytic microorganisms. Therefore, we could perform a complex analysis of the prokaryotic community. However, during analysis of the obtained datasets (the number of sequences in each probe was 1-2 thousand) the emphasis was made on the taxonomic groups of interest, in particular, the phyla Bacteria, Proteobacteria, and Actinobacteria.

Analysis of bacterial communities at the level of phyla and class did not reveal any considerable differences in their composition in endophytic microbiome across experimental variants. The total number of identified nucleotide sequences (reads) belonging to bacteria was low and for different samples did not exceed 100-200. Thus, certain types of endophytic bacteria exist which are widely distributed and are characteristic of a particular sample of seed of conifers. Overall, it should be noted that at the level of phyla dominating position was taken by representatives of the Proteobacteria (about 30-35% of the total) and Actinobacteria (30%) (Figure 1A). The number of phyla such as Firmicutes, Bacteroidetes, Acidobacteria was significantly lower (1 to 5%).

The most interesting results of the microbiome analysis of the seeds of conifers can be found at the level of families (Figure 1, B). For European spruce, the diversity of endophytic bacteria in the composition of the microbiome is low and is determined by two main families: Enterobacteriaceae, and Comamonadaceae. For seeds of *Picea abies* L. from the Republic of Mari-El, representatives from genus *Providencia* (family Enterobacteriaceae) were found as part of the composition of the microbiome. The microbiome of seeds of Scots pine had a similar pattern. As part of the microbiome of *Pinus sylvestris* L. seeds from the Republic of Tatarstan representatives of families such as Enterobacteriaceae, Pseudomonadaceae, Flavobacteriaceae were found, which are also widely detected in various associations with plants, including endophytic associations. It was found that the Gram-positive bacteria were closely related to the orders Lactobacillales and Clostridiales.

The endophytic microbiome analysis of *Pinus sibirica* L. seeds (Figure 1B) revealed its composition of bacteria from families Enterobacteriaceae, Pseudomonadaceae (Gammaproteobacteria) Comamonadaceae, Oxalobacteraceae (ord. Burkholderiales). No significant differences in the microbiome composition of cedar seeds have been identified for seeds from different geographical regions. As a general trend, similarity is found with other microbiomes of conifers seeds with the same dominant components that are characteristic for pine and spruce seeds.

Potential role of microbiome in biocontrol

One of such typical groups of microorganisms can be considered as representatives of the order Burkholderiales: families Comamonadaceae and Oxalobacteraceae. It is known that the members of this order are most commonly found in the composition of endophytic microbiota within tissues of different plants (Frommel *et al.*, 1991; Pillay & Nowak, 1997; Bensalim *et al*., 1998;. Ait Barka *et al*,. 2000; 2002; Compant *et al*., 2005; Berg *et al*., 2006; Shcherbakov *et al.*, 2013). It is possible that the bacterial endophytes of this order also occupying a dominant position in the microbiome composition of conifer seeds are its main structural components. Members of the order which was described above, in particular the genus *Burkholderia*, exhibited their biocontrol properties in *in vitro* experiments (Berg *et al.*, 2006) and in the process of interaction in the plant rhizosphere (King & Parke, 1996; Vandamme *et al*., 2007).

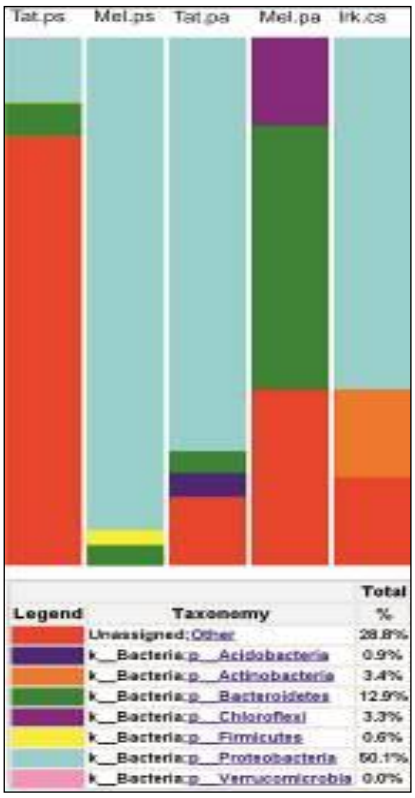

Figure 1A. Taxonomic diversity of endophytic bacteria associated with coniferous seeds of different geographical origin. Taxonomy summary, current level – phyla.

Other potential families providing endophytic biocontrol properties as part of the microbiome of seed endospheres are the families Pseudomonadaceae and Flavobacteriaceae, which genera are known for their biocontrol and plant growth promoting abilities and widely are used in agricultural microbiology for the development of broad spectrum biofertilizers (Shenin *et al*., 1996; Tikhonovich & Provorov, 2009).

Thus, we have not identified certain dependences in the distribution of taxonomic units, depending on the host species or geographical origin of seeds samples, which coincides with opinion of other authors. The microbiome composition of pine is more diverse compared to spruce and cedar which is dominated by one or another species. Rather, certain taxa colonize the endosphere of seeds randomly. Thus, the most likely candidates are typical epiphytes of generative organs of conifers (Cankar *et al*., 2005; Compant *et al*., 2010; Pirttilä, 2011). As a result, the amount of microflora in the endosphere of seeds is negligible (Truyens *et al*., 2015) and it is determined at the level of tens of units sequenced reads. In addition, according to our data, the seed microbiomes are substantially different in their taxonomy from the endophytic microflora of vegetative parts of coniferous plants (Chanway, 1997; Izumi *et al*., 2008; Carrell & Frank, 2014).

Figure 1B. Taxonomic diversity of endophytic bacteria associated with coniferous seeds of different geographical origin. Heatmap showing the most dominant families and their average relative abundances as percentages of all sample 16S rRNA gene sequences recovered in conifer seeds samples.

It is possible that part of the detected bacteria using metagenomic analysis methods has biocontrol properties with a potential to provide protective properties to germinating embryos of conifers (Bacilio-Jiménez *et al*., 2001; Shade & Handelsman, 2012;), as well as to accompany the plant throughout its entire life as endophyte, epiphyte or rhizosphere microorganism (Mitter *et al*., 2013; Truyens *et al*., 2015*).* Other conifers, which do not carry the seed biocontrol agents, may consequently be more susceptible to a variety of phytopathogenic invasions. In this case, the detailed study of the future behavior of bacterial seeds endophytes is required, especially their strategy of plant colonization during germination and their possible effect in biological control of plant pathogens.

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Comparative analysis of the root microbiomes of *Verticillium longisporum* resistant and susceptible rapeseed lines

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Abstract: In a first pilot study we investigated the root-microbiomes of two contrasting double haploid oilseed rape lines which were either resistant or susceptible to *Verticillium longisporum* infection. The two contrasting oilseed rape double haploid lines were obtained from a cross of a susceptible and a resistant parent. We hypothesized that the rapeseed lines establish specific root microbiomes which may be responsible or contribute to the resistance against *V. longisporum.* To get a first impression of differences in the root microbiomes of the two rapeseed lines seeds were germinated and grown for 10 days either in sand or soil. Roots were harvested to study the root microbiomes. First results indicated that the root microbiomes were strongly affected by the growth in sand or soil but also showed rapeseed line specific differences, especially after growth in sand. Interestingly, the root microbiome of the resistant rapeseed line was more diverse than that of the susceptible rapeseed line after growth in soil. Those preliminary data gave a first inside in rapeseed line specific root microbiomes. Currently running studies will further extend the understanding of the role of the rapeseed line specific microbiomes for expression of *Verticillum* resistance.

Key words: rapeseed, *Verticillium longisporum*, root microbiome

Introduction

Agricultural plants harbor a high diversity of microbes colonizing the rhizosphere and endophytic compartment of roots. Several of those root colonizing microbes promote plant growth and prevent fungal and bacterial pathogen invasion (antagonism). Root exudation pattern and other so far unknown plant-derived factors can strongly affect root colonization with specifically adapted microbial communities, which in turn have beneficial effects on the plants. It was demonstrated in several studies that plant species and genotypes as well as the soil types can strongly influence the composition of the rhizosphere and root microbiome (Berg & Smalla, 2009; Bulgarelli *et al*., 2012; Lundberg *et al*., 2012; Berg *et al*., 2014). Here we investigated the root microbiomes of two contrasting rapeseed lines which were either resistant or suseptible to *Verticillium* infections to determine if specifically adapted root microbiomes can be correlated with the resistance phenotype. Furthermore root microbiomes of both rapeseed lines grown in soil or sand were investigated to determine if soil types effects the development of rapeseed line specific root microbiomes.

Material and methods

Rapeseed lines
Two contrasting oilseed rape double haploid lines were investigated in this study, DH 12 (resistant to *V. longisporum*) and DH 147 (suseptible to *V. longisporum*). The two double haploid lines were obtained from a cross of a *V. longisporum* susceptible and resistant parent and selected based on 191 background microsatellite markers for similar genome composition and by two quantitative trait loci-specific markers for contrasting resistance allele composition.

Experimental setup
Rapeseed seedlings were germinated and grown for ten days in sand or soil in the greenhouse. A subset of the plants was harvested for microbiome studies (9 pooled plants per treatment). A further subset of the plants were dip inoculated with a *V. longisporum* spore solution. Mock inoculated plants were used as controls. Differences in the development of the disease pattern were investigated two and four weeks after dipinoculation and growth in a sand/soil mixture.

Microbiome study
Rhizosphere and endophtic root compartments were seperated as described by Bulgarelli *et al*. (2012). Genomic DNA from washed root samples (= endophytic compartment and strongly root attached bacterial biofilms/rhizoplane microbes) were extracted using the NucleoSpin soil DNA extraction kit from Macherey Nagel as described by the manufacturer. DNA concentrations were determined by Nanodrop anaylsis and adjusted to 5 ng/µl for PCR amplifications. 16S rRNA amplicon sequencing and data processing was performed by LGC genomics (Berlin, Germany) by 300 bp paired-end read Illumina MiSeq V3 sequencing. Amplicon libraries were generated with bacterial 16S rRNA gene targening primers 341F and 785R. Comparative microbiome analysis was performed by using SILVA and the SILVAngs pipeline (Quast *et al*., 2013). Statistical analysis of microbiome datasets were done in PAST version 2 (http://folk.uio.no/ohammer/past/).

Results and discussion

Resistance and susceptibility against Verticillium longisporum in contrasting rapeseed lines
V. longisporum infection of ten days old seedlings of DH 12 and DH 147 showed different effects on the development of the plants two weeks after infection. Infected DH 147 plants showed disease pattern typical for *V. longisporum* infection, while infected plants of DH 12 grew in the same manner as control plants. Four weeks after infection DH 147 plant were dead while DH 12 plants did not show any symptoms, indicating the resistance of DH 12 against *V. longisporum.*

Root microbiome of the contrasting rapeseed lines after growth in sand and soil
To elucidate the role of the root microbiome on *V. longisporum* resistance initally the root microbiomes present in the two rapeseed lines without fungal inoculation were compared. Plants were either germinated and grown in sand or soil. Ten days after germination both genotypes were equally developed. For both lines growth in sand and soil was different. Plants grown in soil were always larger than those grown in sand. The root microbiome (bacteria strongly attached to the root surface and colonizers of the endophytic compartment) showed clear differences between plants grown in sand and soil but also between plant lines

(Figure 1). In summary 37 bacterial phyla or candidate phyla were detected. The most abundant phyla in all samples were Proteobacteria, with 60-62% relative abundance if rapeseed lines were grown in soil and >54% if grown in sand. Within proteobacteria Alpha- and Betaproteobacteria dominated the microbiomes. Second and third most abundant phyla after growth in soil were Actinobacteria (6 to 9%) and Bacteroidetes (7 to 10%). A strong rapeseed line specific effect was determined after growth in sand with an unexpected high abundance of Planctomycetales (20%) in the *Verticillium* resistant rapeseed line. Non-metric multi-dimensional scaling at the level of phyla (Figure 1B) and at the level of genera (Figure 1C) clearly showed the differences of the root microbiome for the rapeseed lines after growth in sand and soil. While the overall patterns of the root microbiomes were similar for the two rapeseed lines after growth in soil clear difference in the root microbiomes of the two rapeseed lines were obtained after growth in sand. The difference of the root-microbiomes after growth in soil was less obvious in this analysis. But subsequent rarefaction and diversity analysis showed that the root microbiome of plants grown in soil was more diverse for the resistant line than for the susceptible line. In contrast the diversity obtained for the root microbiomes after growth in sand was the same but as shown before more different among the plant lines.

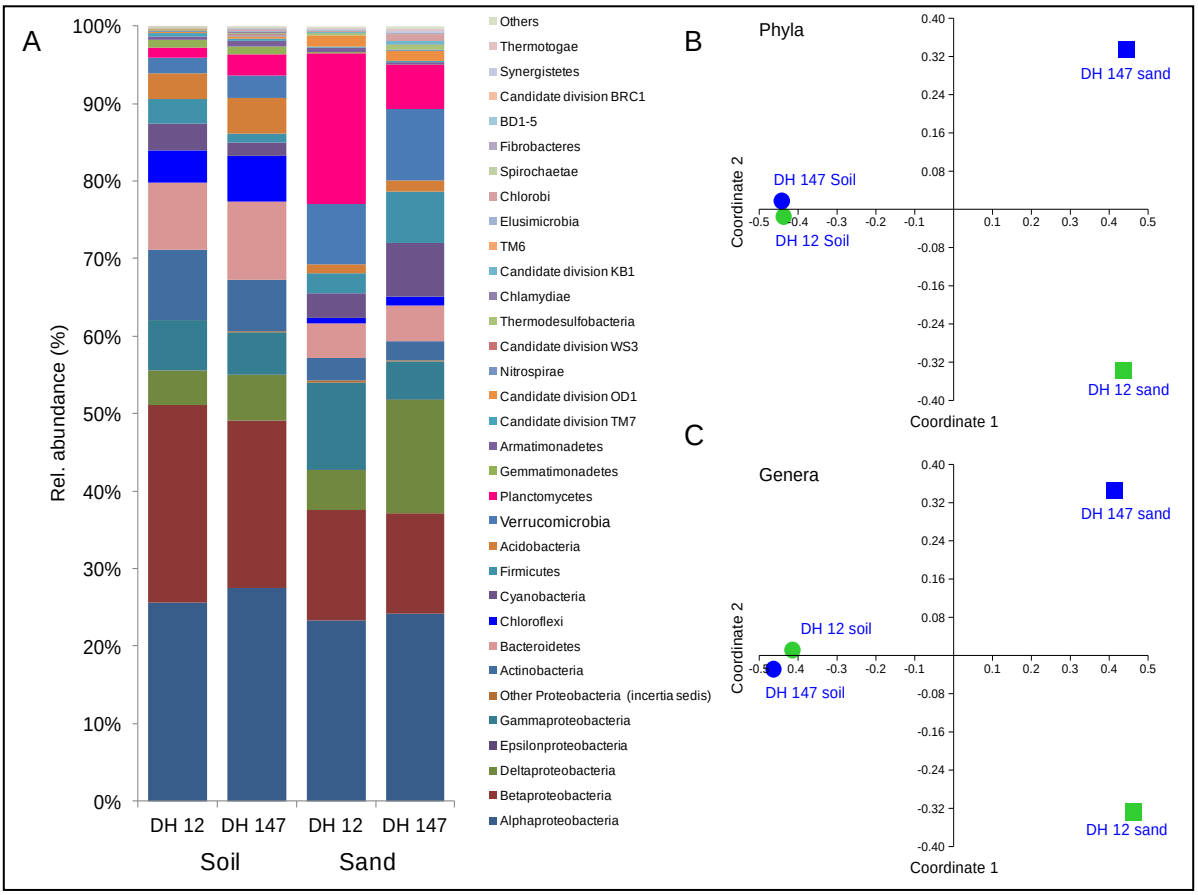

Figure 1. Root microbiomes of 10 days old seedlings (a pool of nine individual plants) from DH 12 and DH 147 grown in soil or sand. A: Relative abundance of bacterial phyla, B/C: Non-metric multi-dimensional scaling at the level of phyla (B) and genera (C). The amplicon datasets contained a total of 391,699 reads with an average length of 415 nucleotides. A total of 42816 OTUs were defined using a sequence similarity threshold level of 98%.

Conclusions and outlook

Here we demonstrated that both, soil type and plant lines, affect the diversity and compositions of root microbiomes of rapeseed lines with contrasting *V. longisporum* resistance. This pilot study indicates that an increased microbiome diversity may be involved in resistance expression against *V. longisporum*. As already shown by Wei *et al*. (2015) species rich microbial communities efficiently prevent pathogen invasion. This correlates well with the finding that the resistant rapeseed line harbours a more diverse root microbiome. Further experimental evidences are however required to proof this finding. Furthermore, microbiome shifts in the presence of *V. longisporum* needs to be investigation. Not only differences in the initial microbiomes but also different responses to the exposure of the pathogen may contribute to differences in *V. longisporum* resistance. As shown by Chapelle *et al*. (2015) the presence of pathogens can strongly affect shifts in the root microbiome by the induction of the expression of oxidative stress response genes in several members of a root microbiota. Furthermore, more resistant and susceptible rapeseed lines are currently investigated to elucidate if the obtained microbiome shifts are rapeseed line (genotype) specific or trait (susceptible/resistant) specific.

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Linking the belowground microbial composition, diversity and activity to soilborne disease suppression and growth promotion of tomato amended with biochar

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Abstract: Biochar, in addition to carbon sequestration, soil amelioration and improvement of plant performance, can significantly reduce plant diseases. Nevertheless, the mechanisms associated with soilborne-disease suppression are not fully understood. This study tested the effects of two biochars at concentration of 0-3% (w:w) on fusarium crown and root rot (FCRR) of tomato caused by *Fusarium oxysporum* f. sp. *radices-lycopersici* (FORL), with an emphasis on mechanisms of disease suppression. Biochar at higher concentrations suppressed FCRR of tomato by up to 79%. Furthermore, biochar significantly reduced the *Fusarium* root colonization and survival in soil. Yet, direct toxicity of biochar to FORL was not observed in *in vitro* assay. Biochar amendment significantly increased the culturable counts of general bacteria, fluorescent *Pseudomonas* spp., *Trichoderma* spp. (well-known biocontrol and plant growth promoting agents) and other microorganisms. Indeed, biochar-stimulated fluorescent *Pseudomonas* have antagonistic activity towards FORL. Illumina sequencing analyses of 16S rRNA gene showed substantial differences in bulk soil, rhizosphere and rhizoplane bacterial taxonomical composition between biochar-amended and control soils. Nevertheless, biochar amendment caused a significant increase in microbial diversity (Shannon's diversity, phylotype richness), microbial activities (respiration rates, dehydrogenase and other enzymes activities) and an overall shift in carbon-source utilization by microbial communities (Biolog Microplates), concurrent with increased plant growth and disease suppression. High microbial diversity and activity in the rhizosphere has been previously associated with soilborne diseases suppression and growth promotion, and this may collectively explain the significant reduction of disease and increase in plant growth observed in the presence of biochar.

Key words: microbiome, organic soil amendments, soil-borne diseases

Evaluation of the antifungal activity of the protein and non-protein extracts of *Trichoderma asperellum* and *Trichoderma atroviride* culture filtrates against *Phytophthora infestans* (Mont.) de Bary

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Abstract: This work aims to study the *in vitro* and *in vivo* antifungal effect of protein and non-protein extracts of the culture filtrates of 12 isolates of *Trichoderma asperellum* and one isolate of *Trichoderma atroviride* against A1 and A2 isolates of *Phytophthora infestans*.

The protein assay revealed significant concentrations (25.36 µg/ml) of extracellular enzyme extract for the TK isolate and a concentration of intracellular enzyme of 46.30 µg/ml for the TM isolate. The study of enzyme activity highlighted a proteolytic activity for all antagonistic isolates with the largest area proteolysis (10.3 mm) recorded for the TH and TM isolates. However, no chitinolytic activity was developed by all antagonist isolates

However, the protein extracts of all antagonist isolates showed a low inhibitory effect on mycelial growth but a moderate effect on sporulation and germination of the two pathogenic isolates. Mycoparasitism or inhibition of pathogenicity of *P. infestans* have not been observed.

Furthermore, chemical analysis by FTIR of butanol extracts of all *Trichoderma* spp. isolates culture filtrates revealed 16 chemical groups with some similarities between antagonist isolates and the dominance of acids, alkanes, aromatic groups and alcohols. Chemical analysis by GC-MS of the methanol and hexane extracts of antagonists culture filtrates revealed 32 metabolites with the dominance of 2H-Pyran-2-one-6-pentyl component (6PP).

The antioxidant activity by UV spectroscopy using the method of trapping of the free radical DPPH showed a moderate reducing effect compared to ascorbic acid for methanol extracts for all isolates of *Trichoderma* spp. culture filtrates.

Indeed, the antifungal activity of the two types of extracts has been confirmed *in vitro* and *in vivo*, on the A1 and A2 *Phytophthora infestans* isolates. The methanolic extracts of culture filtrates of the *Trichoderma asperellum* isolates confirmed the complete inhibition of mycelial growth, sporulation, germination and pathogenicity of *P. infestans* isolates. These results are encouraging to test them in field trials and thereafter develop a biofungicide formulation for the management of late blight of potato caused by *P. infestans*.

Key words: *Phytophthora infestans*, *Trichoderma atroviride*, *Trichoderma asperellum*, chemical analysis, extracts of culture filtrates

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Use of *Trichoderma* spp. isolates in the biocontrol of *Botrytis cinerea*, causal agent of gray mold of tomato *Solanum lycopersicum* (Mill.) in Algeria

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Abstract: The massive use of fungicides in the field of chemical control is ineffective with the emergence of new resistant and aggressive strains of *Botrytis cinerea*, and it is toxic for consumers. In order to develop alternative biological control methods, we conducted *in vitro* and *in vivo* tests to study the antagonistic activity of thirteen isolates of *Trichoderma* spp. issued from Algeria rhizosphere, against four isolates of *Botrytis cinerea* collected from tomato grown under protected conditions in Algeria (Douaouda, Sidi Ghilass, Tipaza city and El Oued city). The antagonistic activity was compared to the activity of a systemic fungicide whose active ingredient is the Procymidone 50% (Prolex).

The direct confrontation of *B. cinerea* with the antagonistic isolates or their culture filtrates showed a significant inhibition of mycelial growth (up to 80%) and a complete inhibition of sporulation, germination, production and germination of sclerotia of *B. cinerea* isolates. Structural changes resulted in the winding, lysis and vesiculation of the mycelium, as well as deformation and digestion of conidia content from the fourth day of confrontation. Also, a reduction in symptoms of gray mold was observed on detached leaves of tomato treated with the antagonist isolates whatever their modes of application (preventive or curative).

Moreover, a biostimulant effect of tomato (germination of seeds and growth of plants) was recorded after the treatment of seeds by these antagonist isolates. Foliar infections, damping-off, and the percentage of colonization of different plant parts by the plant pathogen have been greatly reduced (less than 5%).

The present work has confirmed the biostimulant effect and antagonistic activities of *Trichoderma* spp. against *B. cinerea* in view of their use in the management of gray mold of tomato.

Key words: *Trichoderma* spp., *Botrytis cinerea*, antagonistic activity, *Solanum lycopersicum*

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Testing the efficiency of some fungal isolates and bacteria in the inhibition of the growth of some of the causative fungi of root rots of grains and legumes

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Abstract: Root rot diseases cause losses in production that can reach up to 50% or more in grain and legume production. In the recent years, root rots became one of the most important disease problems in various wheat, lentil and chickpea growing areas in Syria. Their proliferation is increasing due to the difficulty in controlling them with chemicals, to poorly trained farmers and to an absence of crop rotation from year to year, especially for wheat. Therefore it is necessary to develop suitable and alternatives harmless to the environment and human to control the diseases, such as resistant varieties and biological control. In order to obatin healthy and safe products, international organizations are concerned about food security and consumer protection. Research was implemented in the year 2011 at the Agricultural Research Center in al-Qamishli in northeastern Syria, at an altitude of 452 m above sea level in length 41.13° east longitude, latitude 37.03° north. Average annual rainfall is about 440 mm with clay soils Lomé, with a proportion of mud between 54% and 24% silt and sand 22%, pH = 7.2.

The effectiveness of five isolates belonging to the genus *Trichoderma* isolated from soil in the study area and one bacteria belonging to the genus *Bacillus* (Bs10) was tested. Two isolates belong to the species *Trichoderma viride* (TrV4 and TrV5) and three isolates belong to the species *Trichoderma harzianum* (TrH1, TrH2 and TrH3). Inhibition of the growth of the pathogenic fungi *Fusarium culmorum, F. oxysporum, F. moniliforum, F. avenaceum, Helminthosporium sativum, Alternaria tenuis,* and *Rhizoctonia solani,* originally isolated from infected roots of wheat, lentils and chickpeas, was tested. Mycelial plugs of 0.5 cm diameter from each biological control isolate has been deposited 3 cm apart of the pathogenic fungus on a Petri dish, and 3 Petri dishes for each combination was inoculated. In order to study the bacterial antagonism, a bacterial spore suspension at 10^7 spore/ ml was added at a rate of 10 ml to 1 l PDA before hardening. Three plugs of each fungal isolates were placed in Petri dishes. The plates were placed in an incubator at a temperature of 25 ± 2 °C for a period of 9 days. The growth of the mycelium was assessed as colony diameter based on a 1-5 scale with (1) mycelium of the biocontrol fungus covers the entire space; (2) mycelium of the biocontrol fungus covers two thirds of the space; (3) mycelium of the biocontrol fungus covers half the space; (4) mycelium of the biocontrol fungus covers one-third of space and (5) mycelium of the pathogenic fungus covers the entire area.

The results indicated that the biocontrol fungal isolates led to the inhibition of the isolates of *Fusarium* spp. but did not affect the growth of other fungal isolates. The biocontrol isolates did not show any efficacy toward *Alternaria tenuis, Rhizoctonia solani*, while the bacterial isolate led to the inhibition of the growth of all fungal isolates tested, with *Fusarium* spp. the most inhibited and *Alternaria tenuis* the least inhibited and a significant reduction of the production of fungal spores compared to the control.

Key words: Al Qamishli, bacteria, biological control, fungi, root rots, Syria

Antifungal activity of a Moroccan plant extract against pathogenic fungi *Pyrenophora teres*, the causal agent of Net Blotch of barley

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Abstract: The extract of a medicinal plant, *Daphne gnidium*, was tested for its *in vitro* and *in vivo* antifungal activity against *Pyrenophora teres* f. sp. *teres*, the fungi causing net blotch of barley. The extract obtained by plant decoction is tested *in vitro* on the growth diameter of five Moroccan isolates of *Pyrenophora teres* f. sp. *teres*. Twelve concentrations were tested: 0, 5, 10, 15, 20, 25, 30, 35, 40, 45, 55 and 60 g/l. The concentration of 40 g/l of *D. gnidium* extract induced complete inhibition of 4 pathogen isolates *in vitro* conditions. The isolates inhibited by the medicinal plant extract and transferred on fresh PDA media did not restarted. Therefore the effect of *D. gnidium* on *Pyrenophora teres* f. sp. *teres* is fungicidal. The most active concentration of the extract in *in vitro* studies was tested in *in vivo* tests on barley leaves against net blotch. Incidence of net blotch was decreased to a rate of 0 on the scale of Tekauz, while control barley plants showed a rate up to 9 on the severity scale of the disease. This study demonstrated that plants extracts have a high potential to control net blotch of barley. Therefore such natural products represent a sustainable alternative to the use of chemical fungicides.

Key words: Net blotch, *Pyrenophora teres* Drechs. f. sp. *teres*, *Daphne gnidium*, aqueous extract, antifungal activity

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Analysing the bioactive potential of the endomicrobiome of New Zealand's medicinal plant *Pseudowintera colorata*

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Abstract: All land plants are inhabited by micro-organisms. International research has demonstrated that many of these are known to perform beneficial functions and contribute to plant growth and/or production of bioactive metabolites. Medicinal plants are highly valued for their bioactive compounds, and may host unique endophytes capable of producing the same or similar compounds as the host plant. *Pseudowintera colorata* (horopito), an endemic New Zealand plant recognized for its antimicrobial properties, is used in traditional Māori medicine (rongoā). The biologically active chemical constituent polygodial is used for treating candidiasis. This current study explored the bioactive potential of culturable endophytes isolated from horopito and analyzed the structure of the horopito endomicrobiome identifying: (i) a core endomicrobiome that was independent of site and (ii) members of the endomicrobiome affected by site, host physiology and edaphic factors. Plants were sampled from 10 sites across New Zealand. A total of 345 bacteria were isolated from surface sterilized leaves, stems and roots and assessed for their ability to inhibit phytopathogens such as *Neofusicoccum parvum*, *Neofusicoccum luteum*, *Ilyonectria liriodendri*, *Nectria galligena*, *Pectobacterium atrosepticum* and *Pectobacterium brasiliensis* using a dual culture technique. Additionally, the endophytes were screened against the opportunistic human pathogens *Staphylococcus aureus*, *Escherichia coli* and *Candida albicans*. DNA was also extracted from the sterilized tissues and amplified using group specific primers for actinobacteria, α-, β- and γ-proteobacteria, total fungi and arbuscular mycorrhiza. The amplicons were separated using denaturing gradient gel electrophoresis. Analysis of band profiles showed that some bands were consistently present in all plant tissues and some were tissue independent. Further analysis of the bands including sequencing of representatives will reveal species richness and information on correlations according to plant location, tissue type or age.

Key words: horopito, biocontrol, endomicrobiome

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Long term (in)stability of vegetative incompatibility type diversity and hypovirulence in *Cryphonectria parasitica* populations

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Extended Abstract: Chestnut blight is a dangerous disease of the American (*Castanea dentata*) and European chestnut (*C. sativa*) caused by the growth of the fungus *Cryphonectria parasitica* inside the infected trees' bark. The spreading of mycelia inside the plant's tissues causes damage to the bark, cambium and the vascular tissue. If the active growth of the virulent fungus girdles the entire branch or trunk of the tree, distal parts of the plant die off, and the lower dormant lateral buds sprout and grow (Dutech *et al*., 2012). The most effective known way to combat this disease is by means of biological control using a hyperparasite – *Cryphonectria hypovirus* 1 (CHV-1) (Prospero & Rigling, 2013). This virus infects *C. parasitica*, causing morphological shift of the affected mycelia: from the brightly orange to white, which indicates the attenuation of the virulence of the infected strain. Thus, the white colored, CHV-1-infected *C. parasitica* strain is said to be hypovirulent, because the infection induced by such a strain causes only slight, superficial, and non-lethal wounds (Nuss, 2005). The efficacy of the biocontrol mediated by the presence of the CHV-1 infected strains of *C. parasitica* is directly associated with vegetative compatibility (vc) type diversity of the *C. parastica* population, the prevalence of the hypovirulent isolates, and their distribution among different *C. parasitica* vc types. Vegetative compatibility of *C. parasitica* in European fungal isolates is controlled by at least six bialleic loci called vegetative incompatibility (*vic*) loci. The difference in one or more loci obstructs the hyphal fusion of the growing mycelia, which prohibits free transportation of the cytoplasmic elements between the incompatible mycelia, thus preventing the efficient dissemination of the CHV-1 within population (Cortesi *et al*., 2001). Sexual reproduction is the most efficient way the vc type diversity can increase within a population, thus potentially obstructing CHV-1 transmission and biological control of this pathogen.

Cryphonectria parasitica appeared in Croatia in the 1950-ies and rapidly spread over most of the country in the next couple of decades. By the 1980-ies none of the native chestnut populations were free of this disease. The first comprehensive study was carried by Krstin *et al*. (2008) and it showed pronounced differences between populations of *C. parasitica* in Croatia. In our recent study we wanted to discern whether the population structure and CHV-1 distribution changed in the last decade i.e. to see whether the populations' vc diversity increased or decreased and if that affected the prevalence of the hypovirulent *C. parasitica* isolates. We have revisited three chestnut populations in Croatia – two continental: Hrvatska Kostajnica and Ozalj, and one in Istria, approximately 10 km from the Adriatic coast (Buje). These populations were chosen because of the differences in population structure,

hypovirulence prevalence and climate differences between them. In the previous study, sexual structures (perithecia) as well as the presence of two idiomorphs of the MAT gene were found in all those populations, suggesting a potential for sexual reproduction and increase of the vc type diversity (Krstin *et al*., 2008).

In the first study, done in 2006, the coastal population in Istria, Buje, had the lowest number of hypovirulent isolates – only 12.7%. Continental populations had much higher prevalence of the hypovirulent isolates: 44.1% in Ozalj, and 50.8% in Hrvatska Kostajnica. These numbers changed by 2014 when our study was conducted; the proportion of the hypovirulent isolates in Buje increased slightly to 19.0%, while at the same time substantially decreased to 27.8% in Ozalj and to 30.9% in Hrvatska Kostajnica. However, general forest health seemed much better in continental populations than in Buje; while in Buje a lot of completely dead trees were observed, in continental populations signs of healing were observed, despite the reduction of hypovirulent strains' prevalence in populations. Furthermore, the population structure changed in the last decade as we observed an increase of Shannon's diversity index in all three populations: in Buje from 1.69 to 2.46, in Ozalj from 0.63 to 1.78, and in Hrvatska Kostajnica from 1.59 to 2.36. This was mainly because of the appearance of the new, and in some cases, previously unobserved, vc types in all populations in Croatia. The most probable reason for that is recombination during the sexual reproduction of the fungus and, at least to some degree, the influx of the new individuals from the surrounding areas.

We have also determined that the abundance of different vc types varies between populations. However, no significant difference in vc type distribution between virulent and hypovirulent isolates within a population was observed. This indicates that CHV-1 is fairly well represented in most of the vc types in each population. Still, some changes, or rather a drift, in population structure when considering vc types was observed. For example, in Ozalj in 2006 vc type EU-1 was absolutely dominant with more over 87% of the isolates belonging to that particular vc group. This changed quite drastically and in 2014 we found that EU-2 was the most abundant vc type, represented with about 42% isolates, while only 24% of the isolates had EU-1 vc type. Similar observation was made in Buje, where in 2006 about 38% isolates were EU-1, and 25% were EU-17, while in 2014 EU-13 became the most common vc type, represented with about 21% of isolates. Approximately 15% of all isolates belonged to EU-4 vc type. Other vc types were represented with less than 10%. Such dramatic change in the percentages of vc types was not observed in Hrvatska Kostajnica, although, numerous new and rare vc types were identified.

Our results indicate that the chestnut – *C. parasitica*-CHV-1 pathosystem is highly dynamic, i.e. that a dramatic change in vc type diversity and CHV-1 prevalence in populations can occur in less than a decade. It is worth noting that even though the percentages of hypovirulent isolates were reduced in Hrvatska Kostajnica and Ozalj when compared to 2006, thus far these two chestnut populations remained in a fair overall state, while the Istrian population Buje, showed a severe disease symptoms and significant dieback of the trees. This indicates that when both virulent and hypovirulent isolates of the major vc groups in populations are present, the overall prevalence of CHV-1 infection is the most important factor contributing significantly to the health status of the chestnut populations. The trend of reducing hypovirulence prevalence in two populations is somewhat worrying, because in the future, skewing the ratios of hypovirulent and virulent *C. parasitica* isolates in populations might hinder the natural dissemination of the viruses within populations, reducing the CHV-1 prevalence even further and possibly increasing the severity of the disease symptoms in populations, resulting in more dieback and significant loss of chestnuts in the forests, as is observed in Buje.

Key words: chestnut blight, hypovirus, population structure

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Plant endophytes in tomato studied by omic techniques

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Abstract: Endophytic bacteria play, actively or passively, major roles in plants influencing traits as fitness and resistance. As many microbial communities, plant endophytic communities are highly complex and dynamic. For these reasons their study demands the application of next generation sequencing based applications to overcome the obstacle of unculturable bacteria and to portray their extraordinary biodiversity. Moreover, NGS based analyses are consistently integrated with bacterial cultivation and functional characterization of taxa showing instances of association with plant resistance mechanisms. In this way the whole procedure results application-oriented, producing valid results for the employment of endophytes to improve plant productivity in a sustainable way.

From a technical point of view, this project focuses on the use of tomato plants (*Solanum lycopersicum*) as model plants and specifically targets the study of the plasticity of endophytic communities in response to abiotic (drought) and biotic stresses (pathogenic infections: *Verticillium dahliae*, *Fusarium oxysporum*, *Clavibacter michiganensis*).

What is remarkable in this project is the potentiality of whole community surveys for the study of the application of endophytes in biocontrol: not only singular taxa with beneficial effects can be highlighted, but beneficial community patterns and taxa co-occurrences can be detected, characterized and applied as well. Moreover, the nature of these procedures is clearly implementation-prone: the commonly performed investigation of the bacterial community can be expanded to fungal and archaeal endophytes and, when needed, experiments can be implemented with multiple-omics techniques for a profound interpretation of endophytic mechanisms enhancing plant defense.

This project is part of the BestPass International Training Network funded by the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement No 676480. The overarching aim of this network is the production of knowledge about the use of endophytes to improve plant productivity in a sustainability way.

Key words: endophytes, NGS, biocontrol

Contribution to the study of the biological activity of *Mesembryanthemum nodiflorum* L. (Aizoaceae) on phytopathogenic fungi

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Abstract: In the present study, antifungal activity of decoction and aqueous plant extracts from aerial parts of *Mesembryanthemum nodiflorum* have been investigated against five devastating fungal disease agents that cause a major constraint to the cultivation of crops. The effect of different concentrations of plant extracts on the mycelia growth was determined under *in vitro* conditions. In this study it was found that the five fungi tested had shown sensitivity to the extracts. The minimum lethal concentration (MLC) value of *M. nodiflorum* extract varies according to the studied species. The value of MLC was found to be similar for *Fusarium oxysporum* W. L. (Godron), *Sclerotinia sclerotiorum* (Lib.) and *Verticillium dahliae* (Kleb.) (16.25 mg/ml). *Geotrichum candidum* (Link) and *Pythium diclinum* (Tokunaga) had a slightly higher MLC value of 20 mg/ml. The results demonstrated the potential of *M. nodiflorum* for use in bio-protection as antifungal agent against these phytopathogenic fungi.

Key words: plant extract, *Mesembryanthemum nodiflorum*, phytopathogenic fungi, antifungal activity

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Combining cultivation-dependent and -independent approaches to select effective bacterial biocontrol agents

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Abstract: In previous studies, we performed cultivation-dependent and -independent approaches to describe the microbiome in healthy, Flavescence dorée-diseased or -recovered grapevine plants to identify putative biocontrol agents. Obtained results allowed the identification of two strains, colonizing exclusively healthy and recovered plants, that we tested for *in vitro* biocontrol activity. Both strain R8 (*Burkholderia* sp.) and R16 (*Paenibacillus pasadenensis*) demonstrated to control the growth of fungal pathogens of grapevine such as *Botrytis cinerea* and *Phomopsis viticola*. Further studies are planned to determine the strains' efficacy against other pathogens, including phytoplasma, and effective *in planta* biocontrol activity.

Key words: microbiome, endophyte, *Paenibacillus pasadenensis*, *Botrytis cinerea*

Introduction

Currently, description of plant microbiomes is mostly carried out through cultivation-independent techniques, based on molecular biology, that offer a less biased approach and a more complete representation of the microbiome when compared with cultivation-dependent techniques (Bulgari *et al*., 2014). Still, the bacteria that can be used for actual biocontrol against diseases on a large scale are only those that we can effectively cultivate and obtain in large quantity. Normally, a large screening is required to select effective biocontrol agents within strains of bacterial species. In previous studies, an approach starting from the field, observing grapevine yellows affected vineyard sanitary status through years, and analyzing the plant-associated microbiomes with both cultivation-dependent and - independent methods, allowed to detect putative biocontrol agents (Bulgari *et al*., 2011; 2014). Here, we tested the biocontrol activity of such bacteria by *in vitro* competition assays against fungal pathogens.

Material and methods

Bacterial and fungal strains used

As reported by Bulgari *et al.* (2011; 2014), cultivation-dependent and -independent microbiome-assisted screening, carried out on leaf samples of healthy, diseased, or recovered grapevine plants in northern Italy, revealed the exclusive presence of the endophytic bacteria *Burkholderia* sp. strain R8 and *Paenibacillus pasadenensis* strain R16 in healthy or recovered plants. These data suggested their possible activity as biocontrol agents. Two pathogenic fungi (*Botrytis cinerea* and *Phomopsis viticola*) were used for the evaluation of antifungal activity of R8 and R16 bacterial strains.

Dual-culture antifungal assays

Dual-culture antagonism assays were carried out on TGYA plates inoculating 8 x 10^5 CFUs of strain R8 or R16. After two days at 37 °C, 0.5 cm mycelial plugs were added to the plates which were incubated at 25 °C in the dark. As negative control, plates with the fungal strains alone were used. Fungal growth was measured 5, 7, and 14 days post inoculation (dpi) as mycelial growth diameter. Each test and measurement was carried out in triplicate. Growth inhibition percentage (GIP) was calculated as [1-(D1/D2)] × 100, where D1 is the radial colony growth on bacteria-treated plate, D2 is the radial colony growth in the control plate.

Dual-plate antagonism assays

To evaluate the ability of strains R8 and R16 to inhibit fungal growth through the production of volatile compounds, a dual-plate assay was carried out as described by Chaurasia *et al.* (2004). After fungal inoculation, all the plates were kept at 25 °C in the dark and the fungal growth was measured at 14 dpi. Each test and measurement was carried out in triplicate. Growth inhibition percentage determined by volatile compounds (GIPv) was calculated as described above.

Statistical analysis

The SPSS statistical package for Windows, v. 22.0 (SPSS Inc.), was used for all statistical analyses. Normal distribution and homogeneity of variances were verified using the Shapiro – Wilk test and the Levene's test, respectively. GIP and GIPv did not meet the requirements for parametric tests, thus data were analyzed according to the Kruskal-Wallis (KW) non-parametric one-way analysis of variance.

Results and discussion

Dual-culture antifungal assays

Strain R8 reduced the growth of *B. cinerea* by 63% at 5 dpi and 78% at 14 dpi. However, the strain showed little efficacy against *P. viticola*, achieving a maximum of 47% of growth inhibition (Table 1). Strain R16 caused total inhibition of *B. cinerea* mycelial growth at 5 dpi and maintained this effect through 14 dpi. The strain also reduced mycelial growth of *P. viticola* by 82% at 14 dpi (Table 1). Visual effects of the bacteria on the fungi are displayed in Figure 1.

The results obtained for strain R16 are comparable or stronger, than those presented for other known biocontrol agents against *B. cinerea* (Touré *et al*., 2003; Ji *et al*., 2013).

Table 1. Growth inhibiton percentage (GIP) caused by strains R8 and R16 against *B. cinerea* and *P. viticola* at 5, 7, and 14 days post inoculation (dpi) reported as mean value. Within each treatment, different letters indicate significantly different values (KW $P < 0.05$).

	Strain R8		**Strain R16**	
	B. cinerea	***P. viticola***	***B. cinerea***	***P. viticola***
GIP 5 dpi	63 a	37	100	78 a
GIP 7 dpi	68 a	40	100	83 b
GIP 14 dpi	78 b	47	100	82 b

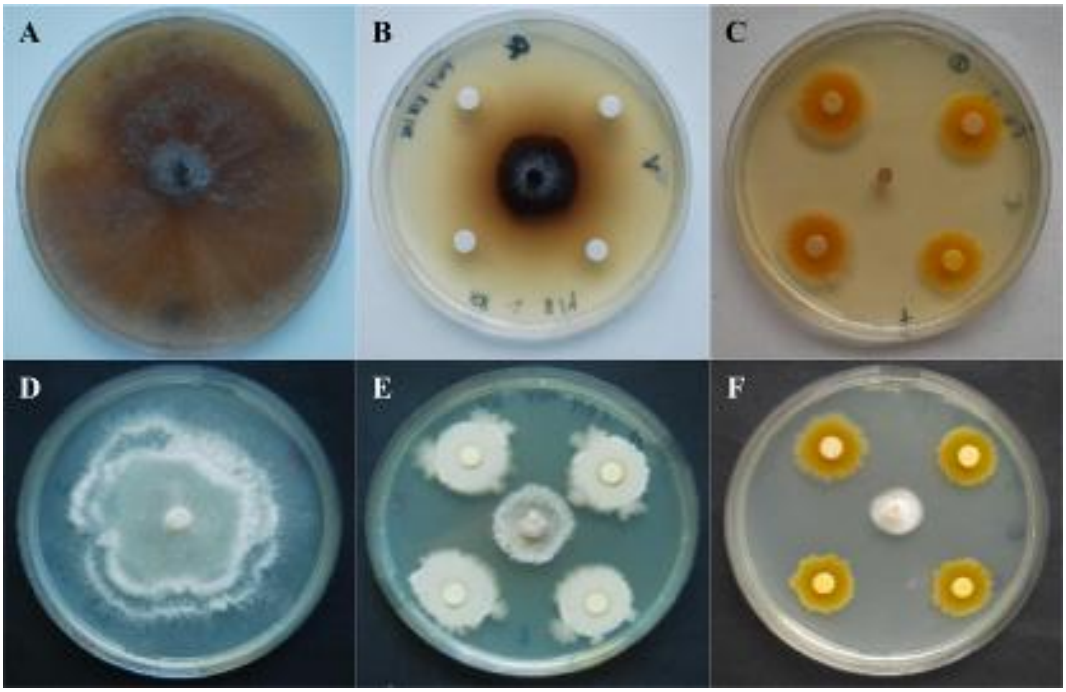

Figure 1. Comparison between dual-culture treatments with strains R8 and R16 and a phytopathogenic fungus. Photographs of one representative plate per treatment, taken at 14 days post inoculation, are reported. A: control *B. cinerea* plate; B: *B. cinerea* with R8; C: *B. cinerea* with R16; D: control *P. viticola* plate; E: *P. viticola* with R8; F: *P. viticola* with R16.

Dual-plate antifungal assays

Strain R8 achieved a mycelial growth inhibition of 72% against *B. cinerea* through volatiles alone. Interestingly, this result is almost the same as the one obtained with dual-culture assays, suggesting that the activity of strain R8 against this pathogen is mediated mostly by volatile compounds. Strain R8 inhibited *P. viticola* by 25%, losing almost half its effect compared to the dual-culture assay (Figure 2).

Strain R16 instead showed a greatly reduced efficacy against both fungi in this assay, achieving 61% and 5% of growth inhibition against *B. cinerea* and *P. viticola*, respectively (Figure 2). While the effect against *B. cinerea* hints at the production of antifungal volatile compounds, the absence of inhibition against *P. viticola* also suggests that these molecules could be specific against some species of fungi.

In conclusion, comparison between culture-dependent isolation of endophytes and culture-independent analysis of microbiome in plants with different sanitary status allowed to identify with great accuracy two bacterial strains that are promising candidates for biocontrol.

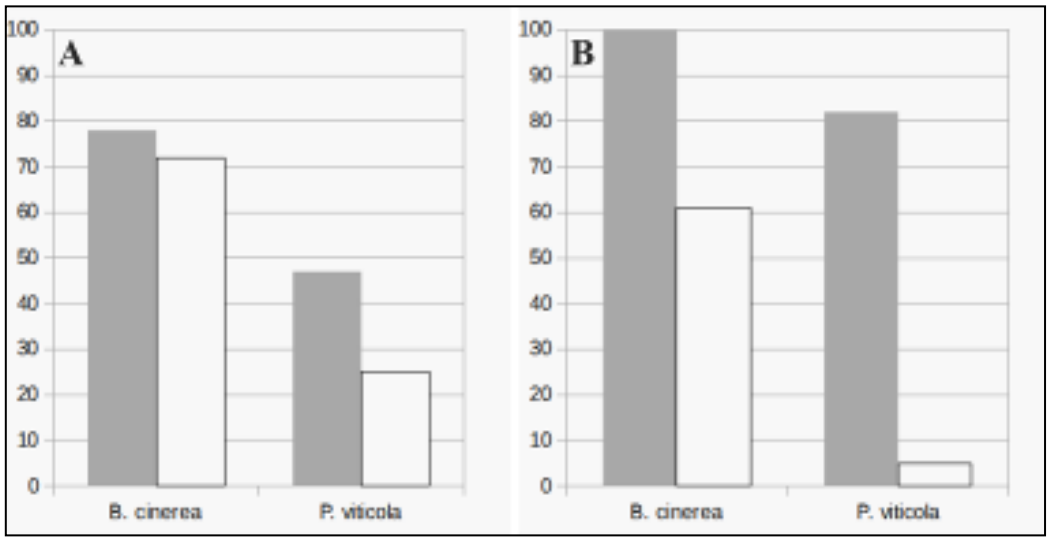

Figure 2. Comparison between GIPv (in white) and GIP (in gray), for R8 strain (A) and R16 strain (B).

Acknowledgements

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The grapevine phyllosphere as potential source for BCAs against downy mildew (*Plasmopara viticola*)

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Abstract: European grapevine cultivars classified as *Vitis vinifera* subsp. *vinifera* are highly susceptible to *Plasmopara viticola*, the causative agent of downy mildew, which is endemic on wild *Vitis* species of North America. This obligate biotrophic oomycete is responsible for relevant yield losses throughout the growing region and demands frequent applications of chemical or copper-based fungicides. The phyllosphere represents a large microbial habitat not only for phytopathogenic but also for beneficial organisms contributing to the health of their host plants. We selected six *Vitis* genotypes from the Botanical Garden in Graz (Austria) with different primary origin, leaf morphological traits, and disease resistance levels and compared their phyllosphere-associated alphaproteobacterial microbiome with that of a highly susceptible cultivar (*V. vinifera* subsp. *vinifera* 'Müller Thurgau'). At two sampling times (June and October), 16S rRNA gene amplicons from total microbial DNA were sequenced using Illumina's MiSeq platform. Resulting reads were assigned to operational taxonomic units (OTUs) and processed by bioinformatics tools. Additionally, we visualized colonization patterns of phyllosphere bacteria by fluorescence *in situ* hybridization in combination with confocal laser scanning microscopy. Efficacy of around 600 bacterial isolates from the six genotypes against downy mildew was tested on 'Müller-Thurgau' leaf discs. Results revealed highly dynamic alphaproteobacterial community structures during the growing season with a significantly higher estimated richness of taxa in spring (Chao1 865.2 ± 70.7 versus 621.0 ± 76.5 OTUs). Distinct alphaproteobacterial communities (beta diversity metrics) were shaped in October, presumably in correlation with host-variety factors. Indicator taxa with significant influence on beta diversity clustering ($P < 0.05$) were identified, e.g. *Novosphingobium* with predominant abundance on highly resistant leaves. Throughout all samples, most abundant bacterial families were Sphingomonadaceae (35.6% average relative abundance), Methylobacteriaceae (22.7%) and Acetobacteraceae (10.0%). Efficacy tests of bacterial isolates showed reduction of disease severity of up to 70.0%. Our data suggest that the phyllosphere of resistant *Vitis* species is a potential source for microbial antagonists against *P. viticola* and will contribute to find alternative solutions for sustainable plant protection in viticulture.

Key words: *Vitis vinifera*, *Plasmopara viticola*, Alphaproteobacteria

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Taking a look at the inside of trees, does irrigation water quality have an effect on fungal endophytes in *Citrus sinensis* (orange) trees?

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Abstract: Water has always been a resource in absence in the Middle East. Water for agriculture, of different origins and quality is used in the Israeli orchards. One of the major water resources for citrus orchards in Israel is treated waste water. Long term irrigation with waste water influence trees health and appearance. It is clear that the water quality plays a major role in the trees health as well as fruit quality and quantity. Many possible explanations for the trees deterioration have been suggested, among them soil physical character changes, plants root damage and root associated microbial community changes. We suggested another, different approach, claiming that high concentrations of solubles in the treated water are changing the endophytes communities in the tree tissues. These changes cause the loss of beneficial endophytes aiding the trees. We have sampled trees from two orange orchards different only by the water used for their irrigation. Both orchards are of the same citrus variety and stock, planted on the same type of soil and climatic area. Fungal endophytic communities among samples from the two watering were compared using two methods; by isolation of fungi from the different tissues and Next Generation Sequencing (NGS). Samples were collected from roots, branches and leaves at summer, fall, winter and spring. All samples (72 samples) were used for fungal endophytes isolation and total DNA extraction. Phenotypic characterization and ITS identification were done to isolates from the different samples. Deep sequencing of all samples was performed by amplification of a 28S rRNA LSU sequence (LROR/LR3) of the fungal endophytic communities. Data was generated by MiSeq technology analysed using QIIME program. Ribosomal Database Project (RDP) was used for taxa identification. Differences between the methods used, and among samples and treatments in fungal composition are observed. Demonstration of these differences, with emphasis on root tissue is presented.

Key words: Citrus, endophytic fungi, treated waste water, Next Generation Sequencing

Introduction

Water has always been a resource in absence in the Middle East. Water for agriculture, of different origins and quality is used in the Israeli orchards. One of the major water resources for citrus orchards in Israel is treated waste water (reclaimed water). Today about 80% of the waste water are being treated and used in agriculture in Israel. It is estimated that by the year 2020 about 60% of the water for agriculture in Israel will be reclaimed water (Cohen *et al*., 2012). Long term irrigation with waste water, of second degree treatment, influence trees health and appearance. It is clear that the water quality plays a major role in the trees health as

well as fruit quality and quantity (Trachisky *et al*., 2013). Many possible explanations for the trees deterioration have been suggested, among them soil physical character changes, plants root damage and root associated microbial community changes. We suggested another, different approach, claiming that high concentrations of solubles in the treated water are changing the endophytes communities in the tree tissues. These changes cause the loss of beneficial endophytes aiding the trees, and may enables opportunistic pathogens to overdevelop in the stressed trees (Ben Hor, 2002; Belesky *et al*., 2009). These weak pathogens may be the ones causing the disease like symptoms that deteriorate the trees and eventually kill it (Belesky *et al*., 2009). Endophytes are microorganisms living within the plant tissue without causing visible symptoms to the host (Lane *et al*., 2000). Endophytes may be fungal or bacterial, many of them have been found to be beneficial to the host, living in mutualistic and symbiotic relations with plants (Liarzi *et al*., 2014). There are many examples of endophytes aiding their hosts against biotic and a-biotic stress (Kannadan & Rugers, 2008), in direct (secretion of metabolites) or in direct (ISR/SAR priming) ways (Liarzi *et al*., 2014). In order to study our hypothesis, calming that watering of the trees with reclaimed water for long term has changed the diversity of endophytes in the trees, we used two methods of identification of the fungal communities in the trees. A classical method based on the isolation of the fungi and identification by molecular methods and a Next Generation Sequences (NGS), a method that have the power to identify un culturabal fungi living in the plant tissue.

The objectives of this study were to: 1) to characterize the calturabale fungal community in the different trees tissue (roots, leaves and branches) of orange trees watered with waste water/fresh water at the different seasons of the year (summer, fall, winter and spring); 2) to characterize the un-calturabale fungal community in the different trees tissue (roots, leaves and branches) of orange trees watered with waste water / fresh water at the different seasons of the year (summer, fall, winter and spring) using NGS method; 3) to compere the diversity of endophytic fungi communities in the different trees from the different orchards found by the two methods.

This work, to our knowledge, is the first that compares populations of tree endophytes between two irrigation methods: reclaimed water and fresh water.

Material and methods

Plant material collection

Two orchard plots of oranges (*Citrus sinensis*) were selected, differing only in irrigation conditions: orchard Mehadrin plot 33 in Bet Dagan area is irrigated with freshwater whereas orchard Mehadrin plot 310, located in Safaria is irrigated with reclaimed water. A total of 72 samples were collected spanning all four seasons from three arbitrary trees within each plot and various plant tissues (leaf, branch and root).

Identification of fungal endophytic populations

Two main experimental methods were used in this study in order to identify the endophytic fungal population and it's composition inside the trees. The classical method, in which we isolated fungi from surface-sterilized wood tissue onto culture medium (Potato Dextrose Agar). The disadvantage of this method was that only laboratory cultivatable fungi could be isolated (it is estimated that approximately 95 percent of plant endophytic fungi cannot be cultivated on artificial food media). Following isolation, fungi classification was performed by both phenotypic identification and molecular sequencing of ITS region in the ribosomal

DNA following comparison to the NCBI database according to their genus. Each tissue was examined in all seasons separately both for freshwater and reclaimed water irrigation.

Another method that was used in this research is advanced molecular method based on the principle of NGS (Next Generation Sequencing) technology named Deep Sequencing. This technique has the ability to sequence millions of sequences in parallel and to identify them using unique databases. Thus, it is possible to compare the populations of endophytes between trees, and more importantly, to compare all of the populations regardless of their cultural ability on artificial medium. In this method, the total DNA was extracted from each tissue separately and the fungi specific area named large subunit (LSU) rRNA on the ribosomal DNA was amplified and sequenced. This method yielded approximately 15,000 unique OTU (Operational Taxonomic Units).

Statistical analysis

Statistical tests such as ANOSIM nonparametric test and - NMDS were used to compare between the different variables that can affect the composition of the endophytic population inside the trees.

Results and discussion

We found that there is a significant difference between the populations inhabiting the tissues, suggesting that each tissue has a unique population composition. This statistical significance indicates that the root tissue has more population heterogeneity among the tissues tested - the variety of endophyte populations is the most diverse relatively to other tissues (Figure 1 and 2). However, when examining the effect of irrigation on the populations we discovered it has only a secondary effect, which is not significant. The statistical data analysis of these results confirmed our results obtained by the classical method – there are different populations in the tested tissues. In addition, during the seasons especially in the leaf and root tissues, there is a significant difference in the variety of the endophytic populations within all the sampled trees (not shown). The irrigation condition parameter results designated lack of similarity between the samples of fresh water and reclaimed water notably in the root tissue. In fact, data analysis using both methods, allowed us to obtain information on the composition of the varied endophyte populations between trees depending on different variables.

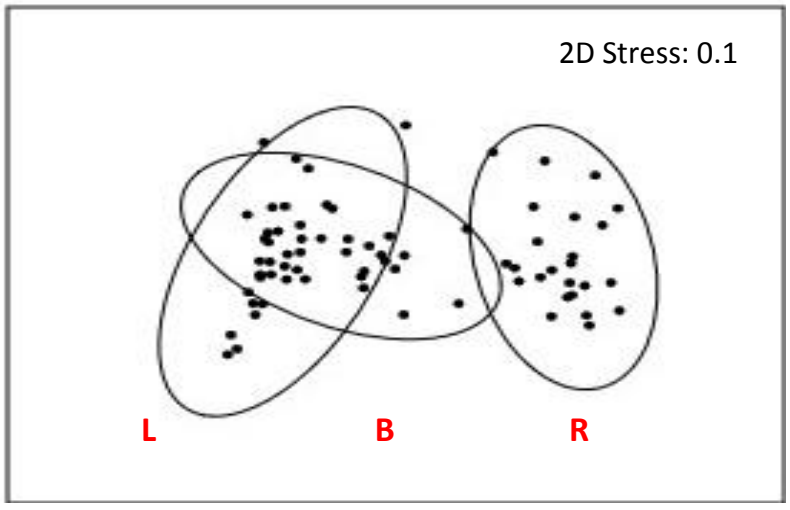

Figure 1. NMDS diagram of the 72 samples according to the plant tissue (culturable).

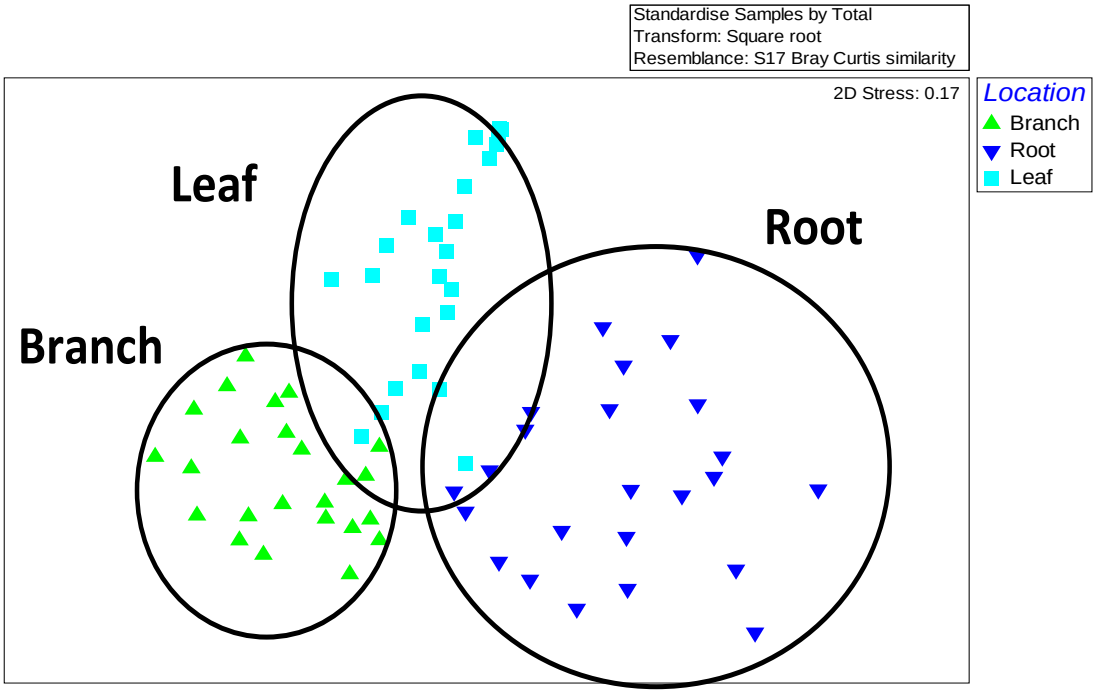

Figure 2. ANOSIM diagram of the 72 samples according to the plant tissue (un-culturable) Global R = 0.736 / p = 0.001.

In conclusion, our research hypothesis was partially confirmed. By analyzing the information gathered, we conclude that the most significant influence on the population composition of the tree is the tree tissue type while the irrigation type (fresh water versus treated sewage water) has only a minor effect prominently expressed in the roots and leaves (Figure 3).

Acknowledgements

We thank "Mehadrin" for enabling us to work and sample their orchards for this project.

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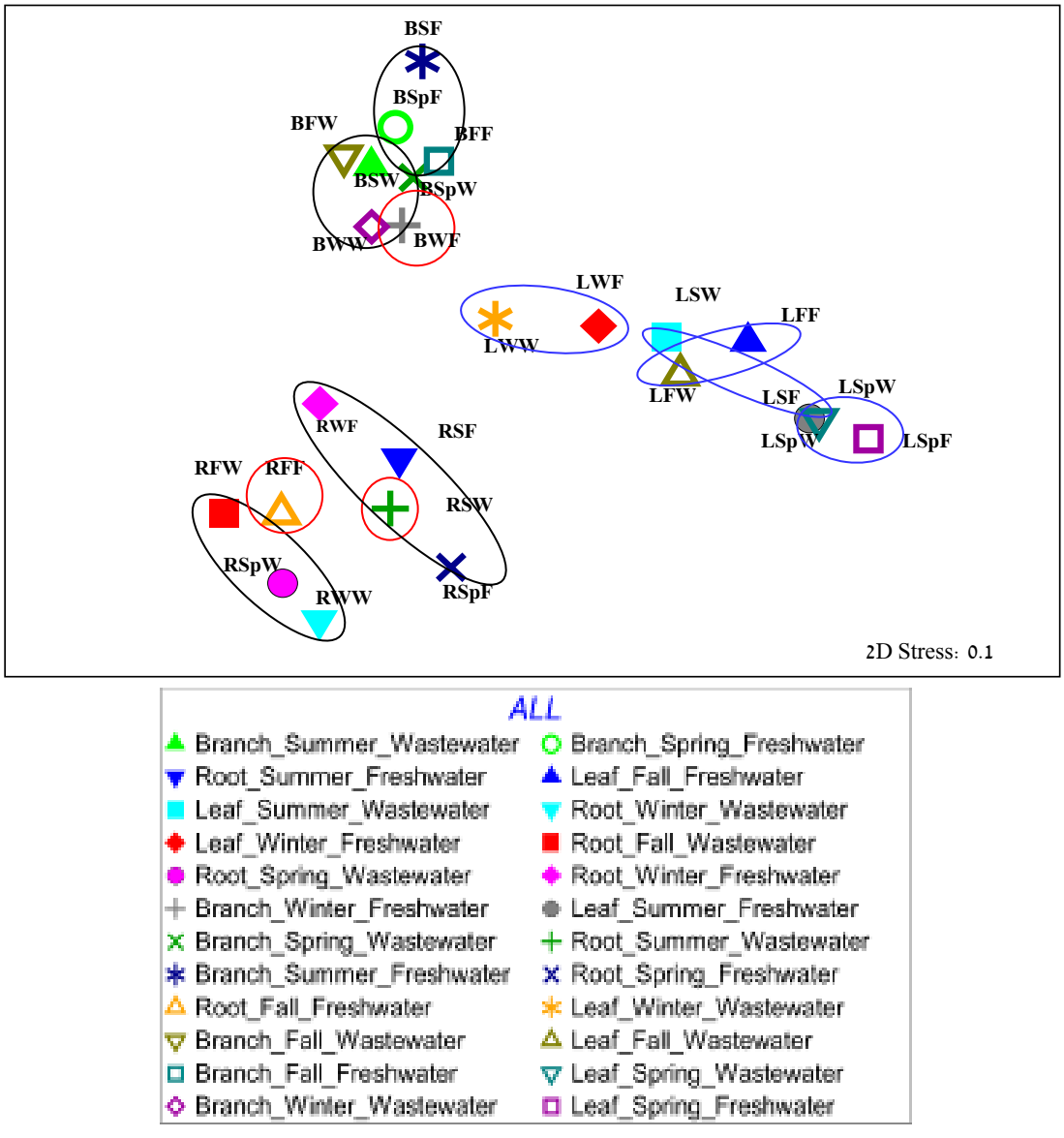

Figure 3. NMDS diagram of samples according to tissue, season and watering type. Each symbol represents avarage of 3 samples. Global R = 0.736 / p = 0.001.

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Comparison of different inoculum methods for infection with *Verticillium dahliae* and *Fusarium oxysporum*

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Abstract: Major crop losses occur worldwide due to salinity and infection with soil-borne pathogens. Among the soil-borne fungal pathogens inhabiting the rhizosphere, *Verticillium dahliae* and *Fusarium oxysporum* are accountable for diseases in a wide range of crops. Establishment of a plant disease by artificial inoculation is crucial for conducting experiments. Studies conducted from a phytopathological point of view focus mainly on the plant parameters and establishment of the disease, while ecological studies investigate the microbial dynamics occurring in the plant as a response to the pathogen infection. There are many phytopathological inoculation methods reported which include injecting the spore inoculum into the plant, in turn injuring the plant tissue. In ecological studies, one of the challenges is to comprehend how resource competition and niche overlap of indigenous microbial communities affect the pathogen invasion success and its distribution. Thus, these methods loose applicability in ecology based studies. This study compares different pathogen inoculation methods for constructing study models more suitable for ecological studies.

The aim of this study is to evaluate and compare different inoculation techniques for the infection of *Verticillium dahliae* and *Fusarium oxysporum* in tomato plants under saline soil conditions. Two different inoculation methods were used, a) direct inoculum addition to the soil, b) root dipping in fungal inoculum. Two- week old tomato (cultivar *Hildares;* 1-2 leaf stage) seedlings grown in quartz sand were inoculated and transplanted into the potting mixture (1:1 quartz sand: coarse sand). The inoculum concentration was 10^7/ml in both cases. The salinity in the plants was increased gradually to EC 10 dS/m by continuously adding a nutrient solution of EC 5 dS/m. Disease severity symptoms like stunted growth, wilting, and browning of leaves were regularly monitored. Plant roots were harvested at 7, 14 and 21 days post inoculation, surface sterilized and colonization by *V. dahliae* and *F. oxysporum* was determined by PCR assays and stem outgrowth tests.

The results of this study will help to develop effective systems to study diseases and their effectual detection which are crucial for the amelioration of the existing biocontrol methods.

Key words: inoculation, microbial community, salinity

Involvement of plant growth-promoting rhizobacteria *Burkholderia phytofirmans* PsJN sigma factors in reducing disease susceptibility of *Arabidopsis thaliana* against a *Pseudomonas syringae* DC3000 virulent strain

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Abstract: Nutrient availability makes plant internal tissues and rhizosphere attractive spaces for microbial colonization. Among bacteria that establish close interactions with plants are the well-known plant growth-promoting rhizobacteria (PGPR), which have a growing importance in sustainable agricultural industry, since they are able to produce beneficial effects on their hosts using mechanisms such as nitrogen fixation, nutrient absorption improvement, phytohormone production, and also by increasing plant tolerance to abiotic stress and reducing disease susceptibility. Furthermore, PGPR interacting with plants need to adapt to constant environmental changes through global gene regulation for successful plant colonization, and later promote plant beneficial effects. In this context, sigma factors are dissociable subunits of RNA polymerase that regulate gene transcription initiation by recognition of specific promoter sequences. Remarkably, plant-associated bacteria carry a high number of these factors, predominantly the extracytoplasmic function sigma factors, the largest and more diverse group, although most with poorly described functions, which are interesting candidates for global gene regulators involved in plant-bacteria interactions. The PGPR *Burkholderia phytofirmans* PsJN possesses a large number of sigma factors and has the ability to colonize and promote growth of agronomically important crops and also of the model plant *Arabidopsis thaliana*. Additionally, it has been reported to promote tolerance to abiotic stresses such as cold and salinity, and stimulates defenses against pathogens such as *Botrytis cinerea*. It also protects *Arabidopsis thaliana* against *Pseudomonas syringae* DC3000 (Timmermann *et al*, unpublished results). The aim of this study was to address the role of sigma factors present in strain PsJN in the induction of protection of *Arabidopsis thaliana* against *Pseudomonas syringae* DC3000. PsJN strain encodes numerous sigma factors, which were inactivated by insertional mutagenesis. Results indicated that a few sigma factors related with motility and biofilm formation could be related to reducing *A. thaliana* disease susceptibility against strain DC3000. These results support a possible role of sigma factors in plant-bacteria interactions, at the level of the rhizosphere colonization and plant beneficial effects.

Key words: PGPR, sigma factor, biocontrol

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Biocontrol potential and efficiency compared of one medicinal plant on the major pathogens of cereals and legumes

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Abstract: In Morocco, wheat and food legumes are important in traditional dishes and for basal alimentation as source of energy. Unfortunately, abiotic and biotic stress are decreasing yield of these crops. Fungal diseases are the most devastating causing very important yield losses. To prevent these yield losses, intensive fungicide application is inevitable which in fact poses a high selection pressure for fungicide resistance. To develop an alternative method against these diseases, we have compared the efficacy of the extract of many medicinal plants. The principal causal pathogens are: *Fusarium oxysporum* f. sp. *ciceri*, *F. oxysporum* f. sp. *lentis, F. culmorum, Pyrenophora tritici repentis* and *Zymoseptoria tritici*. The extract from *Daphne gnidium* used in this study was the most effective. It induced the highest inhibition percentage of radial growth (more than 70%). This result shows a good antifungal activity for limiting and even for stopping the development of the pathogens.

Key words: biocontrol, cereals and food legumes, main diseases

In vitro evaluation of the impact of four Moroccan medicinal plants on five phytopathogenic fungi

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Abstract: The fungal resistance is a major problem in plant protection. There remain few effective antifungal agents against some resistant products. Scientists are looking for new products of natural origin as secondary metabolites of medicinal plants and having an antifungal activity. Morocco is one of the main producers of aromatic and medicinal plants. Our present study proposed to test the antifungal activity of the extracts of four medicinal plants: *Euphorbia* sp., *Equisetum ramosissimum, Eryngium atlanticum* and *Daphne gnidium.* These plants are tested on five phytopathogenic fungi: *Alternaria alternata, Botrytis cinerea, Fusarium oxysporum, Helminthosporium sativum* and *Sclerotinia sclerotiorum.* These four plants do not have the same effect on the five phytopathogenic fungi. The extracts of the decoction of *E. ramosissimum* and *E. atlanticum* are inactive against three fungi. Extracts of *Euphorbia* sp. decoction and *D. gnidium* presented a very good antifungal activity on studied phytopathogenic microorganisms.

Key words: *Euphorbia* sp., *Daphne gnidium, Eryngium ramosissimum, Equisetum atlanticum*, antifungal activity, phytopathogenic fungi

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Elucidating the etiology of apple replant disease: a microbial ecology approach

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Extended Abstract: Apple replant disease (ARD) occurs when young apple trees are planted on a site where the same species or closely related species were grown before. ARD results in a reduction and delay of tree growth as well as decrease of fruit yield and quality. ARD symptoms are stunting of shoots, shortened internodes, deformed leaves and small browned decayed roots.

In the European Union one third of European fruit orchards are planted with apple trees and the land area planted with this crop is decreasing therefore the production. One of the reasons for this reduction in land reduction is ARD. Despite ARD being a worldwide problem known for 200 years, in Europe concerns began only during the 1990s. The land used for fruit crops commenced to be scarce when the Common Agricultural policies of the EU changed, focusing in a more environmentally friendly agriculture, avoiding application of pesticides. Indeed, at that time one of the ways to counteract ARD was the pre-planting application of a range of different fumigants such as methyl bromide, metam sodium, 1,3-dichloropropene and chloropicrin. Although these chemicals help to overcome ARD by enhancing tree growth by killing soil-borne pathogens, they also accumulate in the environment with harmful effects in beneficial microbes such as denitrifiers, leading to low levels of microbial biomass and nitrate. More environmentally friendly though economically not feasible pre-plant treatments are the pasteurisation or gamma irradiation of the soil which do not cause environmental pollution but still diminish the natural soil microbial diversity.

Many studies have been performed in the last decades to understand the etiology of ARD in order to find better management strategies. However, the understanding and unravelling of relationships is difficult since ARD seems to be site-specific and caused by a group of pathogens additionally influenced by abiotic factors.

In early studies it was believed that the causes of ARD where due to several abiotic factors like orchard age, pH, phytotoxins, poor soil nutrition amongst others. Later on some studies showed the pasteurisation, gamma irradiation and fumigation of the soil to lead to a better growth of apple trees suggesting that rather than abiotic factors, biotic factors are the primary causal agents of ARD. Nowadays it is mainly accepted that ARD is caused by a group of microorganisms, the ARD complex, which is site-specific. Therefore, common causal agents are not defined, although some of the causal agents reported are present frequently in ARD soils such as *Pratylenchus penetrans* Cobb within nematodes related with ARD severity, fungi of the genera *Illyonectria* (formerly *Cylindrocarpon*), *Rhizoctonia*, *Fusarium* and oomycetes like *Phytophthora* and *Pythium*. Less investigated is the role of prokaryotes in ARD, although bacteria such as genera belonging to Actinomycetes, *Bacillus* and *Pseudomonas* were suggested in other studies to play a role in ARD.

Current approaches aim to reach suppressive ARD soils by modifying the microbial community structure with biocontrol agents or amendments such as seed meals, animal or green manures. But because there is no consensus about the causal agents of this disease due to its complexity, efforts on overcoming ARD are still needed to reach its aim. Hence, more studies need to be performed to elucidate the causal agents of this serious disease.

The aim of our project is to understand the role of microorganism in ARD, focusing on bacteria and fungi from the rhizosphere and bulk soil as well as root endophytes. Therefore, a greenhouse experiment was conducted in which *in vitro* propagated plants of the sensitive apple rootstock M26 were planted in five ARD soils from different sites in Germany (Ellerhoop, Heidgraben, Ruthe, Meckenheim) and three healthy soils (control) from the same sites where no apple was grown before. In order to confirm that ARD in these sites is due to biotic factors each soil had three different treatment variants: Gamma irradiation, heated to 50 °C for one hour and untreated. After 8 weeks the plants were sampled. Rhizosphere and bulk soil were taken from four containers per treatment in order to isolate bacteria and for DNA-based analysis of the microbial community structure. The shoot length, fresh mass and dry mass of the roots and shoots were determined. For isolation of endophytic bacteria, roots were surface sterilized with sodium hypochlorite and after three washing steps cut into about 1 cm pieces and plated onto bacterial growth media. The outgrowing bacteria were streaked to obtain single colonies, from which DNA was isolated. After performing a 16S rRNA gene PCR the corresponding bands were cut out and purified. The PCR product was then sent for sequencing.

Additionally, samples from the rhizosphere and bulk soil were taken and stored at -20 °C. For the rhizosphere samples, root samples were washed aseptically to remove adhering soil, after root was immediately treated for 3 min with 9 ml 0.3% NaCl per gram of root fresh weight. Subsequently the samples were centrifuged and the obtained pellets were stored at -20 °C for further analysis. Rhizosphere DNA from the pellets and from 0.5 g of bulk soil was extracted and purified. Polymerase chain reaction (PCR) techniques including DNA fingerprinting with denaturing gradient gel electrophoresis (DGGE) for fungi, bacteria and the phylum beta-proteobacteria were performed. Ongoing analyses are the quantification of 16S rRNA and ITS genes conducted by 5´nuclease assays in real-time PCR and 16SrRNA amplicon Illumina sequencing.

Results have shown that the gamma irradiation of the soil led to a significant increase of shoot length, fresh mass and dry mass of the plants. This effect can be observed in ARD soils as well as in control soils.

Up to now, about 12 morphologically different bacterial isolates have been obtained from the plated roots. 16S rRNA of these endophytic bacteria has been amplified and identification by sequencing is in progress.

By DGGE differences in the fungal and bacterial community structure between ARD and healthy soils were observed. UPGMA cluster analysis based on similarities of DGGE fingerprints showed a trend of separate clustering of healthy and ARD soils independently of the soil type. These findings were also seen in the group of beta-proteobacteria in which known degraders of aromatic compounds are found that are common apple tree root exudates (e.g. phloridizin). In future, we will therefore focus on the involvement of beta-proteobacteria in ARD.

In summary, our results indicate the involvement of microbial communities in ARD. To which extent bacteria are causal agents or responders to plant symptoms will be further studied by the sequencing of significant bands on DGGE gels and V3-V4 16S rRNA Illumina Miseq amplicon sequencing. Furthermore, different bacterial isolates could be observed outgrowing from the root pieces. Since most endophytic bacteria are not cultivable, in future analyses, DNA of the surface sterilized roots will be isolated and sent for 16S amplicon sequencing to study the endophytic bacterial community in a culture-independent approach. Other partners within the BonaRes ORDIAmur project will focus on identification of fungal endophytes in a culture dependent as well as a culture independent approach.

Key words: apple replant disease, rhizosphere, endophytes

Controlling the root microbiome by soil management and break crops

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Abstract: Oil seed rape (OSR) suffers from a severe yield decline when it is grown in monoculture or short rotations. Traditionally, oilseed rape (OSR) has been grown in a five-year rotation in combination with cereals. Nevertheless, in modern agriculture, farmers often use only a few economically viable crops (OSR and wheat) resulting in shorter rotations of two to three years. High inputs of fertilizers and herbicides can partially compensate for yield losses, however yields never match those seen in the first year.

Yield decline is likely to be associated with a range of environmental and agronomic treatments including changes in microbial community structure within the soil and OSR rhizosphere. Repeated monoculture could therefore build up a higher abundance of plant related pathogens. In contrast many microorganisms are known to have a positive effect on plant health however these beneficials may be reduced in abundance under monoculture or short rotations.

Until now the complex interactions between soil characteristics, the resultant microbial community composition in bulk and rhizosphere soil, the influence of the plant under different rotations, and their impact on yield decline have been largely unknown.

In a field trial at Rothamsted Research (UK) cropped with OSR continuously since 1991, the influence of different break crops and fertilizer are compared: yield, seed dry weight and oil content were determined.

In addition, amplicon sequencing of 16S rRNA gene fragments are being used to determine the microbial community composition in rhizosphere soil and roots. With this cultivation independent method, it will be possible to identify groups associated with high yields, and potential pathogens which are implicated in OSR yield decline.

Results of this project will help to give concrete practical advice to farmers by generating baseline data regarding microbial community composition under diverse conditions and revealing interactions between plant and environment.

Session 6:
Novel biocontrol strategies

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Fluorescent pseudomonads for joint disease and pest control: just a dream or a real perspective?

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Extended Abstract: Since a couple of decades fluorescent pseudomonads are studied and applied as biological control agents against soilborne diseases and as plant strengtheners and plant growth promoters. Yet, only in the last ten years certain pseudomonads, especially those belonging to the *P. chlororaphis* subgroup have emerged as bacteria displaying not only antifungal but also insecticidal activity (Flury *et al*., 2016; Kupferschmied *et al*., 2013; Péchy-Tarr *et al*., 2008; Ruffner *et al*., 2013). A microorganism which can be used against both, diseases and pests would of course be the ideal biocontrol agent. Interaction of plant-associated pseudomonads with insects is, however, yet a largely unexplored field and we are just at the beginning of exploring insecticidal effects, involved mechanisms and the ecological significance of *Pseudomonas*-insect relationships.

At present we are focusing on elucidating the mechanisms governing insecticidal activity in *P. protegens and P. chlororaphis* and in evaluating their potential for the biological control of pest insects. In order to identify virulence factors we follow two different strategies: a targeted mutational approach and bioassays in combination with comparative genomics using a collection of *Pseudomonas* strains belonging to different phylogenetic groups. The comparative genomics/bioassay approach revealed that potent injectable activity to *Galleria mellonella* and oral activity to *Plutella xylostella* and *Spodoptera littoralis* is restricted to strains belonging to the *P. chlororaphis* subgroup which all produce the Fit insect toxin (Ruffner *et al*., 2013; Flury *et al*., 2016). However, also certain strains belonging to the *P. fluorescens* subgroup which are Fit-non-producers can display a certain level of virulence, but much weaker compared to the Fit-producers. Comparing the genomes of 24 insecticidal and non-insecticidal strains identified a set of 90 genes unique to highly insecticidal strains. Mutational analysis of selected genes revealed that besides the Fit toxin (Péchy-Tarr *et al*., 2008: Ruffner *et al*., 2013), chitinase C and phospholipase C contribute to oral insecticidal activity of our model strain *P. protegens* CHA0 (Flury *et al*., 2016). In parallel we evaluated the contribution of cell-envelope associated components using deletion mutants of strain CHA0. Our latest results indicate that exopolysaccharides as well as the O-antigens of lipopolysaccharides play an important role in insect pathogenicity.

Antimicrobial compounds such as HCN, 2, 4-diacetylphloroglucinol (DAPG), phenazines (PHZ), pyrrolnitrin (PRN), pyoluteorin (PLT) and cyclic lipopeptides (CLP) play an important role in antifungal activity and the suppression of soilborne diseases. We were wondering whether some of these factors could also help pseudomonads to colonize and kill insects. *Galleria* and *Plutella* assays with knock-out mutants of *P. protegens* CHA0, *P. chlororaphis* PCL1391 and *Pseudomonas* sp. CMR12a showed that HCN and the CLPs

orfamide and sessilin, but not DAPG, PLT, PRN and PHZ contribute to insect pathogenicity. Thus, it seems that pseudomonads can use some of these compounds indeed as weapons against both, microorganisms and insects. Interestingly, not only HCN and OFA but also PRN, PLT and PHZ biosynthetic genes were expressed in living larvae during the infection process.

So, what about the host range of insecticidal pseudomonads and their biocontrol potential? So far, we have tested our model strain CHA0 in feeding assays against around 20 insect species belonging to five orders. All tested Lepidoptera and Diptera were highly susceptible. Among the tested Coleoptera some species were susceptible and others not. The tested Hymenoptera were not susceptible at all. However, also in some cases e.g. pot experiments with *Melolontha melolontha* and *Otiorhynchus sulcatus* where no significant larval mortality was obtained, bacteria-monitoring showed that CHA0 could persist in larvae, in the case of *Otiorhynchus* even until the pupae and adult stage. In pot experiments with cabbage and *Delia radicum*, root inoculation with strain CHA0 reduced *Delia* pupation and hatching rates. Insects, which survived until hatching and still harboured the bacteria showed strong deformation of wings, which was also observed for those adults of *Pieris brassicae*, which survived feeding with CHA0 at the larval stage. These results indicate that insecticidal pseudomonads, although very effective against some Lepidoptera, are probably rather opportunistic than primary pathogens on other insects. They colonize and persist in Coleoptera with only little adverse effects, but given a chance, they may become killers, for example if the insect or its gut microflora is weakened. Therefore, a promising strategy is to combine pseudomonads with other entomopathogens. At the moment we test combinations of *P. protegens* and *P. chlororaphis* with entomopathogenic nematodes in pot and field trials against *Diabrotica* spp. on maize. First results show that the two organisms are compatible and have a plant-growth promoting effect. We are currently developing a formulation containing both microorganisms based on alginate beads. Another field trial with wheat showed that the bacteria can also have a systemic effect against insect feeding. Plants survived a heavy attack by the frit fly much better in plots inoculated with pseudomonads. In summary, our studies show that some *Pseudomonas* strains indeed have the potential for pest control, mainly when they are combined with other biocontrol agents.

Key words: insecticidal activity, *Pseudomonas*, combination of BCA's

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Identification and characterization of volatile organic compounds active against barley pathogens

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Abstract: Barley is threatened by various edaphic fungal diseases. Today, since most of the chemicals used for crop protection are being forbidden, there is a growing need of sustainable ways to control these diseases. In this paper, the volatile interactions that take place below ground between barley roots and two pathogenic fungi, *Cochliobolus sativus* and *Fusarium culmorum* were investigated and the effect of fungal volatiles on barley growth and the effect of barley root volatiles on fungal growth were evaluated by cultivating both organisms in a shared atmosphere without any physical contact. We show that the blend of VOCs emitted by infected barley roots decreased *C. sativus* growth by 13 to 17% while having no significant effect on *F. culmorum*. Methyl acrylate and methyl propionate were afterwards identified as the two molecules of the blend responsible for the growth reduction observed. The efficiency of these organic esters on a large panel of pathogens was tested and complete growth inhibition was obtained for five of them. Our results open promising perspectives concerning the biological control of edaphic diseases.

Key words: barley, volatile organic compounds, *C. sativus*, *F. culmorum*

Introduction

Barley has high agronomical significance (123 million tons produced in the world in 2010) and is sensitive to many diseases (FAOstat, 2012). In particular, common root rot caused by *Fusarium culmorum* and *Cochliobolus sativus* is a common barley foliar pathogen worldwide. The disease is particularly aggressive under conditions of high relative humidity and temperature, where yield losses can rise up to 33% (Karov *et al*., 2009). Today, following consumers pressure for an eco-aware and health friendly agriculture, the amount of chemicals pesticides available for cereal protection is decreasing (Hossard *et al*., 2014). There is thus a growing need for new sustainable ways to control cereal diseases.

Plants are able to interact with their environment by emitting volatile organic compounds. Since the 1980's, the effects of VOCs on their biological environment and their potential as bio-pesticides have been on particular interest for the scientific community (Dudareva *et al*., 2006; Morath *et al*., 2012). However, most studies focused on aerial emission and studies of below-ground emissions of VOCs only started recently.

The objective of this paper is to show what kind of volatile interactions can take place between barley and the pathogenic fungi *C. sativus* and *F. culmorum*. The effects of the total VOCs emission from healthy and infected barley root on fungi, and *vice versa* will be assessed. Finally, VOCs with potential fungicidal effects will be identified in the blend of molecules emitted by infected barley roots.

Material and methods

Fungal strains

F. culmorum (MUCL 28166) and *C. sativus* (MUCL 46854) strains were provided by the Belgian Co-ordinated Collection of Microorganisms (BCCM – MUCL) (Louvain-la-Neuve, Belgium). They were stored on PDA medium (Merck KGaA, Darmstadt, Germany) at 23 °C and by cryopreservation at -80 °C.

In vitro co-culture of fungi and barley

In order to investigate the effect of fungal VOCs on barley and *vice versa*, barley and fungi were grown in different compartments of a co-culture device, sharing the same atmosphere. VOCs were thus the only way the two organisms could communicate. Moreover, barley leaves were removed from the co-culture device in order to only take into account the VOCs emitted by the roots (Figure 1).

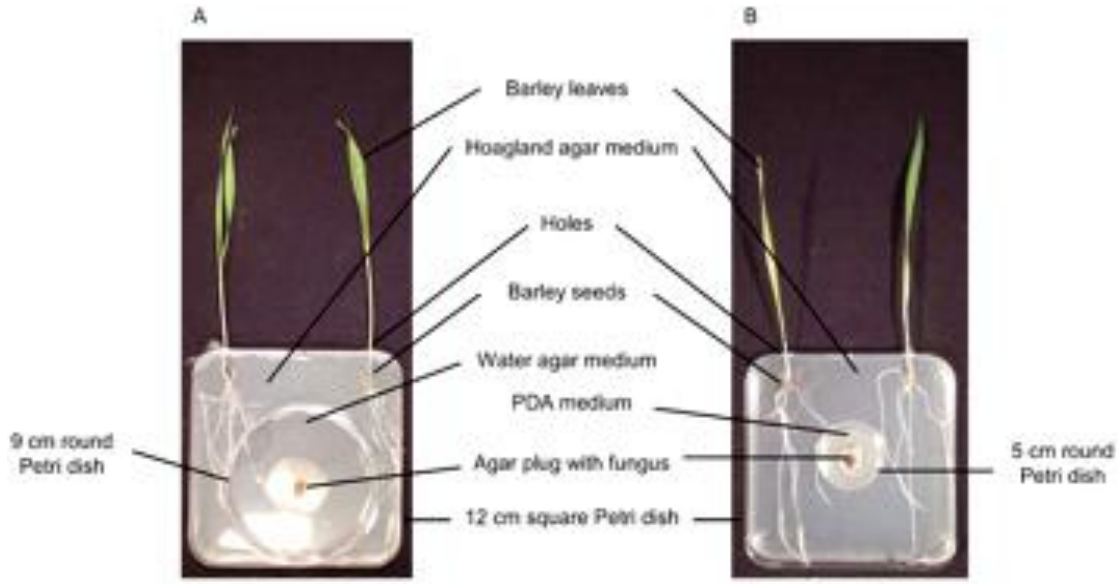

Figure1: Experimental devices for *in vitro* co-culture for the study of the effects of VOCs from non-infected or infected barley roots on pathogenic fungi (A) and for the study of the effects of fungal VOCs on barley (B) (Fiers *et al*., 2013).

Evaluation of the effect of selected VOCs on pathogen growth

Each molecule was mixed with 40 ml of water agar (1% agar (Difco, France)) then poured in cell culture flasks of 600 ml (VWR, Belgium). After medium solidification, a 70 mm disk of an active culture of the pathogen was placed in the center of medium. The cell culture flasks

were placed in a growth chamber under LED light (94 mmol photons/m^2/s) with a 16 h L: 8 h D photoperiod at 22 °C. The radial growth of the fungus was measured each 24 h with a graduated ruler until 240 h.

Results and discussion

Our results show that the VOCs emitted by *C. sativus* decreased barley leaf surface by 19%. This allows us to conclude that fungi can affect plant growth by emitting VOCs and suggest that pathogenic fungi interact with plants through volatiles. This phenomenon could be part of the "pathogenic strategy" of fungi. VOCs emitted by pathogenic fungi weakening plants before any physical contact between the two organisms.

More interestingly, we saw that the diameter of *F. culmorum* grown with non-infected barley roots was slightly larger than when it was grown with infected roots. However, significant differences were observed only punctually. Therefore, it is difficult to conclude that the VOCs released by infected barley roots had a long-term effect on *F. culmorum* growth. However, the diameter of *C. sativus* was significantly smaller (more than 13% 168 hours after infection (hai) and up to 17% 192 hai) when the fungus shared the atmosphere of *F. culmorum*-infected barley roots. Similar results have been obtained when the fungus shared an atmosphere containing VOCs from *C. sativus*-infected roots (13% of growth diminution 192 hai).

In order to determine which VOC of the blend emitted by infected barley roots were responsible for this growth reduction, the separate effect of the major compounds emitted during the interaction were tested. Methyl propionate (MP) and methyl acrylate (MA) were identified as the most efficient compounds responsible for the pathogens' growth inhibition.

The effect of MA was particularly important, going up to 87 and 91% of growth inhibition for *F. culmorum* and *C. sativus*, respectively. For MP also growth inhibition was important, going up to 81 and 91% for *F. culmorum* and *C. sativus*, respectively. The efficiency of these organic esters was then tested on seven pathogenic fungi and two bacteria. Complete growth inhibition was obtained for five of the tested fungi. The effect of the molecules on bacteria was more contrasted but still statistically highly significant.

To go further, we have investigated the mode of action of these molecules, which seem to act on cells membranes and to destabilize ion channels and proton pumps.

In conclusion, the effects of MA and MP observed *in vitro* are promising and the studied esters could be regarded as an interesting and innovative starting point in the development of a sustainable way to control barley's diseases.

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Differential antagonism of individual isolates and mixtures of yeasts against *Fusarium* and *Trichoderma*

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Abstract: The soil microbiome comprises a plethora of tritagonists that can influence the antagonist-pathogen-plant interaction in many ways. Instead of screening for the strongest antagonist, we sought to identify yeasts that differentially suppress soilborne pathogens of the genus *Fusarium* and beneficial fungi of the genus *Trichoderma* in order to improve the biological control of fungal pathogens. We quantified the antagonistic/agonistic activity of 40 yeast isolates against two *Fusarium* and three *Trichoderma* isolates, respectively. This screen identified a broad range of antagonistic activities and a positive correlation between the antagonism of yeasts against *Fusarium* and *Trichoderma*. Combining the six yeasts with the least effect on *Trichoderma* did not change the response against *Trichoderma*, but the mixture suppressed *Fusarium* more strongly than any of the six yeasts did when tested alone. This proof-of-concept experiment indicated that combinations of yeasts may exhibit altered, improved activities for the biological control of soilborne fungal pathogens as compared to single isolates. Mixtures of weakly antagonistic, inactive, or even agonistic microorganisms may thus provide a novel strategy for the biological control of the soilborne pathogen *Fusarium*.

Key words: antagonism, *Fusarium*, soil, *Trichoderma*, tritagonist, yeast

Introduction

Fungi, such as members of the genus *Fusarium*, are the most important plant pathogens in soil, threaten crop production worldwide, and are difficult to control (Koike *et al.*, 2003; Michielse & Rep, 2009; Raaijmakers *et al.*, 2009; Dean *et al.*, 2012). Soil microbiota are essentially involved in soil biological interactions and thereby affect plant growth, development, productivity, and susceptibility to infection by pathogens (Chaparro *et al.*, 2012; Lakshmanan *et al.*, 2014). Nevertheless, microbiomes are still a largely untapped resource for protecting crop plants against pathogens and for increasing agricultural productivity (Schnitzer *et al.*, 2011; Mendes *et al.*, 2013). Considerable efforts are undertaken to harness and use microbiota for novel applications in agriculture (Reid & Greene, 2013; Quiza *et al.*, 2015; Broadfoot, 2016). The composition of soil, rhizosphere and root microbiomes has been elucidated by sequencing-based metagenomics approaches (Daniel, 2005; Berendsen *et al.*, 2012; Turner *et al.*, 2013), but the contributions and functions of most individual species in soil are largely unknown. Soil microbiota thus consist mostly of yet uncharacterized bacteria and fungi that regulate microbial interactions, for which we have recently suggested the term tritagonists (Freimoser *et al.*, 2016). Identifying the most promising tritagonists by functional assays is thus an essential precondition for using and optimising the microbiome in order to increase agricultural productivity in an ecological and

sustainable manner. Usually, those organisms that are most antagonistic in laboratory experiments are considered for applications as biocontrol agents. Here, we aimed at maximising the differential antagonism against pathogenic and known beneficial fungi by combining yeasts that minimally suppressed or even favoured the antagonist *Trichoderma* (and that were only weakly antagonistic against *Fusarium*).

Material and methods

Isolation and cultivation of fungi

Soil or plant material was diluted 10-fold (w/v) with peptone water (1 g/l Bacto Peptone) (Mian *et al.*, 1997). The resulting suspensions were serially diluted and plated on potato dextrose agar (PDA) supplemented with chloramphenicol and tetracycline HCl (5 mg/ml in ethanol or water, respectively) (incubation at 22 °C). Single fungal colonies were transferred to and maintained on PDA agar plates without antibiotics and stored in 15% (v/v) glycerol at -80 °C.

Identification of fungi

Isolates were identified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF) as described (Freimoser *et al.*, 2016) or by sequencing the fungal internal transcribed spacer (ITS) region and assigning a species hypothesis according to the UNITE database (Abarenkov *et al.*, 2010; Koljalg *et al.*, 2013).

Quantification of yeast antagonism against filamentous fungi in vitro

Yeasts were collected from a PDA plate, diluted in water, and adjusted to an OD_{600} of 0.1. 15 µl of this suspension was plated on PDA plates. Filamentous fungi were similarly collected, diluted (OD_{600} = 0.1), and 5 µl were inoculated in the center of the plates. Plates were incubated at 22 °C for 3-15 days depending on the fungal species. Growth of the filamentous fungus in the presence of yeasts (or on empty plates as a control) was quantified before it reached the edge of the control plate with the help of a planimeter. Combinations of yeasts were created by mixing equal volumes of the yeast suspensions (OD_{600} of 0.1) and 15 µl of this suspension was plated on PDA plates.

Results and discussion

The antagonistic activity of 40 yeasts naturally occurring in agricultural environments against two isolates of soilborne *Fusarium* species and three isolates of the genus *Trichoderma* was quantified (relative growth of the filamentous fungus in the presence of each yeast isolate, in quadruplet). The relative growth data for all pairwise comparisons (log_2-transformed) were clustered in order to group yeasts based on the outcome of their interaction with *Fusarium* and *Trichoderma* species (Figure 1A). The majority of yeast isolates reduced the growth of both filamentous fungi (blue colour), but in a few interactions a small stimulatory effect of a yeast isolate was detected (indicated by the pink colour). The clustering analysis identified a group of strongly antagonistic yeasts (highlighted in blue), a cluster that had a stronger effect on *Fusarium* than *Trichoderma* (marked in grey), and a small subset of yeasts that were weakly antagonistic (marked in pink). Overall, the relative growth of *Fusarium* and *Trichoderma*, in the presence of each yeast, showed a positive correlation (Figure 1B). However, several yeasts that either strongly inhibited or did not affect *Trichoderma* at all

exhibited different levels of antagonism towards *Fusarium*, indicating that there might be some degree of specificity in the antagonism of some yeasts against *Fusarium* and *Trichoderma*.

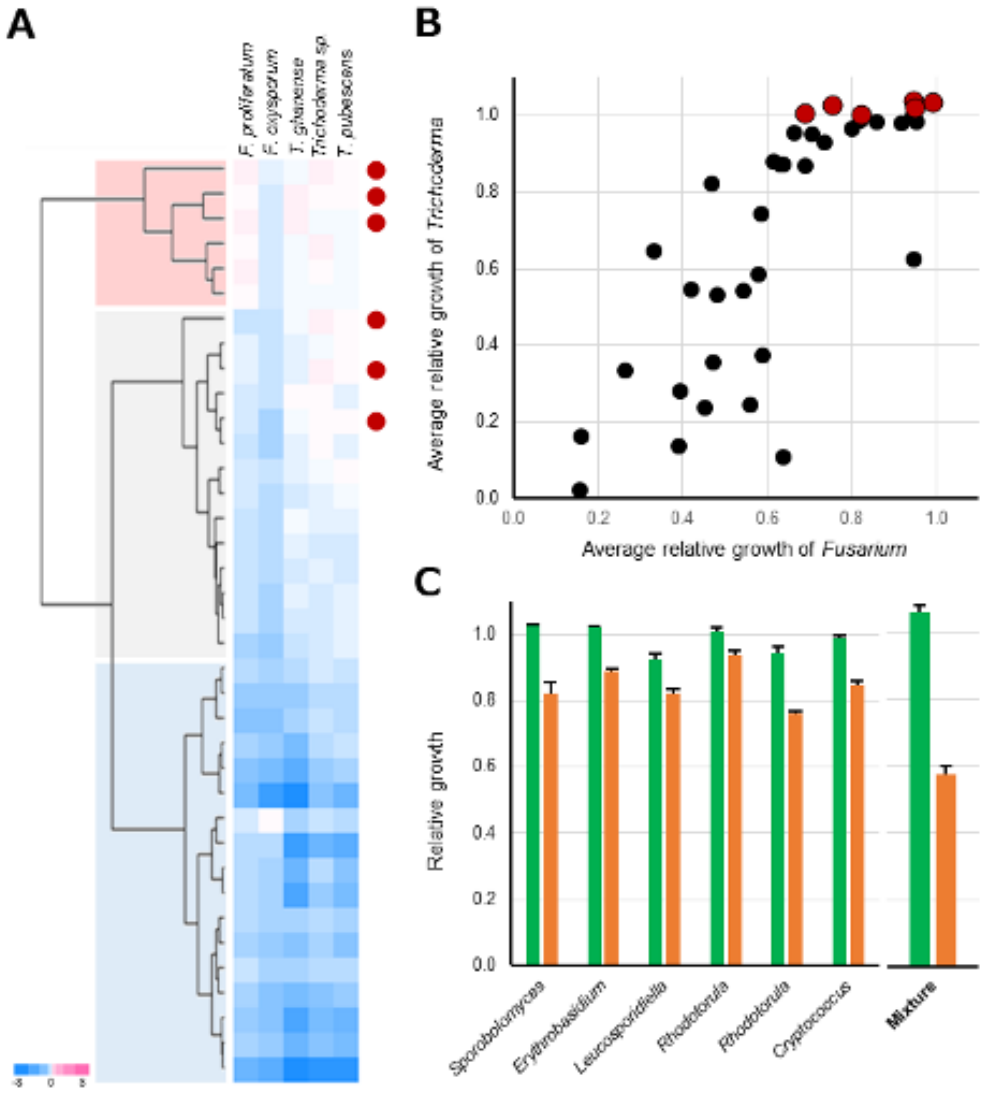

Figure 1. A combination of six weakly antagonistic/agonistic yeasts is more antagonistic against *Fusarium* than the single isolates. A) The competitiveness of 40 yeast isolates (rows) against *Fusarium proliferatum* (from an infected root), *F. oxysporum* (soil isolate), *Trichoderma ghanense*, *Trichoderma* sp., and *T. pubescens* (all soil isolates) was quantified (in quadruplet). The blue colour indicates interactions where a filamentous fungus was suppressed, while pink indicates a stimulatory effect on growth of *Fusarium*/*Trichoderma* by the corresponding yeast. The three main clusters are marked in blue, grey and pink. The six yeasts that were combined in a mixture are marked with a red dot. B) For each yeast isolate, the average for all interactions with species of the genus *Fusarium* (two isolates) and *Trichoderma* (three isolates), respectively, was calculated and plotted against each other. The six yeasts with no detectable effect on *Trichoderma* (marked in red) were tested in combination. C) Relative growth of *Trichoderma* (green) and *Fusarium* (orange) in pairwise competition with the six yeast isolates or in a competition experiment with a mixture of all six yeasts. The mean of four replicates and the standard error of the mean are shown.

In order to improve the specificity and to minimise the antagonism against known beneficial fungi, the six yeasts with the least effect on *Trichoderma* were mixed (one isolate of the genera *Sporobolomyces*, *Erythrobasidium*, *Leucosporidiella*, and *Cryptococcus*; two isolates of the genus *Rhodotorula*). This yeast community, similar to the single isolates, did not inhibit *Trichoderma* to any detectable degree, but the antagonistic activity against *Fusarium* was stronger than for any of the single isolates (Figure 1C). This proof-of-concept experiment documents that communities of weakly antagonistic yeasts can exhibit stronger antagonism than the individual isolates. These results may influence the selection and development of future microbial biocontrol agents and lead to novel biocontrol strategies that rely on microbial communities rather than individual species. Future studies will identify the function of each of the six yeast isolates in the mixture and reveal the mechanism that brings about the altered specificity of the yeast community.

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New formulations for *Candida sake* CPA-1 with biodegradable coatings to improve their survival and efficacy under stress conditions

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Abstract: The biocontrol agent (BCA) *Candida sake* CPA-1 has demonstrated to be effective against several diseases caused by *Penicillium expansum*, *Botrytis cinerea* or *Rhizopus nigricans* on fruit. Nevertheless, to apply the biocontrol agent in the field it was necessary to mix it with a food coating in order to improve survival under stress conditions, as well as adherence and distribution on fruit surface. The objective of this study was to obtain a more competitive formulation at field conditions to be applied independently of any product. To achieve this purpose, the drying process of CPA-1 by fluid-bed spray-drying system together with biodegradable coatings was optimised. This is a very new approach because the novelty of the drying system used and the aim of obtaining a BCA formulation able to form film or coating on fruit surface. Several substances were tested as carriers and binders, and drying temperature was optimised. The addition of protective compounds was also tested to improve survival of CPA-1 during the dehydration process. Biocontrol efficacy on grapes against *B. cinerea* as well as product shelf life were studied. In addition, the improvement of *C. sake* behaviour under stress conditions was also tested. The optimal temperature of drying was 55 °C and two formulations able to develop coating on fruit surface were obtained. One of them was formulated using a combination of soluble and pre-gelatinised potato starch; the other formulation was made with maltodextrin and adding skimmed milk and sucrose as protectant compounds. Formulated products maintained biocontrol efficacy and survived better under stress conditions of temperature and humidity.

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Key words: biocontrol, *Candida sake*, coatings

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Screening criteria for the development of biocontrol products for control of plant diseases

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Abstract: Commercial biological control products for the use against plant diseases have to fulfil a broad range of requirements. Besides their efficacy against the targeted plant pathogens major requirements concern the ecological, toxicological and eco-toxicological characteristics of the used antagonists. Consequently, a broad range of criteria has to be considered during the selection of new antagonists. Screening programs can use a stepwise approach to exclude unwanted candidates in an early stage. The antagonists selected with such a screening strategy fulfilling all the major criteria will combine suitable characteristics for registration and marketing as commercial biocontrol products.

Key words: biological control, plant pathogens, screening, registration, commercialisation

Introduction

The markets for biological control products against pests and plant pathogens increased significantly during the last years and more products are needed for this increasing market demands in the future (Glare *et al.*, 2012). Main drivers for this development are consumers, retailors and regulators demanding more and more sustainable productions systems and lower pesticide residue levels in food and feed. A major step in this development in Europe is the implementation of Directive 2009/128/EC on principles of Integrated Pest Management requiring from growers that non-chemical control measures of pests and diseases including biological control are used if available, before any chemical control measures are taken. This will result in a transition from chemical treatments to sustainable solutions. The implementation of Directive 2009/128/EC will thus increase market opportunities for biocontrol products and will increase the need for the development of new biocontrol products. The market growth for biocontrol products expected for the next years exceeds an annual growth rate of 15%. This includes a growing market for biocontrol products based on antagonistic bacteria and fungi. New screening programs carried out in collaborations between biocontrol industries and research institutes are needed to search for a next generation of antagonists. Such antagonists for use in commercial biocontrol products have to fulfil many different requirements. Besides being active control agents against the specifically targeted plant pathogens, they must be safe and cost effective. The development of new biocontrol products starts with screening programs including hundreds or thousands of candidates. For commercial use, important criteria are market size, efficacy, ecological characteristics, production costs, safety, environmental risks and protection of intellectual property rights. A stepwise screening considering these very different aspects is proposed.

Selection criteria

Screening programs for antagonists of plant pathogens are often focused on testing antagonistic properties *in vitro*, in bioassays and subsequently in crops. However, more criteria must be fulfilled if commercial use is considered: toxicological profiles of an antagonist, technologies for production and formulation and their costs, genetic stability of the antagonist, market size for the product and the possibilities of patent protection for the application (Whitesides *et al*., 1994; Köhl *et al*., 2011). Criteria for antagonist screening for commercial application can be ranked in a stepwise approach to exclude unwanted candidates in early screening steps using inexpensive tests (Figure 1). This will result in less candidates tested in later screening steps using more expensive assays.

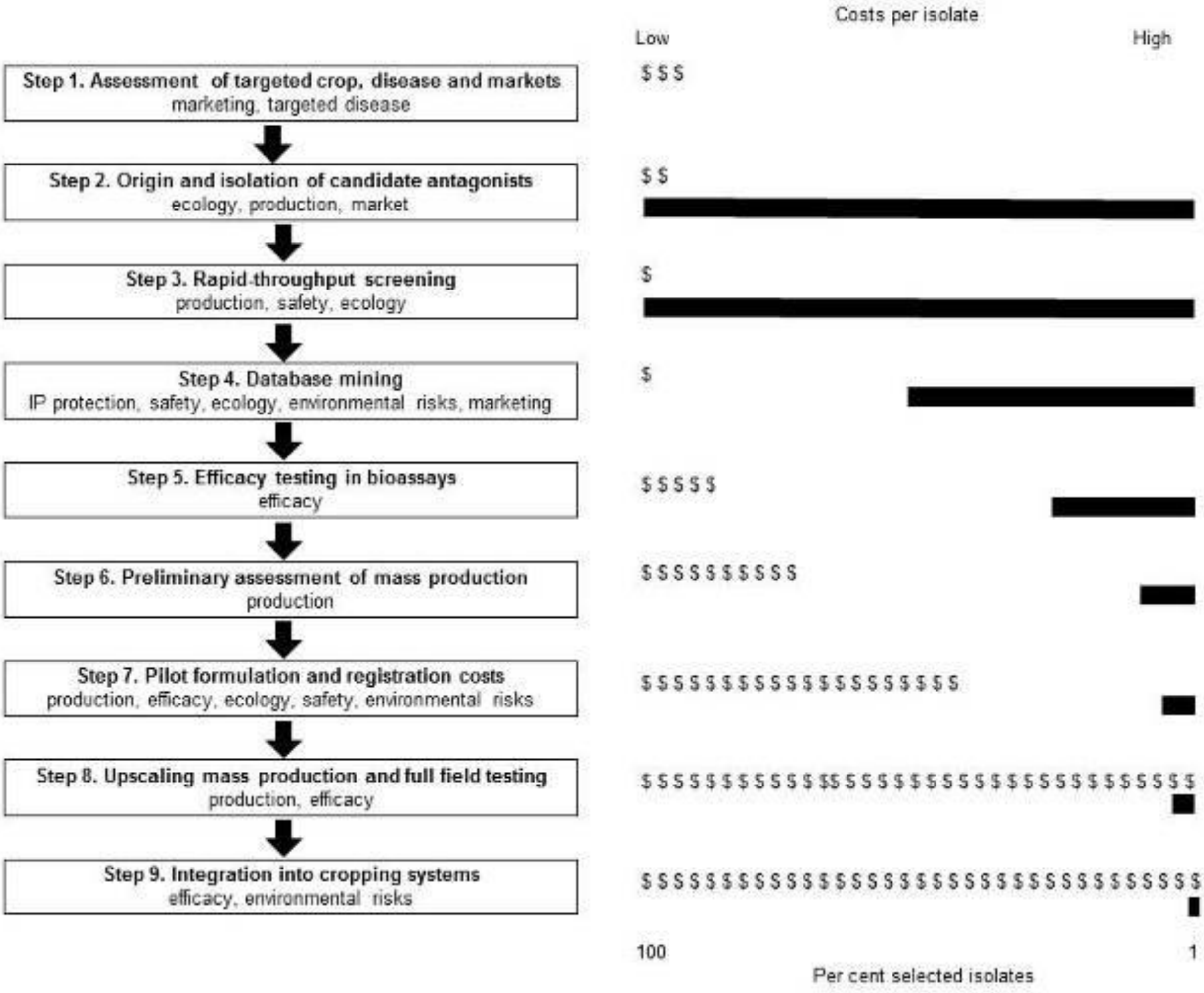

Figure 1. Stepwise screening of microorganisms for commercial use in biological control of plant pathogens. Specific categories of selection criteria are considered for each screening step, screening costs are estimated ($) and the percentage of assessed isolates is given (■). From: Köhl *et al*., 2011.

Screening criteria regarding registration requirements

Before antagonistic microorganisms can be applied as biological control products the antagonistic isolate has to be registered as active ingredient and the formulated isolate has to be registered as crop protection product (Ehlers, 2011; European Commission, 2009). Important criteria are identity, biological properties, toxicological properties and eco-toxicological properties of the strain. The registration procedures are costly and time-consuming. The amount of information from studies needed to fulfil the dossier requirements is often uncertain at the beginning of a registration process.

Knowing and anticipating the dossier requirements already during the development of new biocontrol candidates will help to select antagonists which may require less input, to avoid major difficulties and to allow a more predictable registration process. Screening criteria excluding candidate isolates which potentially cause uncertainties during the risk assessments done by the registration authorities will thus increase the chance that new biocontrol agents will become available for use by growers. Examples for such screening criteria with respect to the registration regulations of the European Union are:

Identification and strain-specific detection. Identification at species level and a strain-specific detection protocol are requirements for the registration dossier. Candidate antagonists with an uncertain taxonomical status should be avoided. An early identification of the candidate isolates at species level will also allow a range of first database-based risks assessments. Often isolates belonging to a species already used in biological control are preferred by industries because existing published knowledge and registration dossiers will be available and possible risks could be evaluated easier by registration authorities. Information on species not used in biocontrol yet will be more limited so that authorities may face more uncertainties leading to more detailed risk assessments. On the other hand, industries may prefer newly described antagonists allowing a strong patent protection.

Population backgrounds. Populations of the antagonistic strains must not persistently exceed the natural background level of the species. Species not present naturally in the compartment should be avoided. Antagonistic species for which knowledge on levels of naturally backgrounds is available should be preferred.

Environmental fate in the compartments water, soil and air. Persistence and spread of the antagonistic strains in the compartments water, soil and air must be limited to avoid environmental risks. In general, persistence and mobility of applied antagonistic strains in the natural environment is limited. Candidate species should be evaluated in literature-based desk studies for possible risks.

Mode of action. The mode of action of the antagonists must be known and described in the registration dossier. Candidate strains with a mode of action based on a metabolite with antibiotic properties will be assessed very thoroughly including detailed toxicological studies. Candidate strains with other mode of actions may require less detailed studies. Screening criteria such as formation of inhibition zones on agar plates may result in candidates with higher requirements during registration. On the other hand, isolates producing no inhibition zones on agar may be preferred to avoid documentation of the possibly involved metabolites and their toxicology.

Human and plant pathogenicity. Microorganisms pathogenic for humans or plants cannot be registered as plant protection products. All candidate isolates should be identified at species level to allow first literature-based risk assessments. Isolates close to known human or plant pathogens should be selected for absence of known virulence genes.

Metabolites of concern. Metabolites of concern must not be produced by the antagonists. Candidate antagonists should be screened for the presence of genes or gene clusters regulating the production of metabolites of concern such as mycotoxins. Such isolates should be excluded in an early stage of a screening program.

Conclusions

The efficacy of antagonists in reducing disease is an essential criterion for the selection of new antagonists. However, many more criteria equally important have to be fulfilled for the development of a commercially viable product. Such criteria belong to very different categories, such as antagonist ecology, (eco)-toxicology, safety and environmental risks, mass production and formulation, but also present and expected market demands, the specific requirements for registration dossiers and the options for the effective protection of intellectual property rights. Considering such a wide range of criteria during screening programs for the development of commercial biological control products against plant pathogens can further increase the impact of the biocontrol science on plant protection. Essential considerations at the beginning of new screening programs are to collaborate with biocontrol industries from the beginning, to consider the relevant commercial questions early during the screening program and to combine the expertise in plant pathology with expertise in several other disciplines, e.g. biotechnology, agronomy, microbiology, toxicology, registration, marketing and product development.

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Trichome O-acyl sugars protects *N. attenuata* against both native fungal pathogens and a specialist herbivore

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Abstract: Plants are simultaneously threatened by herbivores and pathogens, and it has been hypothesized that many plant defenses evolved as dual functions. However, evidences remain scare. In many plants, trichomes are the first layer of protection directly against attacks from herbivores and pathogens. They act not only as physical barriers, but also known as the place to produce and store various defensive metabolites. (Van Dam & Hare, 1998; Fahn, 2000; Roda *et al*., 2003; Schilmiller *et al*., 2008; Last *et al*., 2012). *Nicotiana attenuata*, a wild tobacco species, native in Great Basin Desert, USA, is fully covered with glandular trichomes (Roda *et al*., 2003; Weinhold & Baldwin, 2011). Among many secondary metabolites produced in the trichomes of *N. attenuata*, O-acyl-sugars are the most abundant one, which can be produced up to 1.5 mg/g FW. Studies have shown that this group of O-acyl sugars can act as both direct and indirect defensive against aphids, spider mites and other soft-body insects (Rodriguez *et al*., 1993; Chortyk *et al*., 1996; McKenzie & Puterka, 2004; Weinhold *et al*., 2011). However, whether this group of O-acyl sugars can directly protect plants from attacks from native pathogens and specialist herbivores remains unknown.

To address this question, we deployed natural variations of trichome O-acyl sugars in *N. attenuata.* Screened 26 ecotypes of *N. attenuata* revealed 15 different O-acyl sugars, which can be classified into three structural classes. Two ecotypes, A83 and A84 showed extremely low level of O-acyl sugars. Testing the susceptibility to native pathogens and herbivore showed that both A83 and A84 are highly susceptible to *M. sexta*, a specialist herbivore of Solanaceae family, and native fungal pathogens, *Fusarium brachygibbosum* U4 and *Alternaria* sp. U10. Investigations on other defensive traits suggest that all of known defenses in *N. attenuata* are intact in these two ecotypes, except O-acyl sugars. Tests using F2 population between A84 and a high O-acyl sugar containing genotype (UT) further showed that the level of O-acyl sugars in leaves correlates with susceptibility to both *M. sexta* and the two native fungal pathogens. Furthermore, removing O-acyl sugars from leave surface of *N. attenuata* increased *M. sexta* growth rate as well as the susceptibility to the two native fungal pathogens. Adding O-acyl sugars into artificial diet and germination medium reduced *M. sexta* growth and spore germination of the fungal, respectively. Taken together, we provide the first evidence that leave trichome O-acyl sugars act as dual functions protecting *N. attenuata* from attacks from both native fungal pathogens and specialist herbivores.

Key words: *Nicotiana attenuata*, leaf trichome, O-acyl sugars, native fungal pathogen, native herbivore

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Seed treatment with biological control agents against *Verticillium* wilt in oilseed rape

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Abstract: *Verticillium* wilt caused by *Verticillium* spp. is difficult to suppress and results in severe yield losses in a broad range of crops including oilseed rape (OSR) and cauliflower. The objective of the study was to develop a sustainable seed treatment that will protect oilseed rape and *Brassica* vegetables from *Verticillium* wilt. Preselected *Serratia* and *Paenibacillus* isolates showing antagonistic properties against fungal pathogens were compared for their plant growth-promoting (PGP) potential under a range of plant growth conditions. *Serratia* treatment resulted in a variety of levels of PGP, while *Paenibacillus* strains damaged roots of the seedlings under gnotobiotic soil free conditions. The selected strains from each genus, *S. plymuthica* 3RP8 and *P. polymyxa* Sb3-1, were tested for their PGP potential under sterile and non-sterile soil conditions. *P. polymyxa* Sb3-1 did not have a significant effect on plant growth in non-sterile soil; however it did promote plant growth in sterile soil. *S. plymuthica* 3RP8 had a mild non-significant PGP effect on the seedlings in gnotobiotic soil-free conditions and in unsterile soil. Similarly to the *P. polymyxa* Sb3-1, HRO-C48 significantly increased the weight of the OSR seedlings in sterile soil. This indicated that the choice of growth environments is crucial for the investigation of plant-bacterium interaction. In order to study the biocontrol efficacy of *P. polymyxa* Sb3-1, it was applied to the seeds of OSR in two different concentrations. The resulting one week old seedlings were infested with *V. longisporum* EVL25 using a root dipping method. Both treatments induced a significant increase in the lengths of the seedlings, however no significant reduction of disease was observed. The treatments with *S. plymuthica* 3RP8 did not result in any significant improvement in the seedlings' health. The field trials and screening for new antagonistic isolates are ongoing.

Key words: biocontrol, plant growth promotion, PGP, *Verticillium* wilt, *Paenibacillus*, *Serratia*, oilseed rape

Introduction

The overall objective of this study, which is a part of a BIOCOMES project (www.biocomes.eu), is to develop a sustainable method of protecting oilseed rape and *Brassica* vegetables against fungal pathogens by treating seeds with beneficial bacteria. *Verticillium* spp. induce vascular wilting corresponding with high yield losses within a wide range of dicotyledonous plants, including economically important field crops such as oilseed rape (OSR, *Brassica napus* L.) and vegetables like cauliflower (*Brassica oleracea* L.) (Debode *et al*., 2005). *Serratia* and *Paenibacillus* strains are widely known for their plant growth promoting (PGP) and biocontrol qualities (Petersen *et al.*, 2013; Rybakova *et al.*, 2015a). *Serratia plymuthica* HRO-C48 has been successfully used for controlling *Verticillium*

wilt and other soil-borne fungi as a soil amendment in strawberry fields (RhizoStar®) (Kurze *et al*., 2001). The application of *S. plymuthica* HRO-C48 to the seeds of the oilseed rape was shown to reduce the degree of *Verticillium* wilt in oilseed rape plants under greenhouse conditions (Müller *et al.*, 2008), while one of the main advantages of *Paenibacillus* as a biocontrol agent (BCA) is its ability to build endospores. This ability increases its survival in soil and provides advantages over the non-spore formers in product formulation (Emmert & Handelsman, 1999; Rybakova *et al.*, 2015a).

Material and methods

Bacterial strains and growth conditions

The fungal pathogen used was *V. longisporum* ELV25 Stark (Karapapa *et al.*, 1997) (Messner *et al*., 1996; strain collection TU Graz, Environmental Biotechnology). The bacterial strains of genera *Serratia* (*S. plymuthica* 3Re4-18, *S. plymuthica* HRO-C48, *S. plymuthica* 3Rp8, *S. plymuthica* S13, *S. proteamaculans* SP1-3-1), *Paenibacillus* (*P. polymyxa* Sb3-1, *P. peoriae* GnDWu39, *P. brasilensis* Mc2-9, *P. polymyxa* Pb71, *P. polymyxa* 302P5B5, as well as a rifampicin resistant mutant of Sb3-1 *P. polymyxa* Sb3-1 Rif^R) and *Pseudomonas* (*P. azotoformans* Ru40, *P. brassicacearum CKB26*, *P. protegens* F37, *P. fragi* W31 and *P. fluorescens* F2) were used in this study (Rybakova *et al.*, 2015b and unpublished data). The bacterial strains were routinely grown on Standard I nutrient agar (NA, SIFIN, Berlin, Germany) at 30 °C. *V. longisporum* ELV25 was grown either on the potato dextrose agar (PDA) or in the Czapek Dox liquid culture (Sigma-Aldrich) at 22 °C. When required, rifampicin was added at concentrations of 100 μg/ml.

Estimation of the PGP and biocontrol effect of P. polymyxa Sb3-1

For the PGP experiments OSR (*Brassica napus* l. partim, "Traviata H 605886" (KWS Saat Einbeck, Germany) seeds were treated with each of the bacterial strains or combinations thereof using a bio-priming method as described in Rybakova *et al.* (2015b). For the PGP studies the two week old seedlings were harvested and their fresh weight was estimated and compared with the not-treated control. In order to estimate the biocontrol activity of *P. polymyxa* Sb3-1, the bacterial cells were applied to the seeds in two concentrations ($\log_{10}$ 7 CFU/ml and $\log_{10}$ 5 CFU/ml) using a pelleting method according to the protocol described by Müller *et al.* (2008). The experimental setup for the biocontrol experiments included 15 pots per treatment with one plant per pot. The one week old seedlings were inoculated with *V. longisporum* using a root dipping method. The roots of the one week old seedlings were artificially injured using a scalpel. The seedlings were inoculated by dipping their roots for 30 minutes into 50 ml of Czapek-Dox broth with a one week old culture of *V. longisporum* adjusted to 5×10^6 CFU/ml or sterile water for the control treatments. The plants were then replanted in soil. After the appearance of the first symptoms, the disease reaction of plants was assessed according to the severity of symptoms as described by Müller *et al.* (2008).

Results and discussion

Comparison of the PGP effect of Serratia and Paenibacillus strains on oilseed rape in gnotobiotic soil-free conditions

While *Serratia* treatment resulted in different levels of PGP (105-113% of the fresh weight of the untreated plants), the opposite effect was observed after the *Paenibacillus* evaluation (38-66% of the fresh weight of the untreated plants). Moreover, the root system of the *Paenibacillus* primed seedlings appeared stunted and damaged, while both the control plants and those treated with *Serratia* strains had intact root systems. Among tested *Paenibacillus* strains *P. polymyxa* Sb3-1 caused the least amount of damage to the plant. *S. plymuthica* 3RP8 exhibited a mild PGP effect on the oilseed rape seedlings. Combined with the data for the *in vitro* antagonism against *V. longisporum* (Rybakova *et. al*., 2015b), the *S. plymuthica* 3RP8 and *P. polymyxa* Sb3-1 were chosen for further PGP experiments.

PGP and biocontrol effects of the S. plymuthica 3RP8 and P. polymyxa Sb3-1

The average fresh weight of the plants bio-primed with *S. plymuthica* 3RP8 and *P. polymyxa* Sb3-1 did not differ significantly from those of the unprimed seedlings grown in non-sterile soil. Similarly, *P. polymyxa* Sb3-1 rif^R (Log_{10} 5 and log_{10} 7) applied to the OSR seeds using pelleting did not have a significant PGP effect compared to the not-treated control. On the other hand, when plants were grown in the sterile soil, bio-priming of the OSR seeds with both *S. plymuthica* 3RP8 and *P. polymyxa* Sb3-1 resulted in a significant PGP effect on the plants (160% and 206% of the weight of the untreated control for the Sb3-1 and 3RP8, respectively). This underlines the importance of growth environments in the plant-bacterium interaction studies.

When OSR seedlings were infected with *V. longisporum*, the lengths of those that were treated with two different concentrations of *P. polymyxa* Sb3-1 rif^R applied to the seeds using pelleting were significantly higher than those of the not-treated control (36% and 40% of increase of the plant length in case of treatment with $log_{10}5$ and $log_{10}7$ CFU/seed of *P. polymyxa* Sb3-1 rif^R, respectively). The fresh weight of the plants did not increase significantly due to their having been treated with the Sb3-1 rif^R, and no significant improvement of *Verticillium* wilt symptoms was detected. The treatment of OSR seeds with *S. plymuthica* 3RP8 did not result in any significant improvement in the seedlings' health. These results indicate that the application of *P. polymyxa* Sb3-1 to OSR seeds using pelleting is a promising method for the biocontrol of *Verticillium* wilt needing further optimization.

The field trials with existing BCAs as well as screening for new antagonistic bacterial strains selected from the endophytic community of *Brassica* plants are ongoing.

Acknowledgements

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Plant disease control by metabolites of fatty acids in bacteria

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Abstract: Various metabolites of lipids produced by some bacteria from vegetable oil were shown to control bacterial and fungal plant disease, respectively. We focused on fatty acids produced as metabolites in culture. Cucumber plants were treated with supernatant (metabolite), water (as control), 2,4,5,6-tetrachloroisophthalonitrile (TPN), sorbitan fatty acid ester (PS) or potassium bicarbonate (PBC) and inoculated with *Botrytis cinerea*. In control, PS and PBC treatments symptoms of browning or holes were observed on each leaf. In treatments with the supernatant and TPN no remarkable symptoms were observed. In *Arabidopsis thaliana*, roots were treated with the supernatant, medium and control (water) and *Erwinia carotovora* was inoculated on above-ground parts of the plants. The treatment with each concentration of supernatant reduced the diseased foliage area by 71.4 to 93.0% compared to the control (water). These results indicated that the mechanism of disease control by metabolites from fatty acids was induced systemic resistance.

Key words: induced systemic resistance, fatty acid, metabolite(s)

Introduction

Vegetable oil consists of glycerides (fatty acid ester) and is degraded to some fatty acids (such as oleic acid and linoleic acid) by enzymes (such as lipase or lipoxygenase). The linoleic acid is known as precursor of jasmonic acid (JA) which can induced systemic resistance. Fatty acids are degraded naturally, physically, chemically and biologically. Some oxidized fatty acids such as 9-hydroxyoctadeca-10E, 12Z-dienoic acid were reported to be a plant vitalizer as a rooting or flowering promoter. There are reports that lipid peroxides are generating active oxygen and have antimicrobial activity. In our study, we focused on the biological metabolites produced by bacteria in cultures containing fatty acids and some minerals.

Material and methods

Metabolites in supernatant of culture

Some bacteria were isolated using oil coated agar plates and selected for their high emulsifying capacity. Bacteria were cultured with fatty acids and some minerals. After culturing, the supernatant was collected by centrifuging.

Treatment of cucumber leaves

Cucumber plants were grown for one month in plastic pots and sprayed on the leaves with each agent: Water, 0.08% 2,4,5,6-tetrachloroisophthalonitrile (TPN), 0.14% sorbitan fatty acid ester (PS), 0.16% potassium bicarbonate (PBC), or 0.2% of culture supernatant (including metabolites). After 24 hours, filter disk paper (20 × 20 mm) was dipped into a

spore suspension of *Botrytis cinerea* (10^6 spores/ml) and inoculated on the true leaves and kept under high-humidity condition. The symptom area of the leaves was measured after 3 to 5 days.

Treatments of Arabidopsis thaliana roots

Arabidopsis thaliana (wild type, Col-0) plants were grown for two weeks in soil with celled trays (72 cells per tray). Supernatant (including metabolites) and medium (without biodegradation) were diluted to 0.2, 1 and 10% by adding water. The metabolites solutions, medium solutions and water (as control) were injected into the soil to treat only the roots, respectively. A suspension of *Erwinia carotovora* subsp. *carotovora* (OD_{600} = 0.1) was sprayed onto whole plants after 24 hours and plants were kept under high-humidity and dark condition. Whole plants were observed for areas with symptom after 5 to 7 days.

Results and discussion

Disease control by leaf treatments with metabolites

Disease control by each treatment in cucumber is shown in Table 1. The leaves treated with water (control), PS or PBC showed remarkable symptoms by *B. cinerea*. In treatment with PBC, disease severity of the leaves was similar to the control treatment with symptoms of browning and holes. Holes on the leaves were not observed after treatment with PS. Plants treated with the metabolites and TPN did not show any remarkable symptoms. These results indicated that treatment of metabolites controlled disease by *B. cinerea*. Moreover, inhibition zones indicating antifungal activity were not observed on agar plates.

Table 1. Disease (*B. cinerea*) control by various treatments in cucumber.

Treatment	Symptom area (mm)[1]	Disease severity
Control (water)	15 × 10	Browning and hole
Sorbitan fatty acid ester	12 × 15	Browning
Potassium bicarbonate	15 × 14	Browning and hole
2,4,5,6-tetrachloroisophthalonitrile	None	None
Metabolites (supernatant)	None	None

[1] Length and width of symptoms on leaves was measured.

Disease control by root treatment with metabolites

Table 2 shows disease (*E. carotovora*) control by the different treatments in *A. thaliana*. The treatments with 0.2 and 1.0% medium did not control disease caused by *E. carotovora* and 10.0% of medium slightly controlled the disease by 14.3%. The treatment with supernatant controlled the disease at all concentrations. The disease protection with treatments of the supernatant at concentration of 0.2, 1, and 10.0% was 71.4, 92.6 and 93.0%, respectively. These results indicated that the metabolites controlled soft rot disease by *E. carotovora* by root treatment and that the mechanism was induced systemic resistance.

Table 2. Disease (*E. carotovora*) control by various treatments in *A. thaliana*.

Treatment	Disease protection (%)[1]
0.2% medium	0
1.0% medium	0
10.0% medium	14.3
0.2% supernatant	71.4
1.0% supernatant	92.6
10.0% supernatant	93.0

[1]Disease protection (%) in comparison with disease area of the control treatment.

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Sulphur-containing volatiles: new weapons in the fight against plant diseases

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Abstract: During the last decade, the importance of bacterial volatiles in cross-kingdom interactions has become evident. In addition to promoting plant growth and root development, bacterial volatiles have been repeatedly shown to inhibit the growth of phytopathogenic fungi, although the molecules responsible for this effect are still largely unknown, with the notable exception of hydrogen cyanide. Most research performed so far has dealt with pathogens from the genera *Rhizoctonia* or *Fusarium*, however, at present, hardly anything is known about how fungi-like organisms such as oomycetes react to bacterial volatiles. Our recent work has shown that oomycete pathogens such as *Phytophthora infestans,* causing late blight in potato, are particularly sensitive to the volatiles of potato-associated *Pseudomonas* strains. In a screen aimed at identifying the chemical composition of the volatile blends from those efficient anti-oomycete *Pseudomonas*, sulphur-based compounds were identified as potent inhibitors of all life stages of the pathogen, including mycelial growth, sporangia production and germination, as well as zoospore motility. Some of these sulphur–containing volatiles were able to prevent disease establishment on infected plant material. We are currently investigating i) the genetic determinants leading to the emission of these potent volatiles, ii) the blend of volatile produced by the bacteria on potato plants rather than on cultivation media, iii) the induced physiological changes in the targeted pathogens, and iv) the best application strategy, comparing the strains' effects with that of pure volatiles. When using the volatile-producing strain as a protective treatment, formulation should be designed as to warrant establishment of the biocontrol strains in the phyllosphere as well as boosting *in situ* emission of protective volatiles.

Key words: volatiles, *Pseudomonas*, *Phytophthora*

Biological control of the date palm tree borers, *Oryctes* spp.

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Abstract: The efficacies of the entomopathogenic nematode (EPN) *Rhabditis blumi* and the entomopathogenic fungus (EPF) *Beauveria bassiana* as biocontrol agents were determined against date palm tree borers *Oryctes* spp. (Coleoptera: Scarabaeidae: Dynastinae) in laboratory and field trials during the 2015 season. Laboratory results demonstrated that direct sprays of 1000 infective juveniles (IJs) per ml of *R. blumi* on Arabian Rhinoceros Beetle, *Oryctes agamemnon arabicus* (ARB) caused 71.67% mortality in larvae and 15% mortality in adults. While, treating the food source of the larvae (pieces of fresh tissue of the frond bases) with the same dose and period resulted in 48.33% mortality in larvae and 10% in the adults. Laboratory results also showed that using a concentration of 1×10^9 conidia/ml of *B. bassiana* as direct spray of the ARB larvae led to 66.7% mortality and to 60% mortality if the food source was treated. Field experiments results showed that injection of 50 ml per palm tree with a concentration of 1000 IJs/ml of *R. blumi* inflected about 42% mortality in ARB larvae infesting the tree. Meanwhile, injection of 50 ml at 1×10^9 conidia/ml of *B. bassiana* imposed 50% mortality in larvae. Results of this investigation illustrate the possibility of using *R. blumi* and *B. bassiana* as bicontrol agents against palm borers in IPM programs.

Key words: entomopathogenic nematode, *Rhabditis blumi*, entomopathogenic fungi, *Beauveria bassiana* , biocontrol, endophytes, palm borers, *Oryctes* spp.

Combined control of *Locusta migratoria manilensis* by dissemination of *Metarhizium anisopliae* using *Carabus smaragdinus* as the vector

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Abstract: *Locusta migratoria manilensis* Meyen is one of the most serious pests in the world. *Metarhizium anisopliae* Sorokin is an effective fungal biocontrol agent to control locusts, *Carabus smaragdinus* Fischer is also an important natural enemy which can pose a threat to locusts, while the majority of the biocontrol agents were applied independently. We studied the virulence of *Metarhizium anisopliae* and predation by *Carabus smaragdinus* against *Locusta migratoria manilensis* and their interaction effects on the pest population. Results showed that *M. anisopliae* was harmless to *C. smaragdinus*. Predation rate by *C. smaragdinus* decreased from 64.19% to 61.23% when locusts were treated with *M. anisopliae*, while infection rate by *M. anisopliae* increased from 70.85% to 75.34% in the presence of *C. smaragdinus*. The corrected mortality rate of releasing *C. smaragdinus* combined with spray of *M. anisopliae* was 83.3%, and that of releasing *M. anisopliae*-inoculated *C. smaragdinus* was 100%, which were higher than those achieved with *C. smaragdinus* or *M. anisopliae* alone. The results suggest that control of *L. migratoria* by dissemination of *M. anisopliae* using *C. smaragdinus* as the vector is a potential choice.

Key words: *Locusta migratoria manilensis*, *Carabus smaragdinus*, combined control

Bacteria and bacteriophages based biocontrol product against SRE in potato tubers

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Abstract: Soft rot Enterobacteriaceae (SRE) (pectinolytic *Pectobacterium* and *Dickeya* spp., formerly known as pectinolytic *Erwinia* spp.) are economically important plant pathogenic bacteria of various agricultural crops including potato (*Solanum tuberosum* L.). SRE are responsible for soft rot and blackleg diseases and cause increasing damage in potato production worldwide. Effective management to control blackleg and soft rot disease is lacking and validated detection methods for SRE do not exist. We aim to develop a biological control product containing wild type strains of antagonistic bacteria and lytic bacteriophages to be used against SRE in potato tubers during storage and transit. From 22 antagonistic bacterial strains, characterized in our former studies and belonging to different genera, 15 combinations containing up to 5 strains each will be selected and evaluated. The designated combinations will be tested on potato tubers together with the mixture of SRE pathogens and under disease-promoting conditions of relatively high humidity and temperature. The most promising candidate combinations will be additionally formulated in order to expand their stability and possibility of applications. The formulated product will be tested under natural potato storage conditions and with cooperation with potato growers to adjust to the particular needs of the target group (potato producers, farmers and customers). We expect that the developed biological control product will reduce the potato tuber soft rot incidence up to 50% in comparison to hygienic practices used in potato production so far.

Key words: Soft rot Enterobacteriaceae (SRE), potato (*Solanum tuberosum* L.), biocontrol, antagonistic bacteria, lytic bacteriophages

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N-acyl-homoserine lactones play an important role in the biological activities of the endofungal bacterium *Rhizobium radiobacter* RrF4

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Abstract: The Alphaproteobacterium *Rhizobium radiobacter* F4 (*Rr*F4) was originally isolated from the plant growth-promoting basidiomycete fungus *Piriformospora indica* (syn. *Serendipita indica*) that forms a tripartite Sebacinalean symbiosis with a broad range of host plants. Interestingly, the isolated bacterium showed biological activities widely comparable to those exhibited by *P. indica*, but the mechanism by which these are achieved is not fully understood. Chemical analysis showed that *Rr*F4 produces a spectrum of different N-acyl-homoserine lactones (AHLs) with acyl chains of C8, C10, and C12 and hydroxyl- or oxo-substitutions at the C3 position. To assess the impact of *Rr*F4-produced AHLs on its beneficial activities, the AHL-depleted mutant *Rr*F4NM13 was generated. Our results showed a reduction of wheat biomass in *Rr*F4NM13-treated plants compared to plants treated with *Rr*F4 wild-type. Furthermore, the systemic resistance to *Xanthomonas translucens* pv. *translucens* (*Xtt*) was reduced in *Rr*F4NM13- treated plants compared to control plants treated by *Rr*F4 wild-type. In accordance with these results, quantitative PCR analysis showed that the colonization of wheat roots by *Rr*F4NM13 was reduced at different time points compared with *Rr*F4. Significantly, growth promotion mediated by *Rr*F4 was greatly reduced in *Rr*F4NM13-treated *Arabidopsis* plants. Remarkably, *Rr*F4NM13-treated *Arabidopsis* plants showed a compromised induced systemic resistance (ISR) phenotype upon infection with the bacterial pathogen *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst*) as compared to *Rr*F4. Together, our results show that AHLs are required for inducing the full biological activities conferred by *Rr*F4 on host plants.

Key words: *Rhizobium radiobacter* F4, N-acyl-homoserine lactones, bacterial pathogens

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Efficacy of postharvest treatments by nebulisation of biological control organisms against *Botrytis cinerea* fruit rot on pear

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Abstract: Storage diseases of pome fruits are caused by different fungal species. Disease management to control storage diseases includes several treatments with different fungicides in the weeks prior to harvest. However, residues on fruits become more and more a public and governmental concern. In order to reduce the chemical residue on fruits to a minimum, more research is done on alternative disease management. In this respect, in 2013, a project on 'Nebulisation of biological control organisms in cold storage rooms to control storage diseases', which is funded by Flanders Innovation & Entrepreneurship (vlaio), has started at the pcfruit institute in collaboration with ILVO and the Catholic University of Leuven. Here the efficacy of several biological control organisms (BCOs), which were applied through specific atomization in the cold storage room, was examined against storage diseases. Two groups of fungal pathogens causing storage diseases were monitored. The first comprises the latent fruit rot pathogen, *Neofabraea* spp. The second are the wound pathogens (*Botrytis cinerea*, *Monilinia* spp., *Penicillium* spp.) that penetrate the fruits through accidental wounds, for example during picking. However, not only the efficacy of the BCOs is important but also the homogeneous distribution of the compounds in the cold storage room. The results of these trials will be presented.

Key words: biological control organisms, storage diseases, nebulization

Introduction

Different methods for the control of storage diseases on apple and pear are available for the fruit growers. In general, specific fungicides are applied in the orchard during the last weeks pre-harvest, with the last treatment as close as possible to harvest, depending on the pre-harvest interval of the product. In addition or besides that, postharvest treatments can be executed.

The three registered methods in Belgium are: dipping/showering of pears with Philabuster (a.i. imazalil and pyrimethanil), thermonebulisation of apples with Xedathane-A (a.i. pyrimethanil) or the recently registered dipping of pears/apples in Penbotec (a.i. pyrimethanil). Such postharvest treatments are considered as an alternative for the fungicide treatments during the last weeks before harvest.

However, in practice sometimes longer pre-harvest intervals need to be applied to meet the extra legal residue requirements (max 4 residues or max. 1/3 of MRL's) imposed by retailers. Postharvest control of storage diseases with BCOs can offer an alternative and opens perspectives for a further integrated production.

In the past several yeasts/fungi were selected for the control of storage diseases by pre harvest applications: *Aureobasidium pullulans*, *Metshnikowia fructicola*, *Candida oleophila* and *Bacillus subtilis* (Kurtzman & Droby, 2001; Marrone, 2002; Stockwell & Stack, 2007). These BCOs are active against the major storage pathogens *Botrytis cinerea*, *Penicillium expansum* and *Neofabraea* spp. which cause respectively grey mold, blue mold and Bull's eye rot on apple.

Together with ILVO and KU Leuven, pcfruit started up a research project, which is funded by vlaio. The general objective of this research project is to develop a suitable technique for post-harvest treatments with BCOs during cold storage by cold fogging or vaporization of the product. Here, the results of a trial with natural infestation on pear are presented.

Material and methods

Biological control organisms

The BCOs used in the trial were BCO X4 and BCO X5, at a concentration 20.0 g/l for BCO X4 and 9.0 g/l for BCO X5. The BCOs are under code, because the origin is only known by the producing firm.

Recovery tests

During the trial recovery test were carried out, by taking samples of the fruits after they were treated. Each fruit was placed in a jar with 200 ml 0.1% Tween solution and shaken during 30 minutes at 130 rpm. Afterwards a concentration series was plated on PDA-medium and SAB-medium and incubated at 25 °C. After 2 days the colonies were counted and recalculated to Colony Forming Units/cm² (CFUs/cm²).

Efficacy of postharvest BCO treatments towards fruit rot on pear

For these tests pear fruits (1 bin of 480 kg fruits/object) of the cultivar Conference were used. The different strategies tested were: 1) Untreated control; 2) Water control: a nebulisation with only clear water; 3) A preharvest treatment with Geoxe (a.i. fludioxonil, 0.250 kg/ha LWA) combined with a postharvest BCO X4 nebulisation treatment; 4) A preharvest treatment with Geoxe (a.i. fludioxonil, 0.250 kg/ha LWA) combined with a postharvest BCO X5 nebulisation treatment; 5) A postharvest BCO X4 nebulisation treatment; 6) A postharvest BCO X5 nebulisation treatment. This trial was executed in a small cooling room (80 m³) at 16/09/16, the day after harvest. Each bin was placed individually in the centre of the cooling room. The nebulisation was performed by using the nebulisation-device 'Swingtec Fontan Starlet'. For each treatment an amount of 1 litre solution (neither water of a suspension of the respective BCO) was used for the nebulisation process. After each nebulisation the bin was left for 2 hours inside the cooling room. From each object samples were taken from the upper layer of the bin for recovery tests of the BCOs, 1 day after the treatment. After storage fruit rot evaluations were carried out per layer (4 layers in each bin, numbered 1 till 4 from top of the bin to the bottom of the bin). At the moments of evaluation a recovery analysis was performed on fruits from each layer in the bins.

Results

Efficacy of postharvest BCO treatments towards Botrytis fruit rot on pear

Each pear was evaluated after 6 months of storage (1st evaluation: 02/03/16). Figure 1 shows the results for the first evalation. The colored bars display the efficacy.

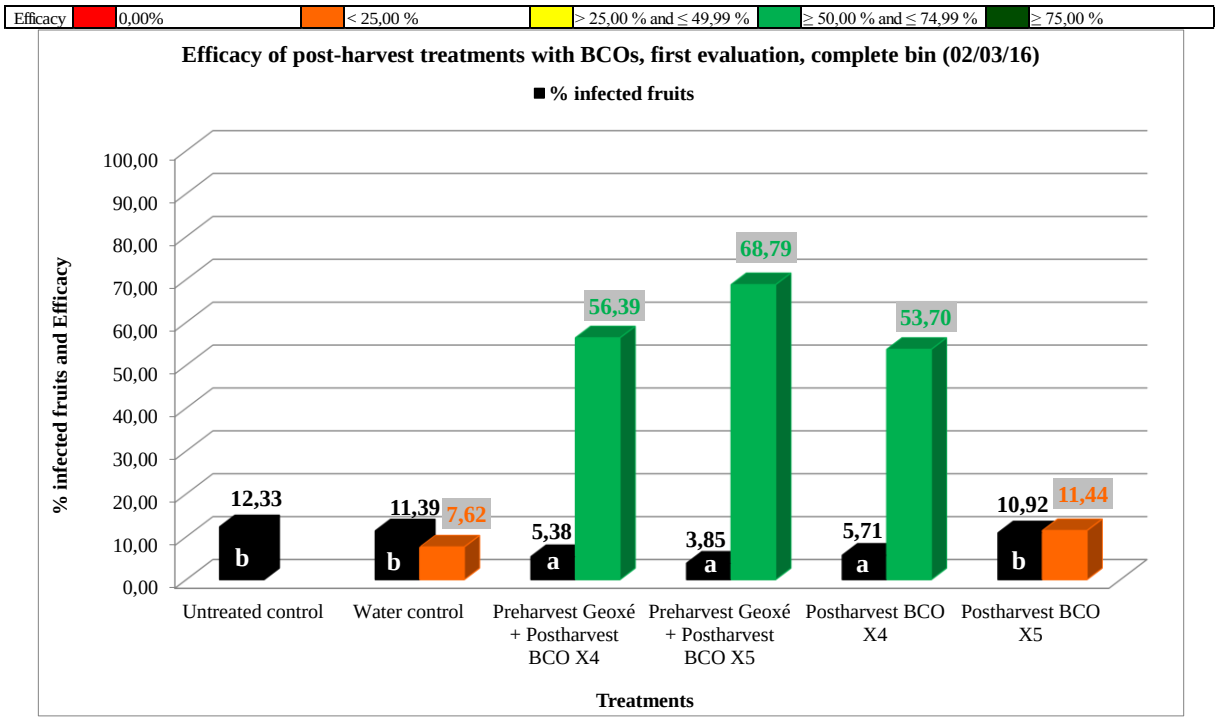

Figure 1. Efficacy of postharvest treatments with BCOs. Legend: Black bars: % infestation with *Botrytis*. Coloured bars: % efficacy.

In the untreated control 1.33% infected fruits were present (Figure 1). The water control had no influence on the amount infected fruits (11.39%). The same tendency is seen for the postharvest treatment with BCO X5, were 10.92% of the fruits were infected and only an efficacy of 11.44% was obtained. This treatment was not significantly different from the water control. The best result was achieved for the preharvest Geoxe treatment combined with the postharvest treatment of BCO X5, with only 3.85% infected fruits leading to an efficacy of 68.79%. But also the preharvest Geoxe treatment combined with the postharvest treatment of BCO X4 had simular results, with only 5.38% infected fruits and an efficacy of 56.39%. A single postharvest treatment of BCO X4 alone gave also very promising results with only 5.71% infected fruits, resulting in an efficacy of 53.70%. All three applications where significantly different form the water control.

Differences in the amount of infected fruits and obtained efficacy was also seen when the different layers in the bins were taken into account. The upper layer (layer 1) had always the least amount of infected fruits resulting in a higher efficacy than the layers more down in the bin (Figure 2).

For the strategy where the preharvest Geoxe treatment was combined with a postharvest BCO X4 treatment the best results were achieved for layer 1 (73.21% efficacy and 5.08×10^3

CFUs/cm²) and layer 3 (56.44% efficacy and 3.72 × 10² CFUs/cm²). The same trend is seen for the strategy with only a postharvest BCO X4 treatment. With also the 1st and 3rd layer having the best efficacy and recovered CFUs/cm². With BCO X5 lower efficacies were obtained. The combination with a preharvest Geoxe treatment led to higher efficacies as the strategy where only a postharvest treatment with BCO X5 was performed.

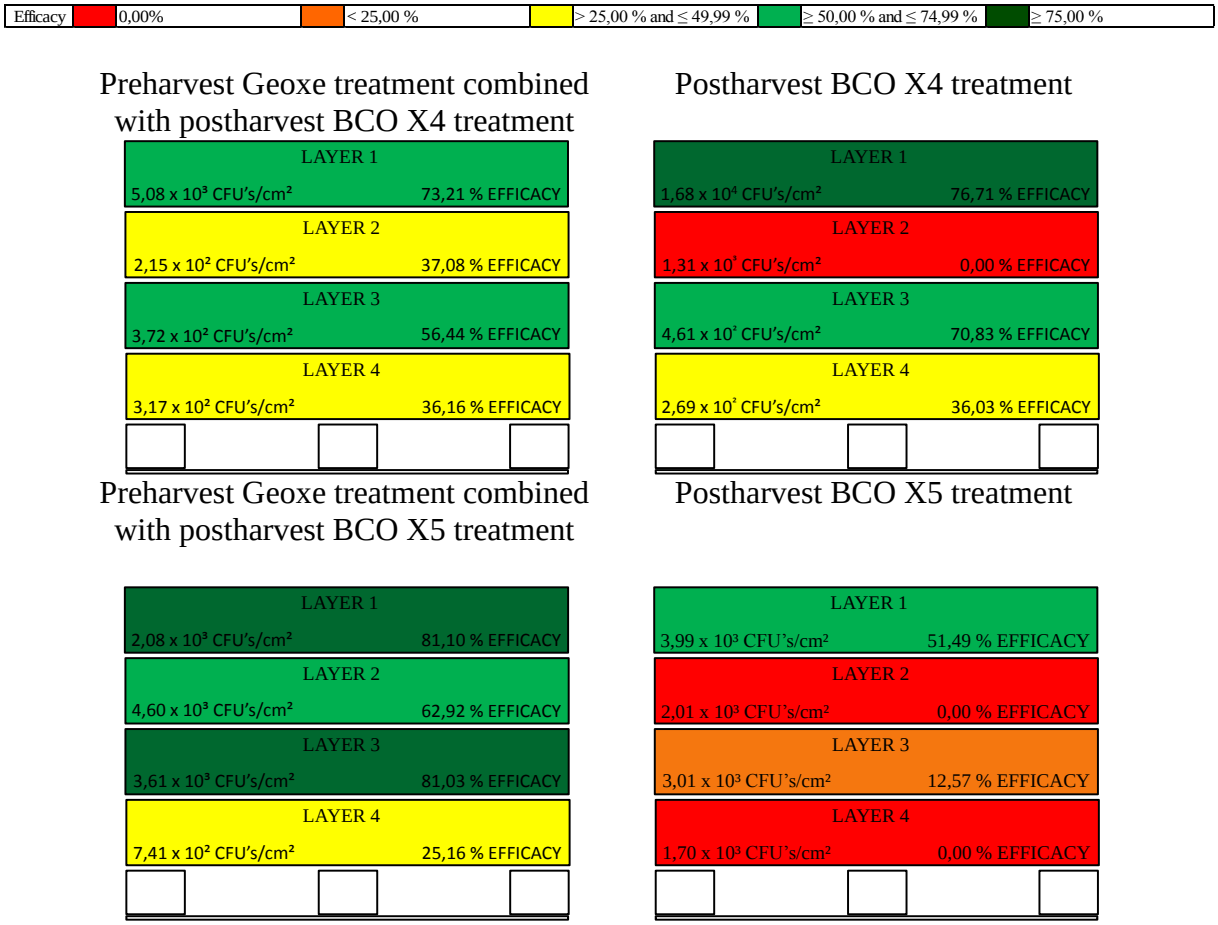

Figure 2. Recovered CFU's/cm² and efficacies per applied treatment schedule and per layer in the bin.

Discussion

Based on the results obtained it can be stated that BCOs can be applied by nebulisation in the cold storage room to prevent postharvest diseases. It is shown that there is a correlation between the number of CFU's and the obtained efficacies. However, research is still necessary to obtain a homogenous distribution in a cold storage room filled with bins.

Acknowledgements

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Control of *Plasmodiophora brassicae* by combining antagonists, organic amendments, and cultivation practices

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Abstract: *Plasmodiophora brassicae* is a common threat to vegetables of the Brassicaceae family. Recent research has shown that *Trichoderma* ssp. are potential antagonists of *P. brassicae*. Furthermore, ridge cultivation and the use of chitin-based fertilizers are potentially able to reduce the disease. In order to control the disease different control measures and their combination were evaluated in a pot and a field experiment. In a first step different biocontrol agents (*Trichoderma* spp., and *Bacillus* spp.) were tested alone and in combination with Biosol (chitin) in a pot experiment in the greenhouse using naturally clubroot infested soil. In this experiment, a synergistic effect between antagonists and chitin-based fertilizer was observed. Of the best performing biocontrol strain, one strain was selected for the field experiment. The field experiment was conducted in a naturally heavily clubroot infested soil. The application of antagonists, application of a chitin-based fertilizer, and the cultivation on ridges were tested alone and in all possible combinations. All control measures reduced the disease, where ridge cultivation had the most disease suppressive effects. Additional disease suppression by combined measures was observed. To control clubroot it is worthwhile to combine different preventive control measures.

Key words: *Plasmodiophora brassicae*, *Trichoderma* spp., cultivation practice

Introduction

Many vegetable crops belong to the Brassicaceae family. One of the most important disease of vegetables of this family is the soilborne pathogen *Plasmodiophora brassicae* (clubroot). Symptoms are yellowing, wilting, formation of galls on the roots, until death of the plant. Resting spores of *P. brassicae* can survive for a long time in the soil. Control measures are at the moment very limited as chemical control is not possible.

Trichoderma and *Bacillus* species are well-known biocontrol agents. Nevertheless, only limited knowledge is available about their potential to control clubroot in the field (Cheah *et al*., 2000). Beside the use of biocontrol agent, chitin-based fertilizers are known to suppress *P. brassicae* (Heller *et al*., 2007). It is assumed that chitin supports and enhances populations of chitin-degrading microorganisms. These organisms are then responsible for the disease suppressing effect of these fertilizers. Another possibilty to control clubroot is the cultivation on small ridges in order the reduce soil wetness. Often the biocontrol activity of antagonists can vary to a large extent. Combination of different preventive control measures potentially circumvent this constraint. Therefore our investigations aimed at reducing clubroot infestation and enhancing plant growth by combining the application of antagonist, the use of a chitin-based fertilzer and the cultivation on small ridges.

Material and methods

Pot experiment

In the pot experiment, four commercially available products were used, from the genus *Trichoderma* «Trianum-P» and «Prestop» and from the genus *Bacillus* «Rhizovital» and «B.a. 42». All microorganisms were applied according to the manufactures recommandations to the plantlets two days before transferring the plants to the pots. The soil used originated from a field very heavily infected with *P. brassicae*. As chitin-based fertilizer «Biosol» at a rate corresponding to 300 g/m^2 was applied to the soil before potting. A susceptible chinese cabbage cultivar was used. Plants were grown for 4 months in the greenhouse. At the end of the experiment plant growth parameters were evaluated. Each treatment consisted of 6 replicates.

Field experiment

The field experiment was performed in 2015 on a very heavily infested field. Prior to the establishment of the experiment the spatial distribution of the disease was evaluated using a chinese cabbage crop. A susceptible broccoli cultivar was chosen for the experiment. As biocontrol agent a *Trichoderma* strain (Trianum-P) was used. *Trichoderma* was applied at a rate of 1.5 g/m^2 to the plantlets two days prior to planting them into the field. The chitin-based fertilizer Biosol was applied at a rate of 300 g/m^2. The ridges were about 10-15 cm high. After two months plant growth parameters were measured. The roots were evaluated for clubroot infestation by using a scale from 1-5. From each plot 10 plants were evaluated. All treatments consisted of four replicates.

Results and discussion

Pot experiment

All antagonists were able to significantly enhance plant weight, but differed in the extent. The application of Trianum-P led to an increase in shoot fresh weight from 82 g (control) to 278 g. When the Biosol was added to the soil, shoot weight was doubled. The combined application of Biosol and antagonists had an additional effect on plant growth. A similar pattern was found concerning root weight. For both parameters the combination of Biosol and Trianum-P showed the best effects (Figure 1).

The different biocontrol strains had not the same effect on plant growth, indicating the need of testing different biocontrol agents for specific purposes. Concerning shoot weight this differences were equalised by the co-application of a chitin-based fertilizer. Therefore combining different measures of control might balance positive effects of biocontrol organisms under different conditions.

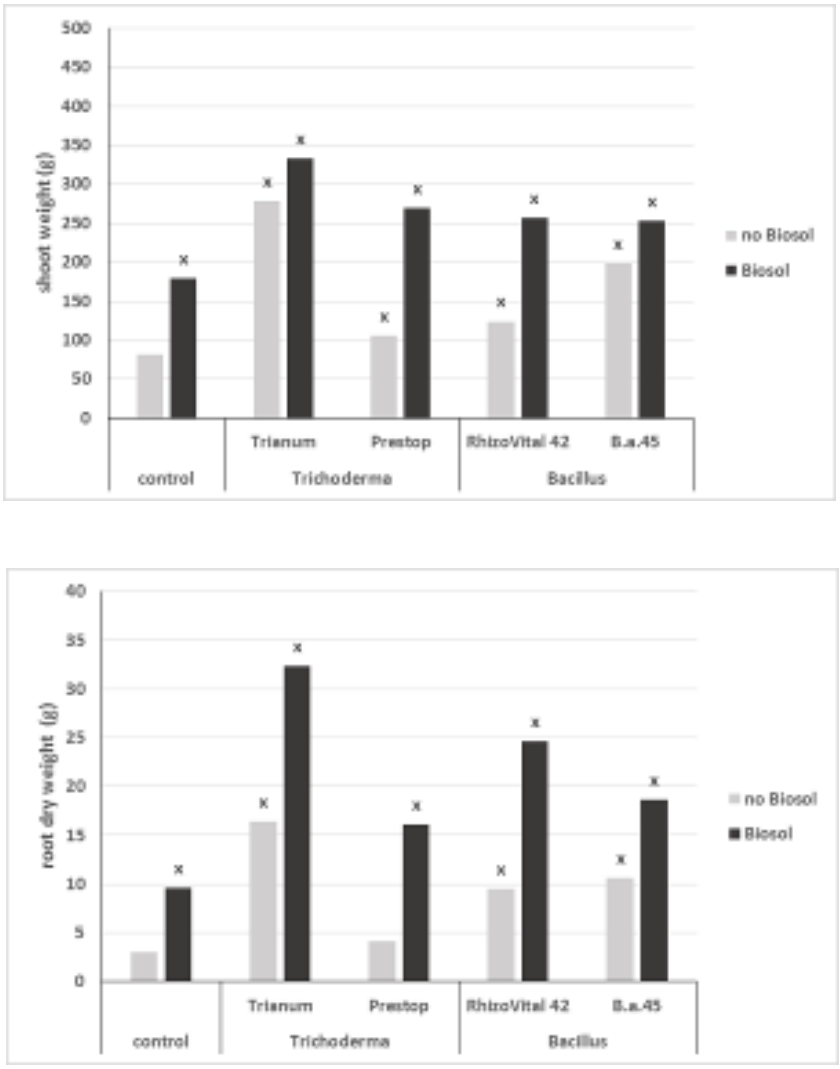

Figure 1. Influence of antagonists on shoot fresh weight (left) and root dry weight (right) of Chinese cabbage grown in a *P. brassicae* contaminated soil. Bars represent the mean of six replicates. x = significantly different to the untreated control (Fisher's LSD $P \leq 0.05$).

Field trial

Plant weight of broccoli was significantly enhanced when the plantlets were inoculated with *Trichoderma* and the broccoli was cultivated on a ridge in comparison to the untreated plants (Figure 2). All treatments reduced incidence of clubroot on broccoli. The highest effect was found when all factors were combined. Interestingly the increase in plant fresh weight by *Trichoderma* was more pronounced when the plants were cultivated on ridges.

In untreated control plots most roots were heavily infested with *P. brassicae* and rotten at the end of the experiment. Most treatments were able to significantly reduce clubroot infestation. Cultivation on ridges showed the most pronounced effects. The healthiest plant were observed when all measures of control were combined (Figure 2).

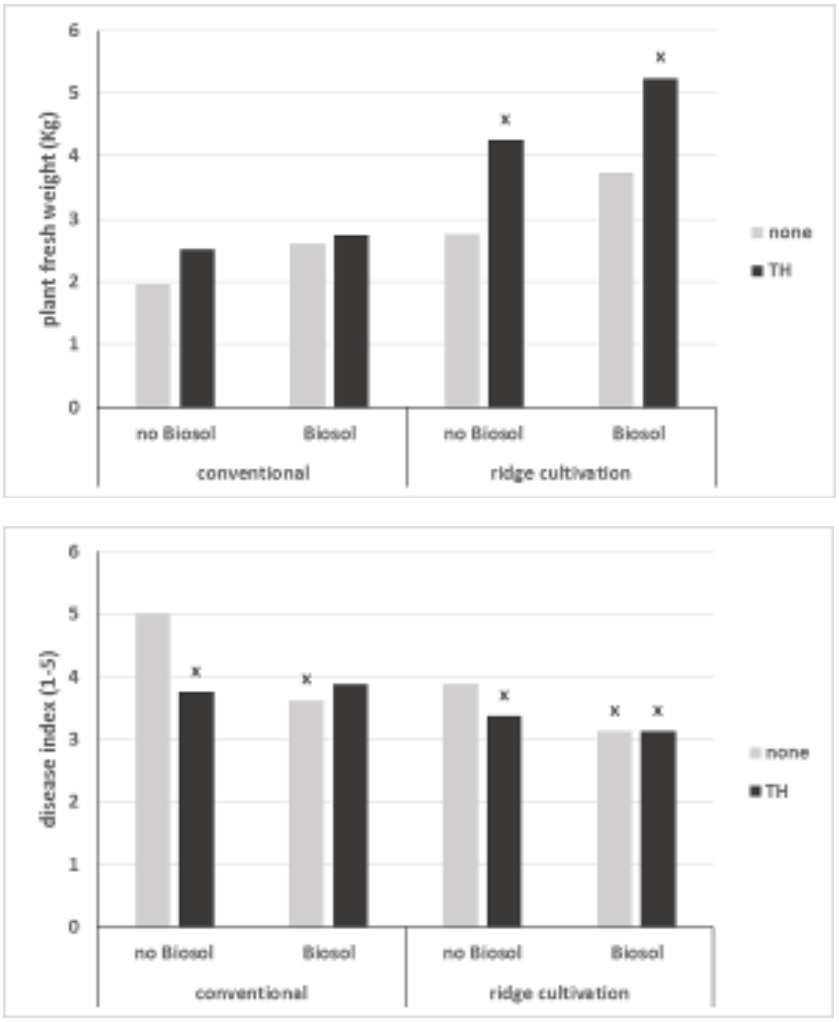

Figure 2. Influence of *Trichoderma* (TH), the amendment of the chitin-based fertilizer Biosol, as well as the cultivation on ridges on plant weight (left) of broccoli and the infestation with *P. brassicae* (right) in a heavily infested soil. Bars represent the mean of 10 plants in four replicates. x = significantly different to the untreated control (Fisher's LSD $P \leq 0.05$).

In contrast to the pot experiments, fertilisation with Biosol did not enhance plant fresh weight when the plants were cultivated conventionally, but had a positive effect when broccoli was cultivated on ridges. The mechanisms behind this observation are still unclear.

All tested control measures were able to reduce disease severity to a similar extent in the field, but combinations had in most cases an additional beneficial effect. This opens novel opportunities for the further development of biocontrol strategies for clubroot disease control. Combining antagonists with specific cultural practices and/or organic fertilizers might stabilise and increase biocontrol activity of the applied beneficial microorganisms.

This year further experiments will be conducted in order to support these findings Furthermore, some microbial populations such as e.g. *Trichoderma* will be determined to understand better the mechanisms behind the observations made. Additionally in a long-term field experiment it will be evaluated if the combination of these control measures will delay the increase of *P. brassicae* populations in a very narrow crop rotation.

Overall we conclude that the combination of antagonists, organic amendments, and cultivation practices will enable growers to produce Brassicaceae vegetable crops also in heavily clubroot infested soils.

Acknowledgements

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Search for microorganisms which can disrupt communication between plant pathogenic bacteria causing hairy roots disease in greenhouse vegetables

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Abstract: Hairy roots disease is an important problem in cultivation of greenhouse vegetables (tomato, aubergine and cucumber). Infection is caused by rhizogenic bacteria from *Agrobacterium*/*Rhizobium* group. It has been shown that infection process is regulated by environmental factors and quorum sensing mechanism. Rhizogenic bacteria produce acyl homoserine lactones (AHL) as signal molecules, which enable cell-to-cell communication. Disruption of this communication process will most likely lead to lower infection and symptoms. There are several possible routes of quorum quenching. One of them is degradation of pathogenic bacteria's signal molecules by other microorganisms present in the root environment. Bacteria capable of AHL degradation were previously isolated from natural environment such as soil and water. In this research AHL degrading bacteria were successfully isolated from greenhouse growing substrate rockwool. Application of these micro-organisms in greenhouse practice is also discussed.

Key words: hairy roots, quorum quenching, lactonase

Introduction

Bacterial infection of plant roots, resulting in hairy roots, is a persisting problem for many greenhouse crops, in particular tomato, cucumber and aubergine, in the Netherlands and Belgium. Presently, no curative measures are available to inhibit disease progression once the plant has been infected. Unfortunately, even strict hygiene measures give no full guarantee that the symptoms will not occur. Therefore an alternative prevention strategy is needed.

Rhizogenic bacteria are gram negative bacteria, which produce acyl homoserine lactones (AHL), which enable "communication" between bacterial cells, termed quorum sensing (Whitehead *et al*., 2001). In rhizogenic bacteria, and closely related crown gall bacteria, infection process is controlled by environmental factors (such as pH, production of phenolic compounds by plant) and quorum sensing (Haudecoeur *et al*., 2010). Virulence genes, which are located on a root-inducing (Ri) or tumor-inducing (Ti) plasmids, are only expressed after a threshold concentration of AHL's is reached (Lang & Faure, 2014).

In recent years strategy of quorum quenching (disruption of quorum sensing) has gained more attention, due to increasing resistance to antibiotics among bacteria. There are several possible routes for quorum quenching. In this reach we focus on possibility of quorum quenching by plant pathogen's AHL degradation or inactivation by microorganisms present in the root environment. Some microorganisms produce enzymes capable of AHL degradation

(Fetzner, 2015). It is possible that these microorganisms play an important role in prevention of bacterial diseases of plants, such as hairy roots. These bacteria were previously isolated from soil and water.

Material and methods

Rhizogenic bacterial strains
Rhizogenic bacterial strains used in this study were: *Rhizobium radiobacter* (strain NCPPB 4043, isolated from cucumber) and an isolate WUR1 from diseased tomato plants obtained in Wageningen UR Greenhouse Horticulture laboratory.

Production of acyl homoserine lactones (AHL) by rhizogenic bacteria
To assess if isolated strains of rhizogenic bacteria produce acyl homoserine lactones (AHL) signal molecules an indirect method was used with AHL biosensor strain *Agrobacterium tumefaciens* NTL4 (pCF218) (pCF372) (Kawaguchi *et al*., 2008).

Isolation of bacteria from rockwool mats
Bacteria were isolated from used rockwool mats (after tomato cultivation for 6 months) Rockwool samples of 1g were taken aseptically. They were placed in 100ml of sterile phosphate buffer and incubated on orbital shaker for 30 minutes (at 125 rpm) to allow bacteria to dislodge from rockwool surface. Aliquots of resulting solution were plated on nutrient agar. Growing colonies were subsequently transferred onto an agar plates to obtain pure isolates.

Detection of lactonase genes in bacterial isolates from rockwool
Fifty isolates from rockwool were tested for presence of genes encoding the lactone degrading enzymes (genes aiiA, qsdA and qsdB). Bacterial genomic DNA was isolated using from 24h old cultures using DNeasy Blood and Tissue kit (Qiagen). Primer sets and PCR protocol proposed by Yin *et al*. (2010) and Tannieres *et al*. (2013) were used. Isolates possesing lactonase genes were further identified on basis of 16S rDNA gene sequence.

Potential for acyl homoserine lactones degradation by bacterial isolates from rockwool
To determine if bacterial isolates from rockwool are capable of degrading AHL's we performed the tests (in triplicate) with AHL biosensor strain NTL4 according to protocol described by Jafra & van der Wolf (2004). Acyl homoserine lactones produced by rhizogenic bacteria and N-3-oxo-octanoyl-L-Homoserine lactone (3-oxo-C8-HSL) (Sigma Aldrich) were used as substrates for biochemical tests of AHL degradation.

Results and discussion

Production of AHL's by rhizogenic isolates
Both tested rhizogenic isolates (NCPPB 4043 and WUR1) are producing the acyl homoserine lactones (Figure 1).

Figure 1. Production of AHL by rhizogenic isolates as determined by indirect method of cross streaking with AHL biosensor *A. tumefaciens* NTL4. Production of blue dye by strain NTL4 is an indication of the AHL presence in the medium.

Lactonase genes presence and AHL degradation by bacterial isolates from rockwool

In DNA of seven rockwool isolates aiiA gene was detected. No isolate tested positive for qsdA or qsdB gene. Results of PCR reaction amplifying aiiA gene are shown in Figure 2. No PCR products were detected for qsdA and qsdB genes.

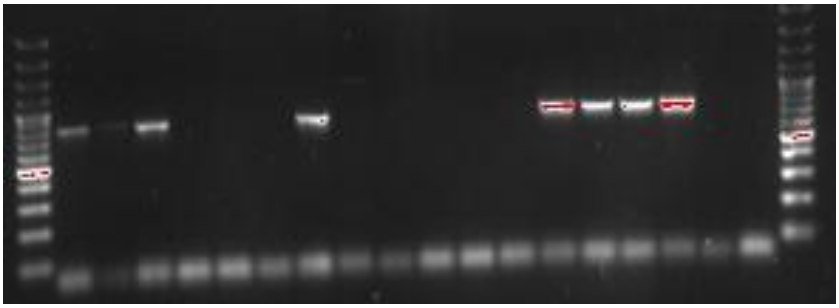

Figure 2. Products of aiiA gene amplification from DNA of rockwool isolates.

Four isolates (ST1, ST2, ST12 and ST14) were used in biochemical tests to determine their ability to degrade or inactivate the AHL's produced by strain *A. radiobacter* NCPPB4043 and rhizogenic strain WUR1. Degradation or inactivation of pure N-3-oxo-octanoyl-L-homoserine lactone (3-oxo-C8-HSL) was also tested. An example of the results is shown in Figure 3. Blue dye production is an indication that AHL substrate was not degraded by tested microorganisms. Isolates ST1, ST2, ST12 and ST14 were capable of complete degradation/inactivation of all three AHL substrates (3-oxo-C8-HSL and mixes of AHL produced by rhizogenic isolates NCPPB4043 and WUR1). Additionally we tested other three isolates (ST7, ST8 and ST9) if they can degrade or inhibit AHL's in biochemical test, but possibly possess other AHL degrading enzymes that the ones for which we have tested.

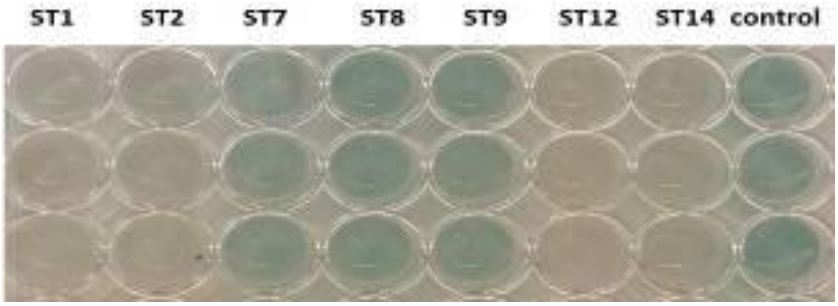

Figure 3. Indirect test of AHL degradation by isolates from rockwool with AHL biosensor strain *A. tumefaciens* NTL4 (substrate N-3-oxo-octanoyl-L-homoserine lactone)

Results show that there are microorganisms present in used rockwool mats (from tomato cultivation), which are capable of degradation/inactivation of signal molecules (AHL's) used by rhizogenic plant pathogenic bacteria as means of communication. Microorganisms with this ability were also successfully isolated from other environments, such as soil (e.g. Jafra & van der Wolf, 2004; Fetzner, 2015). Their application as potential biocontrol agents was implemented against pathogens such as *Pectobacterium carotovorum* in potato (Jafra & van der Wolf, 2004). Isolates obtained from rockwool represent *Bacillus* spp. This is of an importance because representatives of this bacterial genus are capable of surviving adverse environmental conditions by forming endospores. Fetzner (2015) noted that this quality makes AHL degrading isolates attractive candidates for agricultural applications, because they would presumably be able to survive in soil or substrate for longer time.

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Are *Sphagnum*-species potential antagonists of pathogens?

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Abstract: Bryophytes (nonvascular plants such as mosses and ferns) are considered as promising sources of antibiotics and biologically active compounds in nature. Mosses, especially the *Sphagnum* species, constitute a large part of photosynthesizing biomass in Northern Hemisphere and they serve as bioeconomically important but rarely used resource. The widespread *Sphagnum magellanicum* was used as a model species to investigate antioxidative capacity and antagonistic properties of *Sphagnum* moss. Two different bacterial biosensor strains *(S. aureus* 8325-4/pAT19-luxABCDE-hlaPr-frp and *E. coli* K-12/pCGLS-1) were used to evaluate the effect of alive *S. magellanicum* on growth of bacteria. Furthermore, extracellular peroxidase was shown to be activated with chitosan treatment (fungal cell wall component). The results indicated antagonistic potential of *Sphagnum* against bacteria and fungi.

Key words: *Sphagnum magellanicum*, biosensors, peroxidase, antioxidative capacity

Introduction

Antibacterial and antifungal properties of *Sphagnum* species are well known but the mechanism needs to be evaluated. Already native Indian and Maya cultures used *Sphagnum* sp. in their natural medicine (Ando & Matsuo, 1984). *Sphagnum* sp. contain several phenolic compounds which are part of the defense mechanism (Montenegro *et al.*, 2009). Naturally low pH value avoids growth of acid sensitive bacteria, such as several food spoiling bacteria (Borsheim *et al*., 2001). Cell wall structure of *Sphagnum* contains sphagnan which is a weak acid and might be a reason for the antibacterial property of mosses (Stalheim *et al*., 2009). Moreover, in vascular plants one of the defense responses is activation of peroxidases which can be elicited with fungal cell wall constituents such as chitin ($C_8H_{13}O_5$). Chitosan (a residue of chitin) treatment has been shown to cause a rapid increase in peroxidase activity in the culture medium of *Physcomitrella patens* indicating that peroxidases have antifungal activity also in mosses (Lehtonen *et al.*, 2009).

Antifungal microorganisms form part of the defense of the plant against fungal pathogens (Weller *et al*., 2002). *Sphagnum* sp. form acid (pH 3.5-5.0), nutrient poor, wet carpets which are an extreme habitat for micro-organisms. *Sphagnum* carpets are colonized by diverse bryophilous ascomycetes but no substantial fungal diseases are known (Döbbler, 1997). *Sphagnum* mosses harbor antifungal bacteria that take part in the pathogen defense (Bragina *et al*., 2012). For instance, the moss *Sphagnum rubellum* Wils. is colonized by specific bacterial communities with an high proportion of antagonistic species (Opelt *et al*., 2007).

The antagonistic potential of *Sphagnum* sp. and associated bacteria are important for biotechnological applications, i.e. biological control of plant pathogens or the isolation of bioactive compounds.

The aim of the present study was to evaluate the bioactive properties of a widespread model species *Sphagnum magellanicum*. Antioxidativity tests, FRAP and ORAC, proved that *S. magellanicum* contain antioxidative capacity especially in methanol extraction with high phenol content. Furthermore, peroxidase activity of *S. magellanicum* was assessed and shown that peroxidase activity can be induced by fungal cell wall component (chitosan).

By using bacterial biosensor strains (*S. aureus* 8325-4/pAT19-luxABCDE-hlaPr-frp and *E. coli* K-12/pCGLS-1) we investigated the efficiency of *S. magellanicum* on metabolism of bacteria. Genetically engineered bacterial cells performed an inhibitory zone, measured based on bioluminescence using in vitro imaging station (IVIS) (Mannisto *et al*., 2014). There are no or few studies that have examined the diffusion of antibiotic substances from living moss materials in real-time on bacterial plates. The results of the present study suggest high potential of *Sphagnum* sp. for biotechnological applications.

Material and methods

S. magellanicum moss mass (Parkano, Finland) was extracted by using various solvents (methanol, water and acetone). Antioxidant activity of the different extractions were measured with FRAP (ferric reducing antioxidant power) and ORAC (Oxygen Radical Absorbance Capacity) methods (Apak *et al*., 2013). The total phenolic content was determined by Folin-Ciocalteau method (Viitala *et al*., 2011).

In FRAP test the extracts (150 µl) were allowed to react with 2850 µl of the FRAP solution for 30 min in the dark. Readings of the colored product [ferrous tripyridyltriazine complex] were then taken at 593 nm.

In ORAC test peroxyl radical was generated using 2,2'-azobis (2-amidino-propane) dihydrochloride which was prepared fresh for each run. Fluorescein was used as the substrate. Fluorescence conditions were as follows: excitation at 485 nm and emission at 520 nm. The standard curve was linear between 0 and 50 µM Trolox. Different reaction mixtures were analyzed by Thermo Scientific Varioskan Flash multimode reader in 96-well-microplates.

Extracellular peroxidase activity measurement based on oxidation of 2,2′-azino-bis3-ethylbenzthiazoline-6-sulphonic acid (ABTS) according to Lehtonen *et al*. (2009) with some modifications. Mosses (1 g FW) were purified with deionized water and cut into pieces. 1 ml chitosan (1 g/100 ml, pH 4) (Sigma) was pipetted on the samples on petri dishes (controls contained 1 ml water) and incubated 1 h, 2 h and 24 h. After chitosan treatments, 1 ml of deionized water was added and the samples were centrifuged at 11000 rpm for 3 min. The peroxidase reaction mixture (volume 1 ml) contained phosphate-citrate buffer (phosphate 29 mM, citrate 23 mM), ABTS (2,2′-azino-bis3-ethylbenzthiazoline-6-sulphonic acid, 0,5 mM) and H_2O_2 (0.5 µM). Mixture was pre-warmed at 35 °C and H_2O_2 was added just before the measurement. Peroxidase activity was based on oxidation of ABTS as katals (mol/s) per 1 g of FW. Absorbance of the reaction buffer (ABTS and H_2O_2) and supernatant mixture was recorded at 0, 15 s, 30 s, 60 s, 90 s, and 120 s intervals in wavelength 405 nm.

To follow the antimicrobial activity of the *S. magellanicum* and its ability to release antibiotic substances against the pathogens, two continuously light producing biosensor strains were used. Bioluminescence *Escherichia coli* (K12 + pcGLS11) and *Staphylococcus aureus* (pAT19 luxABCDE-hlaP + frp) strains with luciferase gene construct were used (Tienaho *et al*., 2015). The tests were made by two layer technique on agar plates. First

12.5 ml of L-agar (15 g/l) with ambicillin was poured on the plates. Piece of the moss was settled on the hard agar and 12.5 ml of soft (48 °C) L-agar (7.5 g/l) with ampicillin and biosensor bacterias was poured over the plate with moss. The method is explained more precisely in Mannisto *et al.* (2014).

Results and discussion

S.magellanicum contains compounds with antioxidative properties (Figures 1 A-C). Methanol extractions of *S. magellanicum* had high contents of phenolic compounds and methanol extraction was active in FRAP and ORAC tests (Figures 1 A-C). Antioxidative capacity correlated with the total phenol content.

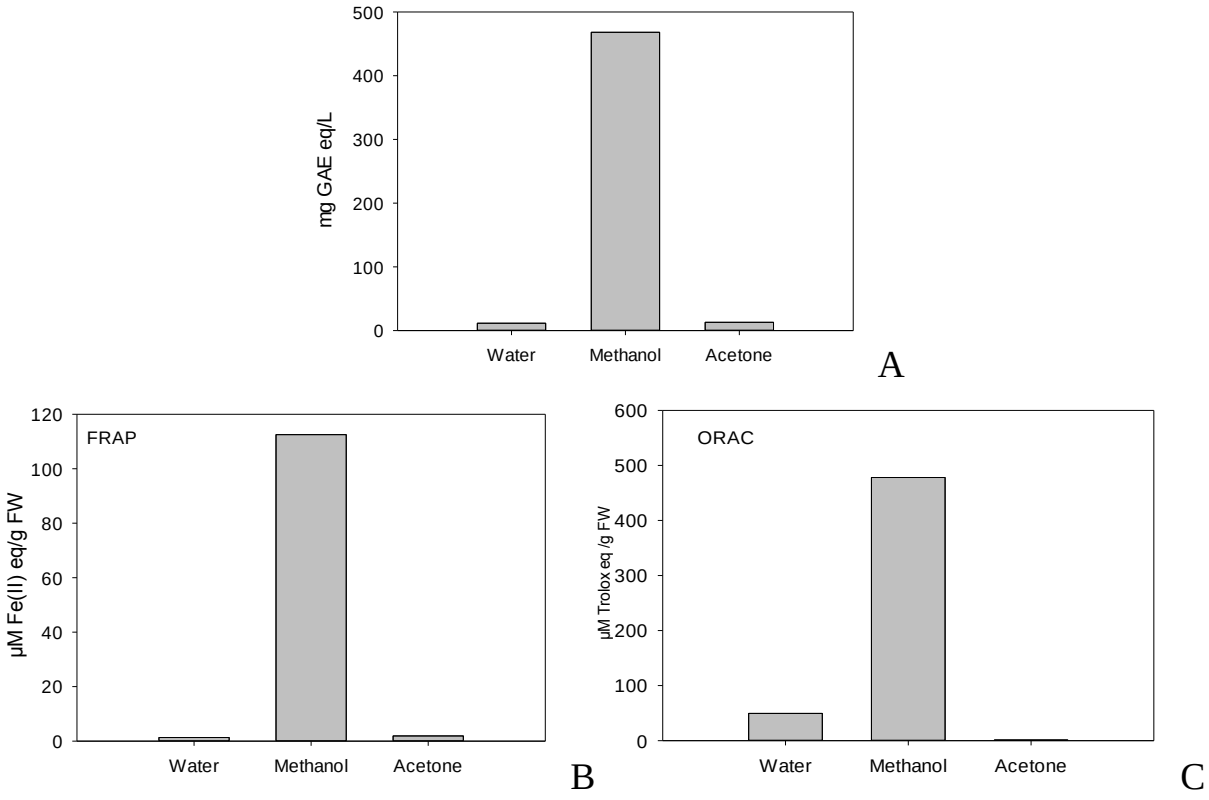

Figure 1. A. Total phenol content and antioxidative properties of *S. magellanicum* measured by B. FRAP and C. ORAC tests from water, methanol and acetone extractions.

Fast induction of peroxidase activity might be part of the defense against fungal invaders. Chitosan treatment induced fast extracellular peroxidase activity of *S. magellanicum.* Peroxidase activity was on higher level in chitosan treated medium than in control treatment one hour after the treatments (Figure 2). This indicates that fast peroxidase activity play a role in defense responses against fungal invaders in *S. magellanicum.* The phenomenon has been shown with other moss species *Physcomitrella patens* (Lehtonen *et al*., 2009).

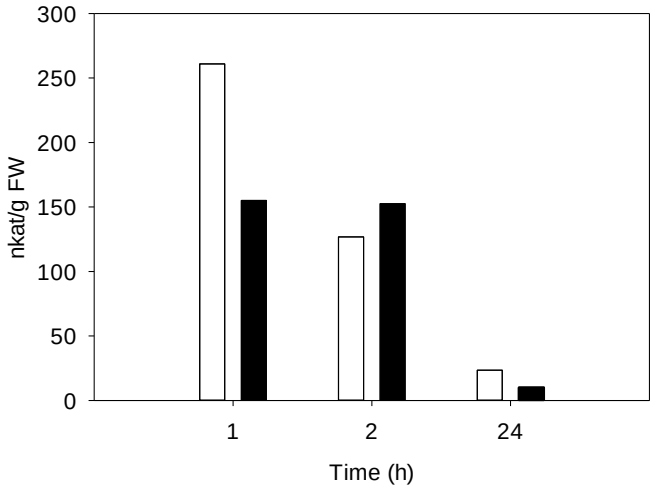

Figure 2. Extracellular peroxidase activity of *S. magellanicum* after 1, 2 and 24 hours after treatment with chitosan (open bars) and pure water (control) (black bars).

Continuously light producing biosensor strains of *E. coli* and *S. aureus* were used to identify antagonistic property of *S. magellanicum* against human pathogens. *E. coli* is a gram-negative, anaerobic bacterium that is commonly found in the lower intestine of warm-blooded organisms (endotherms). Mostly harmless *E. coli* strains can occasionally cause serious food contamination. *S. aureus* is a gram-positive coccal bacterium that is frequently found in the respiratory tract and on the skin. The emergence of antibiotic-resistant strains of *S. aureus* such as methicillin-resistant *S. aureus* (MRSA) is a worldwide problem in clinical medicine.

Both continuously light producing biosensor strains had highest light intensity strait next to the *S. magellanicum* (Figure 3). This indicated that the moss secreted substances to the growth media that affected the metabolism of bacteria. The antimicrobial effects took place kinetically during few hours after which the inhibitory and stress zones substantially increase (Mannisto *et al*., 2014). The changes can be detected by measuring light emission levels described by the changes in colors. Formation of zones usually began as appearance of a red or yellow edge around the material as the bacteria in contact with the increase of the antibiotic concentration produce strong light emission. As diffusion proceeds, the edge expands and moves by time (Mannisto *et al*., 2014). As antibiotic substances begin to affect bacterial metabolism, and whether the amount is still too small to shut down all defense mechanisms, the first reaction is seen as increase of luminescence levels, in colors of red and yellow.

In the present study appearance of red and yellow were considered to indicate bacteria being still alive, but already in contact with subinhibitory concentrations of antibiotic substances (Figure 3). Green light production was considered as unaffected bacteria. The phenomenon can be construed as bacterial "emergency shut-down" as bacteria turn off all unnecessary metabolic pathways when trying to survive antibiotic attack (Mannisto *et al*., 2014). This allows more energy in the form of ATP to be consumed by luminescence pathway, and light levels stay high for a while. Thus, *S. magellanicum* is antagonist against pathogens and has a high potential for different biotechnological applications.

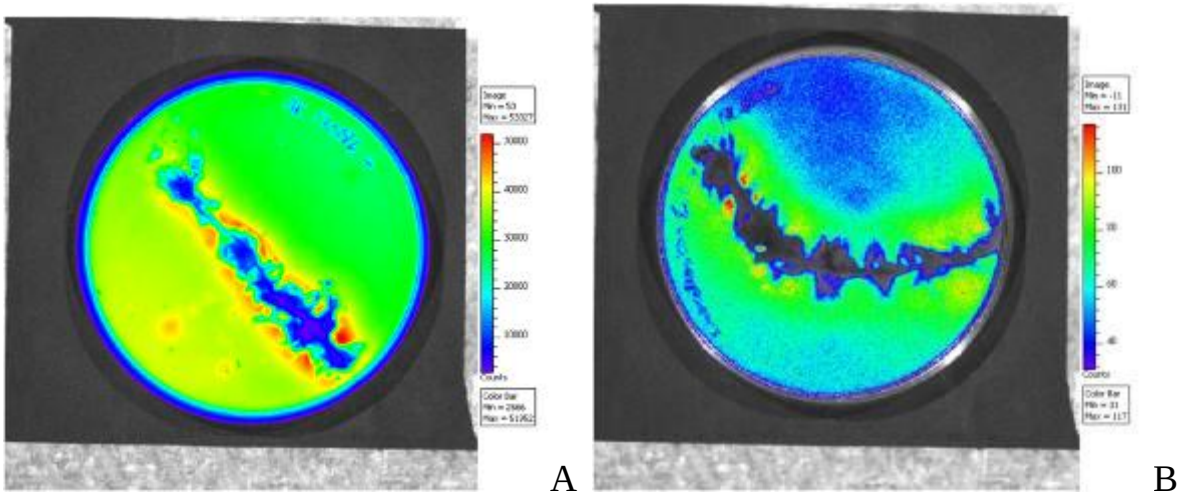

Figure 3. Continuously light producing biosensor strains a) *E. coli* K-12/pCGLS-1 and b) *S. aureus* 8325-4/pAT19-luxABCDE-hlaPr-frp with *S. magellanicum* on agar plates after over-night incubation.

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Extremophile plants as source of biopesticides against European damageable plant pathogens

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Abstract: The use of plant-derived products in postharvest disease management may be a valid alternative to conventional chemical treatments (Pane et *al.*, 2016). Unfavorable environmental conditions (such as salt and drought) increase production and accumulation of reactive oxygen species (ROS). Consequently, extremophile plants have developed adaptive responses including the synthesis of specific bioactive molecules used for medical and nutritional purposes (Ksouri et *al.*, 2012). In that context, the main objective of the present study was the identification of effective plant extracts and essential oils from extremophile plants against the most important plant pathogens in Europe (in term of loss, treatment necessity and/or cost).

The study began with the selection of four endemic medicinal species suspected to be antimicrobial due to their wealth of phenolic and terpene compounds, such as flavonoids, phenolic acids, and coumarins (Ksouri et *al.*, 2012). Each of the aerial plant materials was grounded and macerated with solvent (methanol or chloroform) for 24 h. The solvent was then eliminated along rotavapor. The yield of plant extract varied between 1.56 and 6.7%. Kinetics of growth of the 3 pathogens cultivable in liquid medium was determined before testing the impact of plant extracts and essential oils. Methanolic and chloroform plant extracts (EM1, EM2, EC1 and EC2) and essential oils (EO1, EO2) were compared for their antifungal potential. The chemical composition of the essential oils obtained by hydro-distillation from the aerial parts was analyzed by GC/MS. Yield of essential oil varied between 0.7 and 1.2%. Therefore, antifungal activity of plant extracts and essential oils was evaluated using ELISA microplates with a blocked randomized design, as described previously (Parisi et *al*., 2013).

The results obtained showed that EM2 at 7 mg/ml has a very high fungistatic activity against *Fusarium culmorum, F. oxysporum* and *Penicillium italicum.* It was characterized with a high amount of polyphenols, flavonoids and condensed tannins. Statistical analysis showed that the efficiency of methanol extracts significantly differed from those of the chloroform extracts. In addition, essential oils significantly reduced spores germination in a dose-dependent manner. Their fungistatic activity reached 100% at 6000 ppm. The GC-MS analysis showed that their major components with recognized fungicide and antimicrobial activity were 1,8-Cineole with 54.6% for *Rosmarinus officinalis* and Carvacrol with 32.87% for *Thymus algeriensis*.

In conclusion, this work allowed us to open new perspectives on the application of extremophile plant extracts as novel biocontrol strategy against plant pathogens.

Key words: essential oils, pathogens, polyphenols, fungistatic activity, biopesticides

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In vitro control of *Mycosphaerella arachidis* Deighton, the early leaf spot disease pathogen of groundnut, by extracts from six medicinal plants

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Abstract: Ground nut (*Arachis hypogaea*) is one of the most popular commercial crops in Nigeria. Its successful production has been drastically affected by early leaf spot disease caused by *Mycosphaerella arachidis* Deighton. *In vitro* control of the pathogen by six medicinal plants (*Entada africana, Vitex doniana, Lawsonia inermis, Azadirachta indica, Acalypha hispida* and *Nuaclea latifolia*) was assessed in this study. The extracts of the plants were prepared using cold and hot water and alcohol. The pathogen was isolated from ground nut infected with early leaf spot disease. The results revealed a significant difference ($P < 0.05$) in yield of extracts between cold water, hot water and alcohol extracts. A significant difference ($P < 0.05$) was observed in percentage concentrations of the various phytochemical constituents present in the extracts. Flavonoids percentage concentration was the highest (0.68-1.95%) followed by saponin (0.09-1.53%) in *N. latifolia* extracts. Steroiods had the lowest percentage concentrations (0.00-0.09%) followed by terpenoids (0.02-0.71%) and proanthocyannin (0.05-0.86%). *N. latifolia* extracts produced the highest percentage concentrations (0.07-1.95%) of all the phytochemicals followed by *A. indica* (0.05-1.64%) and lowest concentrations were obtained in *A. hispidia* (0.09-0.87%) and *V. doniana* (0.00-0.88%). The extracts inhibited spore germination and growth of *M. arachidis*. The inhibition by alcohol extracts was high and significantly different ($P > 0.05$) from cold and hot water extracts. Alcohol extract of *L. inermis* gave 100% spore germination inhibition followed by *N. latifolia* and *A.indica* with 97.75% and 85.60% inhibition, respectively. Therefore, field trials with these six medicinal plants on the control of early leaf spot disease of ground nut are recommended.

Key words: plant extract, phytochemicals, inhibition

Introduction

Groundnut *Arachis hypogaea* is one of the most popular commercial crop in Nigeria which accounted for 70% of the total Nigeria export earning between 1956 and 1967 and in 2002 the production was 23,390,000 mt. Groundnut is an important legume crop used as a food, oil and cash source, for making margarine, candy, salted groundnut, crackers/cookies, salad oils and soaps. The production started to decline from peak productions of the 1960s due to severe biotic constraints, which included diseases of fungi and viruses (Godfrey & Olorunju, 2009). Early leaf spot disease caused by the fungus *Cercospora arachidis* S. Hori teleomorph *Mycosphaerella arachidis* Deighton) is one of the major destructive diseases of groundnuts worldwide (Ogwulumba *et al*., 2008). Leaf spot diseases cause nearly complete defoliation,

30-70% loss in pod yield and reduction in the kernel quality (Reddy *et al.*, 1997). Control of *Cercospora* leaf spot (CLS) diseases in Nigeria has depended on some cultural practices, multiple applications of fungicides and development of cultivars tolerant to this disease. Effective and long-term control of leaf spot disease can be achieved by applying recommended fungicides at their recommended time intervals. However, repeated application of fungicides could lead to reduced efficacy of the fungicides due to a gradual loss of sensitivity in the target pathogen population. It could also contribute to higher production costs and environmental pollution (Ambang *et al*., 2008). Therefore, the search for alternatives to chemical products such as the use of natural biocides of plant origin is the most promising option for a safe and sustainable agriculture. Plants produce several secondary metabolite compounds with antimicrobial activity that are specific against a particular pathogen or may have a broad spectrum and can be used for control of fungal diseases in crops (Ambang *et al*., 2010; Ogwulumba *et al*., 2008; Kishore *et al*, 2001).

Therefore, the objective of this work was to study the potential of crude extracts of *E. africana, V. doniana, L. inermis, A. indica, A. hispida* and *N. latifolia* in the control of CLS epidemiology on groundnut with different levels of sensitivity. The results of this study could lead to cheap and efficient methods of plant protection against parasites and contribute to increased crop yield.

Material and methods

Collection of plant materials

Wild and locally cultivated plants of *E. africana, V. doniana, L. inermis, A. indica, A. hispida* and *N. latifolia* were collected in Lapai, Lapai Local Government in Niger State, Nigeria.

Preparation of plant extracts

The fresh leaves of the plants were washed using sterile distilled water (SDW) and were air dried at room temperature (28 ± 2 °C) for four months. The materials were separately ground in porcelain mortal and later with a blender (model MC – BL1242), sieved and stored in air tight bottles. Aqueous and ethanol extraction were made.

Isolation of fungi strain (Mycosphaerella arachidis)

Groundnut plants infected with early leaf spot were carefully identified and collected during the cropping season of 2011 from experimental farm of Agricultural Science Department, Ibrahim Badamasi Babangida University, Lapai Niger State Nigeria. The pathogen was isolated and identified using both morphological and microscopic characteristics. The pathogen was stored in slants on PDA at -4 °C.

Pathogenicity

Pathogenicity test was conducted to confirm the authenticity of the pathogen as causative organism (*M. arachidis*) of early leaf spot disease of groundnut.

Phytochemical screening

Phytochemical test was conducted to qualitatively verify the presence or absence of secondary metabolites in the extract of the plants earlier collected.

In vitro antifungal screening tests
Spore germination assays were conducted according to Kishore *et al*. (2001). Agar well diffusion assay were conducted accoirding to Okigbo & Ogbbonnaya (2006).

Results and discussion

Pathogenicity test
The result of the pathogenicity test confirmed *M. arachidis* as the causative organism.

Phytochemicals
The results of the phytochemical screening showed the presence of secondary metabolites such as alkaloids, saponins, proanthocyanins, steroids, flavonoids, tannins and terpenoids in the plants sampled. The result was in agreement with earlier work of Abiodun *et al*. (2011) who reported on some of these metabolites in different plants extracts.

In vitro antifungal screening
Ethanol extracts of the medicinal plants (Figures 1-3) gave the highest spore germination and radial growth inhibition followed by hot water extracts. Overall, the potency of the extracts on the radial growth of *M. arachidis* was significantly different ($P < 0.005$). The fungitoxic actions of these leaf extracts were due to the presence of metabolites. They also have been reported to inhibit spore germination and radial growth of different pathogens (Soetan, 2008; Ishida *et al*., 2009; Obasi *et al*., 2010).

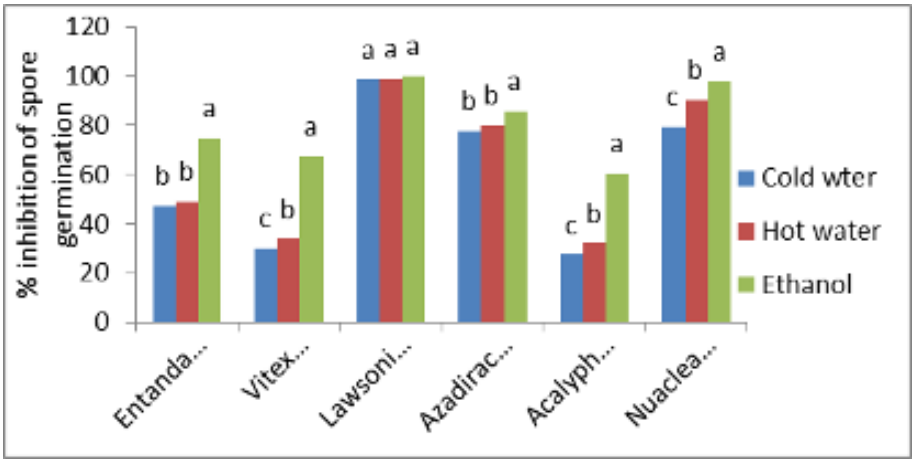

Figure 1. Percentage inhibition of spore germination of *Mycosphaerella arachidis* D. by leaf extracts of six medicinal plants. Bars with the same letter are not significantly different ($P < 0.05$).

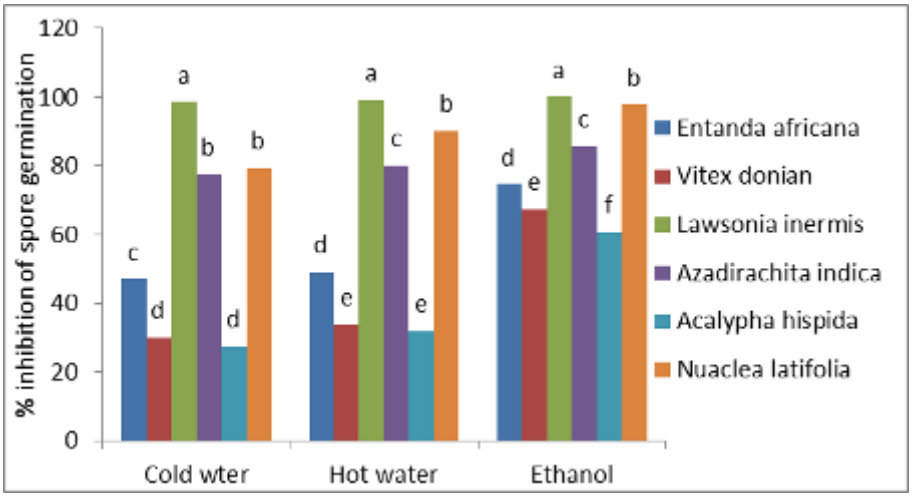

Figure 2. Effect of different extracts of six medicinal plants on *Mycosphaerella arachidis* D. Bars with the same letter are not significantly different ($P < 0.05$).

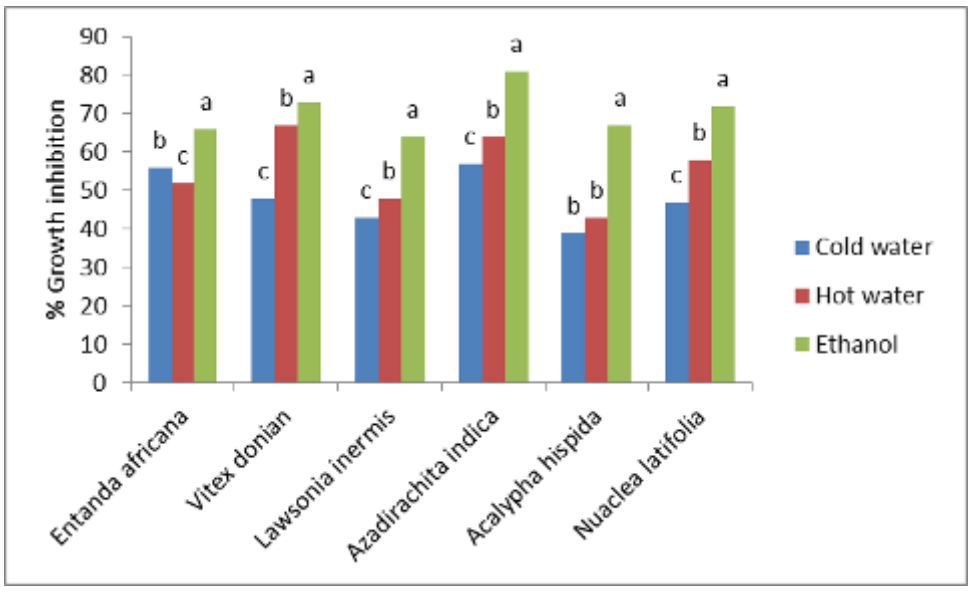

Figure 3. Percentage radial growth inhibition of *Mycosphaerella arachidis* D. by leaf extracts of six medicinal plants.

Conclusion

This research has shown spectra of antifungal activities of extracts from six medicinal plants and provides support to some traditional uses of these medicinal plants. Since these plants are easy to obtain and the extracts could easily be made via a simple process of maceration or infusion, they could therefore be cheaper substitutes for conventional fungicides in controlling various plant diseases.

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Biological efficiency of polyethylene plastics and Idefix (65.6% cupric hydroxide) injection against tomato bacterial leaf spot (*Ralstonia solanacearum*) and their effects on soil microorganisms in Burkina Faso

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Abstract: A study was conducted at Kou Valley, Burkina Faso, on the biological efficiency of polyethylene plastics and ground injection with Idefix (cupric hydroxide, 65.6%), a fungicide and bactericide, against tomato bacterial leaf spot which causes big losses to farmers. Secondary effects on soil microorganisms were also studied. The numbers of bacteria (*R. solanacearum*) were evaluated on SMSA medium. The level of infection development was evaluated and soil microorganisms were quantified on culture media. The efficiencies of polyethylene plastics varied from 0% to 97% and those of injection method with Idefix from 0% to 43%. The number of ammonifying bacteria, nitrifying bacteria, and cellulolytic bacteria and microscopical fungi was higher during tomato cultivation than at the period before tomatoes were planted. The use of polyethylene plastics resulted in a 100% yield increase in comparison with the untreated control. The ground injection method with Idefix resulted in a yield increase of 87% in comparison with the untreated control. The application of black or white polyethylene plastics allows an integrated pest management of tomato bacterial leaf spot without secondary effect on the human health and the environment.

Key words: polyethylene plastics, cupric hydroxide, bacterial leaf spot, tomato

Burkholderia phytofirmans PsJN confers grapevine resistance against *Botrytis cinerea* by a better ressource mobilization rather than a direct antimicrobial effect

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Abstract: Plant growth-promoting rhizobacteria (PGPR) are of great interest since they are beneficial naturally occurring soil bacteria that colonize plant roots and confer beneficial effects. They can increase yield, stimulate plant growth, reduce pathogen infection, and reduce biotic and abiotic stresses. Among these PGPRs, endophytes are defined as those bacteria that are able to colonize the internal tissue of the plant without causing external signs of infection or negative effects on their host. *Burkholderia phytofirmans* PsJN, classified as an ePGPR, was first isolated from surface-sterilized onion roots infected with the mycorrhizal fungus *Glomus vesiculiferum*. This rhizobacterium significantly promotes growth and protects the grapevine against biotic (grey mould disease) and abiotic (cold) stresses. Mechanisms implied in cold tolerance induced by PsJN were elucidated, however, the protective effect induced by the PGPR against *B. cinerea* remains elusive. To unravel the mechanistic pathways involved in the observed resistance, different traits of the tripartite interaction between *Vitis vinifera* L., *Botrytis cinerea* and *Burkholderia phytofirmans* were highlighted. Among these aspects, direct antimicrobial action of PsJN, the ability of the bacterium to prime defense responses and carbohydrate metabolism of grapevine will be discussed.

Key words: grapevine, *Burkholderia phytofirmans* PsJN, biocontrol

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Effect of nettle manure and bio-compost extracts on *in vitro* and *in vivo* mycelial growth on *Botrytis cinerea*, causative agent of grey mold

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Key words: *Botrytis cinerea*, grey mold, biocontrol methods

Introduction

Botrytis cinerea (teleomorph *Botryotinia fuckeliana*) is a major crop pathogen infesting more than 220 hosts worldwide. It is the causative agent of grey mold disease witch cause important damage in vineyards, strawberry and tomato. In Algeria, these crops showed a significant increase regarding productivity factors (growing area, production and yield). Chemical control is often used with the risk of resistance development to fungicides in *Botrytis cinerea* populations. Worldwide, important losses are estimated at more than 2 billion dollars per year only for vineyards for the control of the disease. As a part of a broad project on integrated pest management (IPM), the aim of this work is to develop alternatives methods of *B. cinerea* control based on the effect of natural components used in organic agriculture and agro ecological systems on the growth of the fungus.

Material and methods

Nettle manure and bio-compost were tested. The following tests were conducted: *i) in vitro* mycelial growth on agar medium containing several dilutions of each component and ii) *in vivo* effect of the two components applied before, as preventive, and after, as curative, on detached leaves of tomato which were artificially inoculated with *Botrytis cinerea* isolates (Mouria *et al*., 2013; Mac Cartney *et al*., 2002). The severity of symptoms (Decayed Leaf Area) is noted 5 days after inoculation with *B. cinerea* by adopting the rating scale of Elad & Shtienberg (1994). Nettle manure was obtained by macerate and filtration as described by Bertrand *et al*. (2007). The compost extracts were prepared according to the method of Welzien (1992). Nine isolates of *Botrytis cinerea* were selected regarding to their high degree of aggressiveness.

Results and discussion

Nettle manure caused a 57 to 74% reduction of mycelial growth *in vitro* compared to the negative control. Similar observations were made for inoculations on detached leaves showing a reduction of 50% of the lesions for preventive or curative treatment. The biological

compost reduced mycelial growth *in vitro* by 73 to 85%. Inoculation of detached leaves resulted in a reduction of the lesion development of more than 60% with better efficiencies for curative treatments than preventive treatments.

These results of a preliminary study in Algeria suggest that components used in organic agriculture or empiric agro ecological systems have positive effects in the control of grey mold disease. More criteria are under study to confirm the efficiency of these components to allow their introduction in integrated pest management programs in intensive production and widely in different agro ecological systems in Algerian regions.

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Replacing chemical seed treatments by a tailored mixture of microbial strains to secure germination of Styrian Oil Pumpkin

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Abstract: In 2015, the cultivation area of the Styrian Oil pumpkin reached its highest level to date with 32,175 ha in Austria and the market for this high-value crop is still increasing. Styrian Oil pumpkin is highly susceptible to fungal and bacterial pathogens particularly during germination. Thus, commercially available seeds must be treated with synthetic fungicides including Fludioxonil, Mefenoxam and Captan for the conventional market and with copper-based strippers for the use under organic farming systems. Sowing of completely untreated seeds entails a high risk for farmers and can result in a total loss. As the registration for copper-based strippers will presumably phase out for organic farming in Austria, alternative seed treatment technologies are requested. The biological product 'Peposan' contains a mixture of plant-beneficial bacteria and one fungus and secures germination of Styrian Oil pumpkins and enhances harvest yields. A stabilization of the promoting effect under varying field conditions is achieved by customized formulation and seed treatment techniques that enable an establishment of the microbial strains on the germinating seed and in the seedling bed. After field trials in five consecutive years at nine different sites a mix of three bacterial strains, *Serratia plymuthica* 3Rp8, *Serratia plymuthica* S13 and *Paenibacillus polymyxa* PB71 and the fungal strain *Trichoderma velutinum* G1/8 was shown to consistently evoke a germination performance similar to synthetic or chemical fungicides.

Key words: Styrian Oil pumpkin, fungicides, biocontrol strains, formulation techniques

Resistance management in *Helicoverpa armigera (Hübner)* by recombinant Cry1Ac – entomocidal toxin of *Bacillus thuringiensis*

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Abstract: *Helicoverpa armigera* (Hübner) is one of the polyphagous pests causing severe damage to agricultural crops. Insecticidal Cry protein of *Bacillus thuringiensis* proved successfully to control *Helicoverpa armigera* in the field. Resistance management of *Helicoverpa armigera* against Bt toxin is urgently needed in second generation of transgenic Bt crop. Recombinant toxin Cry1Ac was prepared by domain shuffling between *cry1Ac* to *cry9Aa* by *in vitro* recombination approach-overlap extension PCR (OE-PCR) technique with six base pairs homology at 3'ends of *cry1Ac* and *cry9Aa* genes. Furthermore, recombinant *cry1Ac* was cloned in pBluescriptKS (+) followed by sub-cloning in pET- 28a (+). Recombinant Cry toxin was expressed in *E. coli* BL21 (DE3) plys and subsequently purified by His-tag purification. Insect bioassay analysis revealed that LC_{50} of recombinant Bt toxin was around five fold higher than parental toxin against *Helicoverpa armigera* and could be used as potent biological control agent.

Key words: insect resistance management, Cry toxin, domain swapping, overlap extension PCR

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Dynamics of signaling and signal perception in microbe – host interactions

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Abstract: To better understand beneficial interactions of rhizobacteria with plants, it is necessary to include widely distributed small molecules, which modulate the primary microbial-associated molecular pattern (MAMP)-triggered innate immune response of plants. Besides signaling molecules affecting phytohormone regulons or other secondary metabolites with e.g. antibiotic activities, *N*-acyl homoserine lactones, which are widely found in Gram-negative bacteria as quorum sensing compounds, have profound effects on plant development and systemic resistance.

Key words: N-acyl homoserine lactones, phytohormones, plant growth promotion, immune response, disease control, stress resistance, quorum sensing

Introduction

The plant microbiome has important roles to support plants´ performance and health (Berendsen *et al*., 2012; Turner *et al*., 2013). The challenge of adaptation to changing environmental conditions has led to the development of the hologenome or holobiont view of higher organisms including the host and its associated microbiome as most successful life strategy (Zilber-Rosenberg & Rosenberg, 2008). There is still much to discover regarding organismic and functional diversity within the plant microbiome, since many organisms are difficult or even impossible to be isolated in pure culture. A profound comprehensive understanding of molecular signals within the interactions between the plant microbiomes and their hosts is still rather at the beginning.

Plant growth promotion by rhizosphere associated, root surface or endosphere colonizing microbes is well documented (Dessaux *et al*., 2010; Lugtenberg, 2015). Benefits of these microbe-plant associations can in particular be observed when plants are challenged by limiting nutrient supply, such as phosphate and nitrogen limitation, by abiotic stress, or when attacked by pathogens. Plant beneficial microbes colonizing effectively root surfaces or the inside of roots and shoots can assist plant physiological processes, e.g. by their ability to stimulate root growth, root hair development by their phytohormone production, which is a quite widely distributed phenomenon in microbe-plant interactions. This stimulatory effect on root morphology causes improved exploration of soils for nutrients and water which has analogy to the enlargement of the explored soil volume by mycorrhizal hyphae. Besides the auxin indole acetic acid and related compounds, the microbial production of gibberellin and cytokine is a well-described trait in plant-associated microbes (Spaepen, 2015). The production of a number of volatile compounds, such as 2,3-butanediol or acetoin have marked

influence on plant development and its immune system (Song & Ryu, 2013). Furthermore, the modulation of ethylene production of plants by root associated bacteria has a special role in this hormonal interplay between rhizosphere microbes and roots (Glick, 2014). Ethylene is increasingly produced by plants at several stress conditions, such as salt stress, which causes an inhibition of root growth. It has been found for a number of salt-tolerant bacteria that they have the ability to hydrolyze ACC, the precursor of ethylene, and in this way relieve the growth inhibition in the plant. Many salt tolerant PGPR, but also other beneficial rhizosphere bacteria, possess this ACC-deaminase activity. It has also been suggested, that external supplementation of indole acetic acid by root colonizing bacteria can also help plants to overcome salt-induced inhibition of the plant´s auxin biosynthesis. The quorum sensing autoinducers of *N*-acyl homoserine lactones (AHLs) of Gram-negative bacteria have recently been identified as additional signaling molecules between rhizosphere bacteria and plants (Hartmann *et al*., 2014). Although the response of plants towards microbes is mostly steered by MAMPS (microbial associated patterns), small secondary metabolites like antibiotics but also AHLs are perceived by plants, obviously because of the intimate coevolution of plants with microbes. Therefore, the perception of AHLs by plants will be in the specific focus of this contribution.

Perception of *N*-acyl homoserine lactones by plants and the dynamics of this interaction

In many Gram-negative bacteria, luxI-luxR type quorum sensing (QS) systems are frequently present; *N*-acyl homoserine lactones (AHLs) are known as luxI-type signals which are produced in an auto-inducing manner to sense the density of the population (Fuqua & Greenberg, 2002). The length of the fatty acyl group varies from 4 to 18 carbon atoms and hydroxyl- or carbonyl-group substitutions are found at the C3-position of the AHLs. Specific gene expression is activated or suppressed by binding and releasing the AHL-luxR transcription factor from specific gene promoter regions. AHL-dependent QS-responses are auto-induced global regulons, affecting major interactive traits, like swarming motility, chemotaxis, bioluminescence, plasmid conjugal transfer, biofilm formation, antibiotic biosynthesis, and the production of virulence factors in plant and animal pathogens (Eberl, 1999). AHL auto-inducers also convey information about the surroundings and habitat quality of the cells and thus, AHLs have a central role in optimizing the expression of their genetic repertoire (Hense *et al*., 2007). Moreover, AHL signal molecules not only allow bacterial populations to interact with each other but also with their eukaryotic hosts. AHLs with carbon chain length of C12 and C16 induce specific and extensive changes in the proteome profile of *Medicago truncatula*, as was first shown by Mathesius *et al*. (2003). Also in the interaction with different AHLs of plant growth promoting rhizobacteria (PGPR), beneficial effects due to their AHL-production were reported. In tomato plants it was demonstrated using AHL-deficient mutants of *Serratia liquefaciens* MG1 and *Pseudomonas putida* IsoF, that a ISR-like response towards the leaf attacking fungus *Alternaria alternata* was dependent on short side chain AHLs (Schuhegger *et al*., 2006). It could be proven *in situ* by using specific GFP-labelled AHL-reporter bacteria that AHLs were indeed produced on the root surface, when AHL-capable bacteria are colonizing the root surface (Gantner *et al*., 2005). In *Arabidopsis thaliana*, short side chain AHLs induced phytohormonal changes in the plants and caused an enhancement of root growth (von Rad *et al*., 2008). It was later shown that this root growth stimulatory effect is mediated through a protein G coupled receptor. Furthermore, the application of C4-HSL (homoserine lactone) causes an increase of calcium in plant cytoplasm

which results from the influx from extracellular medium via plasma membrane calcium ion channels. Radiolabeling studies showed that more water soluble short carbon chain AHLs, such as C6- and C8-HSL, can be taken up and transported inside barley plants while the long carbon chain AHLs, like C12- or 3-oxo-C14-HSL cannot be transported (Sieper *et al*., 2014). However, long carbon chain AHLs can cause resistance to plant pathogenic bacteria and fungi via AtMPK6 by a salicylic acid/oxylipin pathway in *Arabidopsis* and enhance free phenol and local cell wall-thickening in barley (Schenk *et al*., 2014; Schenk & Schikora, 2015). Therefore, long carbon chain AHLs are considered as inter-kingdom communication mediators which causes a priming effect towards biotrophic and hemitrophic plant pathogens in a wide variety of plants (Schikora *et al*., 2016).

Plants are also quite active in this interplay with bacterial QS compounds of the *N*-acyl homoserine lactone type. Some plants, like e.g. most legumes, have lactonase activities in their root tissues, which cleave bacterial AHLs before they can be effectively taken up into the plant (Sieper *et al*., 2014). Also AHL-mimicking molecules are excreted by plants which interfere with the bacterial signal system and destroy the coordination of bacterial actions in the rhizosphere (Kalia, 2012). So-called quorum quenching bacteria, which efficiently degrade AHL, were already quite successfully applied to neutralize the QS-signaling of pathogenic rhizosphere bacterium (Cirou *et al*., 2011). In addition, there are lactonase activities found in AHL-producing bacteria, like in *A. tumefaciens* or in *P. putida* IsoF (Fekete *et al*., 2010), which are tightly regulated. It is hypothesized that these AHL-lactonase activities have the purpose to quench the AHL-signaling in situations, when no signals should be sent. Thus, the signaling character of the AHL-production is enhanced.

To further understand the role of AHL-signal molecules of beneficial bacteria in the interaction with plants, AHL-deficient mutants of the plant growth promoting endophytic bacterium *Acidovorax radicis* strain N35 (Li *et al*., 2011) were recently studied in comparison to the wild type. Interestingly, the AHL-deficient mutant caused higher flavonoid production in the plants and other defense priming responses as compared to plants colonized by the AHL-producing wild type (Shengcai *et al*. submitted). This can be taken as signs of induced defense reaction which are lowered in plants colonized by the AHL-producing endophytic bacterium.

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Between field trials and large scale field application – the registration process for biocontrol products and its challenges

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Abstract: To bring microbial biocontrol products to the field application they have to be registered as plant protection products. There are a lot of different legislatives all around the world, all of them have in common that they try to evaluate the risk of such kind of products for human and environment on a scientific sound basis. The discussion still focuses on the possibility of relevant metabolites to be produced by all kind of microorganism, but also the antibiotic resistance genes and their horizontal transfer become a topic. Furthermore, the safety of all plant protection products is not only evaluated by the authorities, but also by the "consumer" (retailers, NGOs, press releases), and their opinions are not always based on scientific knowledge. It can happen that products pass the registration process, but diffuse fear about the use of microorganism-based products on food leads to restrictions by retailers or farmer organizations. Most of the time this is due to a lack of understanding of fermentation methods, and a lack of knowledge of basic microbial ecology as such.

Key words: registration, microbial food safety, preharvest interval

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MIP diversity from *Trichoderma harzianum*, and transcriptional regulation during its mycoparasitic association with *Fusarium solani* in olive trees

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Abstract: The "*Fusarium* rot" is a severe cryptogamic disease of olive trees (*Olea europea*) caused by the soil-born fungus *Fusarium solani*. Control of this disease predominantly depends on the use of pesticides, although an operational biocontrol by antagonism exists for decades. Because of multiple environmental and societal concerns, this alternative approach now enters a new era. It can involve fungal species of the *Trichoderma* genus exhibiting mycoparasitic activity. Recently, a new pathogenic *F solani* strain has been isolated from the soil of a Tunisian olive plantation. The mycoparasitic behavior of *Trichoderma* species against this new agent is unknown. The infestation modes of *Trichoderma* spp. on some pathogenic fungi are well described. However the mechanisms related to solutes absorbotrophy are paradoxically not well known. It has been well established that various permeases are involved, but among several large transmembrane families, the Major Intrinsic Protein (MIP) has not yet been studied in a mycoparasitic context.

Our aim is double: First, to test *T. harzianum* mycoparasitic behavior on *F. solani*, and second, to characterize the MIP diversity and their modulation during myco-heterotrophic activity.

Results show that *T. harzianum* exerts growth inhibition and potential mycoparasitism on *F. solani in vitro* (Petri dish), as well as a protective role against the *Fusarium* rot disease *in planta*. Secondly, *T. harzianum* exhibits 8 MIP members: 4 aquaglyceroporins, 3 aquaporins and 1 X-Intrinsic Protein. This XIP draws particular attention because it is the only member shared with plants, and its biological functions remains entirely unknown. Lastly, 6 members from these 3 MIP subclasses showed significant transcriptional modulations during the confrontation between *T. harzianum* and *F. solani*.

These preliminary results provide information on the MIP diversity in *Trichoderma*, and the modulation of their expression during mycoparasitic interactions. A better knowledge of their role in this context may help optimizing the biocontrol of cryptogams in olive trees.

Key words: *Trichoderma*, *Fusarium*, MIP

Screening of rhizospheric petroleum hydrocarbon degrading bacteria

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Abstract: Soil contamination by crude oil is a common problem worldwide. Utilization of microorganism to remediate soil pollution is known as bioremediation. Twenty-one species of bacteria were isolated from contaminated soil and screened for its ability to degrade crude oil in 0.5 and 1% (w/v) crude oil in Bushnel Haas Mineral salt and agar media and to produce biosurfactants. Bacterial populations increased and oil concentration did not affect bacterial numbers. Nine strains showed best growth in1% crude oil and haemolyzed blood. Growth of the bacteria increased optical density and decreased pH during the incubation period. For 2 bacterial strains, protein concentrations were 0.028 and 0.025 mg/ml after one week and 0.045 and 0.021 mg/ml after two weeks of growth. Production of protein increased with bacterial populations and was taken as indicator of successful bioremediation. Bacterial isolates had emulsifying efficiency and can produce active compounds reducing surface tension from 56.4 N/m to 37.24 and 34.13 N/m, respectively. Further identification of bacteria revealed that the two strains were *Streptomyces minutiscleroticus* and *Bacillus anthracis.* The two strain facilitated bioavailability and enhanced biodegradation process.

Evaluation of antagonistic mixtures to control *Neofusicoccum australe* and *Diplodia seriata* on grapevine pruning wounds

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Abstract: Grapevine (*Vitis vinifera* L.) is one of the most important fruit crop in Chile. Nevertheless, the crop is affected by different species of fungi of the *Botryosphaeria* genus. The control is done mainly by chemicals, although the use of biocontrol agents appears as a new alternative. The aim of this study was to evaluate the biocontrol ability of bacterial and fungal antagonist mixtures to control *Neofusicoccum australe* and *Diplodia seriata* on pruning wounds on canes and vine plants cvs. Cabernet Sauvignon and Chardonnay. Two experiments were conducted, one on not rooted vine canes under greenhouse conditions and the other in vineyards located at the Metropolitan and Valparaíso regions of Chile. Pruning wounds were treated with the bacterial mixtures Bac1 and Bac3, the fungal mixture Fun1, a commercial biofungicide and methyl thiophanate (MT). The wounds were inoculated with conidia of *N. australe* and *D. seriata*. Length of lesions produced by both pathogens and the colonization level on asymptomatic tissue was evaluated. The length of the lesions in the different treatments was highly variable and no significant differences were determined. The lowest percentages for both pathogens' colonization on asymptomatic tissue were obtained with MT and Fun1, on canes under greenhouse conditions and vineyards under field conditions.

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Key words: *Botryosphaeria*, grapevines, biocontrol

Biocontrol microorganisms of *Botryosphaeria* spp. elicit defense and growth promotion in vine seedlings

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Abstract: Grapevine (*Vitis vinifera* L.) is one of the most important fruit crop worldwide. The "dead-arm disease" greatly affects commercial cultivars, being *Botryosphaeria* spp. among the causal agents. The bacterial mixture Bac3 and the fungal mixture Fun1 show biocontrol activity on *Neofusicoccum australe* and *Diplodia seriata* that infect pruning wounds on vine plants cvs. Cabernet Sauvignon and Chardonnay. However, it is unknown if they are able to elicit resistant mechanisms and/or to improve growth of plants. Vine seedlings of both cultivars obtained from seeds, were used as model. Seedlings cultured in the presence of the mixtures Bac3 and Fun1, were used to evaluate growth promoting effect and control seedlings were used for elicitation experiments. These included mechanical damage and seedling inoculation with the biocontrol mixtures, with further quantitation of Phenylalanine ammonia-lyase (PAL), β-1,4-chitinase (Chit) and β-1,3-glucanase (Glc) activities, in time course experiments run up to 120 hours post-inoculation. Results showed that: a) Bac3 and Fun1 significantly increased fresh and dried weight of seedlings from both cultivars; b) seedling inoculation with Fun1 resulted in PAL, Chit and Glc activation at 12, 24 and 24 hours, respectively, in both cultivars, being PAL and Chit induction always higher in the Chardonnay cv.; c) seedling inoculation with Bac3 only induced PAL and Glc at the same time periods as for Fun1 inoculation, in both cultivars. Thus, it may be proposed that the biocontrol mixtures Bac3 and Fun1 show growth promoting and elicitation of defense effects on vine seedlings.

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Key words: *Botryosphaeria*, vine seedlings, biocontrol, elicitation, growth promotion

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Characterization and biocontrol properties of *Lactuca sativa* rhizosphere microbiota in an aquaponic system

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Abstract: Aquaponics is an integrated recirculated system that combines aquaculture and hydroponic plant production. The dissolved nutrients generated by the fish rearing after bacterial activities are used by the plants for their growth. This uptake reduces the accumulation of some molecules which allow a longer water recirculation. When comparing growth conditions between aquaponics and hydroponics, we observed much lower concentration of several key nutrients in aquaponics. Nevertheless, several experimentations, including ours, report similar or better yield in aquaponics compared with hydroponics. One explanation of this phenomenon could be linked to microbial actions. Another possible involvement of microorganisms in aquaponics could be a suppressive action against plant diseases. A first article (Gravel *et al*., 2015) opens the hypothesis of a protective activity of fish effluents microbiota against plant pathogens. For example some soilless systems already show a suppressive effect towards certain root plant pathogens that prove the existence of beneficial microorganisms in hydroponic systems (Postma *et al*., 2008). Nowadays, microorganisms related to aquaculture and hydroponics are well characterized but very little is known about the taxonomy of the microbiota associated with aquaponic system and their roles in the interaction microbiota-plants and microbiota-plant pathogens. The first aim of this study is to characterize the rhizosphere microbiota (metagenomics) of lettuces growing in an aquaponics system. A NGS approach targeting the 16S, 18S and ITS rDNA will be carried out to evaluate the bacterial, fungal and protozoan composition and diversity. The second aim is to assess the resistance of aquaponics lettuces following a pest introduction. Yields, symptoms and microbial changes due to the pest will be recorded and analysed to determine if aquaponics systems provide a better plant protection than hydroponics. Depending on the results, the final steps will be to identify the optimal conditions for the management of the potential beneficial microbiota or isolate a beneficial microorganism.

Key words: aquaponics, suppressive microflora, biocontrol, metagenomics, microbiota, lettuce

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Repeated applications of a non-pathogenic *Streptomyces* strain enhance the development of soil suppressiveness to potato common scab

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Abstract: Disease suppressive soils represent an attractive option for combating diseases such as potato common scab that lack effective control methods. Common scab is an important disease of potato (*Solanum tuberosum* L.) caused by a number of thaxtomin-producing *Streptomyces* species. It degrades the crop quality by producing lesions on the tuber surface and thereby reduces the market value of the crop. Although knowledge on common scab has expanded considerably over recent years, effective management of the disease remains elusive. The development of common scab suppressive soils has been documented in multiple locations, and non-pathogenic *Streptomyces* are suggested to play a role in natural disease suppression. Suppressiveness to common scab can develop as a result of continuous crop monoculture, but the duration required may be years and may not be a practical management method in commercial potato production. We investigated a possibility to enhance the development of soil suppressiveness to common scab using a non-pathogenic *Streptomyces* strain isolated from a scab lesion. The application of the *Streptomyces* strain reduced disease severity in average by over 40% on a susceptible potato cultivar and nearly eliminated scab symptoms on a tolerant cultivar in the field experiments of over 3 years. Scab suppression was evident after a single application and persisted in the soil for two years at least after repeated applications. The development of scab suppression was associated with the changes in microbial diversity and composition in potato tubersphere, which were observed in response to *Streptomyces* applications.

Key words: potato common scab, *Streptomyces* spp., disease suppressive soil

Attitudes of farmers towards biological control principles in Lublin

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Abstract: Biological control is a component of an integrated pest management strategy. It is defined as the reduction of pest populations by natural enemies and typically involves an active human role. Keep in mind that all insect species are also suppressed by naturally occurring organisms and environmental factors, with no human input. This is frequently referred to as natural control. This guide emphasizes the biological control of insects but biological control of weeds and plant diseases is also included. Natural enemies of insect pests, also known as biological control agents, include predators, parasitoids, and pathogens. Biological control of weeds includes insects and pathogens. Biological control agents of plant diseases are most often referred to as antagonists. For many years the use of chemical agents such as pesticides and herbicides has been effective in controlling the many varieties of pests that infest both agricultural crops and backyard gardens. However, these pests are gradually becoming resistant to these agents, because the agents themselves are acting as selective factors making the pests better and better able to resist and persist. As a result, the use of biological controlling agents is increasing.

This study was conducted to determine the socio economic characteristics of farmers, attitudes of farmers towards biological control principles in Lublin province. Data for the study were collected through interview using a questionnaire method from a randomly selected sample of 110 farmers. The first part of research included the independent variables, while the second part included scale to measure. The attitude of farmers towards biological control principles were assessed by summing up positive, negative and neutral reactions. To achieve this, a five point Likert scale was used, while the scoring was reversed for unfavorable statements. A total of 12 questions was asked, the maximum and minimum scores were 60 and 12, respectively.

The majority of farmers (78.6%) were between 30-56 years, 13.7% with primary education and most of the farmers (about 61.4%) were married. Averages of farming experience, family size and farm size were: 12 years, 5 persons, 13.6 ha, respectively. The findings revealed that majority (77%) of the respondents showed neutral attitudes towards biological control principles.

In conclusion:

1. The neutral attitude is evidenced in the responses towards biological control principles. It may be due to the lack of extension services in this region in the subject of principles of biological control.
2. There was a significant relationship between attitudes of farmers towards biological control principles and some variables such as farm size, farming experience, age or education level, while there was no significant relationship with family size or marital status.

Biological control against the *Fusarium* wilt of pea (*Pisum sativum*) using non-pathogenic *Fusarium oxysporum*

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Abstract: In our study, 36 isolates of *Fusarium oxysporum* and 10 isolates of *Fusarium solani* were isolated from the rhizosphere, collars and stems of infected pea plants and of other plants apparently healthy. Pathogenicity tests carried out with the pea variety Merveille de Kelvedon which is classified among the most susceptible varieties to *F. oxysporum* f. sp. *pisi* allowed us to identified 21 strains of *Fusarium oxysporum* f. sp. *pisi* (FOP), 5 strains of *Fusarium solani* f. sp. *pisi* (FSP) and 3 strains of non-pathogenic *Fusarium oxysporum*. Statistical analysis were used to classify isolates into 6 groups with regard to their aggressiveness.

Biological control of FOP and FSP through the use of non-pathogenic isolates of *Fusarium oxysporum* showed that they have an antagonist effect *in vitro* through competition for space and *in vivo* by reduction of the disease index through induction of pea resistance. These results provide evidence of the antagonistic activity of the non-pathogenic strains of *F. oxysporum* isolated from the rhizosphere in controlling *Fusarium* wilt caused by *F. oxysporum* f. sp. *pisi* and *Fusarium* root of peas.

Key words: Pea (*Pisum sativum* L.), biological control, *Fusarium oxysporum* f. sp. *pisi*, *Fusarium solani* f. sp. *pisi*, non-pathogenic *Fusarium oxysporum*

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Effects of arbuscular mycorrhiza fungi and *Striga hermonthica* seed bank size on parasitism and growth of sorghum

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Abstract: The witch weed *Striga hermonthica* is proving to be a nightmare for smallholder farmers in the Sahel and Savannah regions of Africa. The present study was undertaken to investigate the effects of *Striga hermonthica* seed bank size and mycorrhizal levels on sorghum growth parameters and *Striga* parasitism. A pot experiment was undertaken where different *Striga* seed banks were established by mixing the parasite seeds (2 and 10 mg) in each pot. Sorghum cultivar Wad Ahmed were sown (4 seeds/pot), inoculated by AM inoculums (5, 15 and 25 g/pot) and reduced 10 days later to two plants per pot. Significant difference was observed between treatments in sorghum growth parameters including plants height, number of leaves and dry weight of shoots and roots. Mycorrhizal fungi (*Glomus* sp.) had significant effects ($p \leq 0.05$) on total aboveground *Striga* emergency as well as on *Striga* dry weight. From these results, it appears that the number of emerged *Striga* per plant was often low (4 plants/pot) where the sorghum was infested by lowest size seed bank and higher level of AM inoculums. Number of emerged *Striga* was consistently higher in plants inoculated by low AM inoculums (5 g/pot), compared to the other levels of AM inoculums. *Striga* emergency was highly dependent on the size of the parasite seed bank and on the mycorrhiza level. Sorghum shoot and root dry weights were the most affected parameters. It is also evident that the size of the parasite seed bank had negative affected in AM root colonization percentage. Therefore, the size of the parasite seed bank and the level of inoculated AM fungi are key factors in crop yield and in the efficacy of control. These factors should be taken into account in experimental evaluation and intervention measures.

Key words: seed bank density, mycorrhizal level, sorghum, *Striga hermonthica*

Screening and evaluation of chitosan from different sources for the control of yam anthracnose caused by *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc.

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Abstract: Chitosan has been reported to have fungicidal property to some pathogen species as well as an elicitor of resistance, particularly of Systemic Acquired Resistance (SAR). This study was conducted in the laboratory and screenhouse of the Department of Pest Management, College of Agriculture and Food Sciences, VSU, Visca, Baybay City, Leyte from September 2012 to March 2013 to screen and evaluate the efficacy of chitosan from different sources and to determine the most effective concentration and spray interval of these chitosans against the disease in *in vivo* experiments with yam.

Three concentrations of irradiated chitosan (100 ppm, 200 ppm, and 300 ppm) produced inhibition zones which were generally higher than in the control treatment. The treatment with 300 ppm irradiated chitosan had the largest zone of inhibition (13.8 mm), followed by the treatments with 100 ppm crab (7.6 mm), 300 ppm dried *Fomes* (5 mm), 200 ppm shrimp (2.6 mm) and 200 ppm commercial chitosan (1.4 mm). Spray application of chitosan at 7 days interval resulted to lower severity rating compared to 14 days. Crab chitosan at 100 ppm produced a lower severity rating compared to untreated control and was a little inferior to that of fungicides. However, at 14 DAS it was observed that the different treatments were not significantly different which each other. Yam plants sprayed with chitosan, most probably induced SAR which had an effect comparable to the effect of fungicides. The application of chitosan solution sensitized the yam plants to respond rapidly to the attack of anthracnose pathogen which most probably induced SAR leading to low disease severity.

It is recommended to conduct field experiments using irradiated chitosan and compare it with commercial chitosan. Protein expression profiles of yam with induced SAR should be established to identify proteins differentially expressed by SAR induction against yam anthracnose.

Key words: chitosan, SAR

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Endophytic plant growth-promoting bacterium *Kosakonia radicincitans:* An integrated fermentation and formulation

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Abstract: Plant growth-promoting bacteria (PGPB) such as the endophyte *Kosakonia radicincitans* have been extensively studied for their ability to directly or indirectly promote the growth of plants. Development of a novel biofertilizer based on *K. radicincitans* is challenging because of its missing sporulation capability resulting in high viability losses during drying, limited shelf life and difficult application of the formulated "active ingredient". To address the drying problem we chose a biotechnological approach by combining fermentation and formulation to enhance drying resistance of the Gram negative endophyte by manipulating the culture conditions.

Our current research project aims at developing novel integrated submerged fermentation and formulation approaches for *K. radicincitans* DSM16656^T beads or seed coatings that will enhance drying resistance, shelf life and thus efficacy as a plant growth-promoter.

K. radicincitans was raised in baffled shake flask cultures. A screening of carbon and nitrogen sources as well as C:N ratio, micronutrients, initial pH, viscosity and shear forces was carried out. The produced biomass was immobilized in beads based on a broad variation of biopolymers. Beads were developed by using different adjuvants such as nutrients, swelling agents, fillers and drying protectants.

Here we present first data on submerged mass production of endophytic PGPB *K. radicincitans* to enhance drying resistance by variation of culture conditions. Additionally, results regarding the performance of bead prototypes and the viability of cells after drying will be shown.

Key words: endophyte, cultivation, encapsulation

Development of application protocols for BCAs against soil-borne diseases is a top priority research need from practice: Recommendations of the EIP-AGRI Focus Group on soil-borne diseases

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Abstract: The European Innovation Partnership for Agricultural productivity and Sustainability (EIP-AGRI) was launched in 2012. This initiative is part of EU's growth strategy for this decade. The EIP-AGRI supports the cooperation between research and innovation partners through an interactive approach. To deal with specific questions, EIP-AGRI sets up Focus Groups. From November 2014 to September 2015, the EIP-AGRI set up the Focus Group 'IPM practices for soil-borne diseases', bringing together 20 experts (growers, researchers, advisors, policy makers) from several European countries. With the phase out of methyl bromide and the increasing limitation of the use of other chemical soil fumigants, the development of alternatives to suppress soil-borne diseases (caused by fungi and nematodes) in vegetable and arable crops has become an urgent matter. Cross-fertilization of scientific and practical knowledge of different crops and agricultural systems can provide new insights to tackle this problem. Based on literature and a questionnaire among the experts, *Fusarium* spp., *Verticillium* spp., *Rhizoctonia solani*, *Meloidogyne* spp. and *Globodera* spp. were considered the most important soil-borne pathogens in Europe. The group agreed that the traditional curative approach ('reactive approach') of crop protection needs to give place to a new integrated approach based on a good soil health strategy, designed to build resilience of the soil ecosystem. Only by integrating knowledge related to physical, chemical and biological characteristics of soils, soil health can be achieved. IPM (Integrated Pest Management) for specific soil-borne diseases might accomplish implementation when incorporated into a broad soil health strategy. According to this approach, the IPM practices for soil-borne diseases suppression include: prevention, monitoring, crop rotation and additional measures. The use of biological control agents (BCAs) is an important additional practice. The group regarded BCAs as one of the most promising innovations because they are relatively easy to apply in commercial conditions. However, most of their failures in controlling the pathogens are linked to their misuse (e.g. late application, concentration in soil below the threshold of activity, etc.). This Focus Group recommended the development of good protocols for BCA's use as a top priority research need to increase the success of their application in practice.

Other recommendations and more information about the IPM practices (advantages, fail factors and challenges) and ideas for Operational Groups are available online at https://ec.europa.eu/eip/agriculture/en/content/ipm-practices-soil-borne-diseases-suppression-vegetables-and-arable-crops

Key words: biological control, IPM, European Innovation

Measuring gene expression levels in the plant-associated bacterium *Ochrobactrum* sp. A44 – an RT-qPCR based assay for monocultures and complex samples

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Abstract: *Ochrobactrum* sp. A44, a bacterial isolate from the rhizosphere of potato (*Solanum tuberosum* L.), can inhibit tuber tissue maceration caused by the pectolytic plant pathogen *Pectobacterium wasabiae* 3139 (formerly *Pectobacterium carotovorum* subsp. *carotovorum*). In *P. wasabiae*, the expression of genes crucial for disease onset is dependent i.a. on the presence of a threshold concentration of signal molecules from the group of acyl homoserine lactones (AHLs). Enzymatic hydrolysis of AHLs by A44, mediated by the AiiO protein, is considered the central mechanism of the biocontrol activity of this strain. For future investigation of this phenomenon, we have developed an RT-qPCR based assay for measuring gene expression levels in A44 strain. In the era of omics, RT-qPCR is still a method of choice for investigation of small sets of genes in multiple conditions. As no data were available concerning suitable reference genes for data normalization in *Ochrobactrum* spp., we selected 11 candidate genes and evaluated the stability of their expression in A44 cells grown in 11 different setups. The culture variables included temperature, growth phase, medium composition and pH, as well as the presence of AHLs. Analysis of the results using the geNorm module in qbase+ software (Biogazelle) revealed that genes most stably expressed in A44 strain are *gyrB*, *rho* and *rpoD*, making them good reference targets for RT-qPCR analysis. Further, we have designed primers enabling specific amplification of the three established reference genes form A44, but not from other microorganisms, including the related *Ochrobactrum* spp. We plan to apply the developed method to measure gene expression levels of A44 strain in microbiologically complex samples.

This study was funded by a grant from the Polish National Science Centre (2014/13/B/NZ9/02136).

Key words: RT-qPCR, *Ochrobactrum*, reference genes

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Screening of oilseed rape endophytes for biocontrol of *Phoma* stem canker and *Sclerotinia* stem rot

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Abstract: *Phoma* stem canker disease (anamorph *Phoma lingam*, teleomorph *Leptosphaeria maculans*) and *Sclerotinia* stem rot (*Sclerotinia sclerotiorum*) are important diseases of oilseed rape (OSR) (Crumble & Notsch, 1999). We isolated endophytic fungi and bacteria from roots and stems of healthy and diseased oil seed rape, and confirmed their identity via morphotyping and sequencing of the ITS-region (fungi) or via MALDI-TOF (bacteria). Screening against *Phoma lingam* was performed on cotyledons of seedlings according to Delwiche (1980) modified by Zhao (2001). Screening against *Sclerotinia sclerotiorum*, was done as following: After emergence of OSR seedlings in horticultural substrate, 2 g of millet inoculum and 0.9 g of overgrown OSR leaves were spread in the upper part of the substrate. Disease was assessed as 0 = healthy, 1 = girdled hypocotyl (often with white mycelium coming out), 2 = wilted and 3 = dead.

The large majority of endophytic fungi belonged to Ascomycetes, Pezizomycotina group. Basidiomycetes (*Cryptococcus* and not identified), *Fusarium*, *Monographella*, *Alternaria* and *Eudarluca* were only isolated from diseased plants. *Leptosphaeria*, was isolated from stems of both healthy and diseased plants. *Cladosporium*, *Botrytis* and *Periconiella* were detected only in healthy plants, with *Cladosporium* being the prevailing species. None of the fungi tested was effective against *Phoma lingam* and *Sclerotinia sclerotiorum*.

The majority of bacterial endophytes were gamma-proteobacteria of the genera *Pseudomonas*, *Stenotrophomonas*, *Enterobacter* and *Serratia*, mostly isolated from roots. In stems, Bacilli prevailed. None of the dominant groups was exclusively confined to healthy or diseased plants. Seven gamma-proteobacteria slowed down *Sclerotinia* disease development over one month, but only two of them offered significant long term protection over three months. One of these two isolates was the only one that showed also efficacy against *Phoma lingam*. Three of the seven antagonistic strains originated from healthy roots, four from diseased roots.

Key words: *Sclerotinia*, endophytes,oilseed rape

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The *Rhizoctonia solani* AG1-IB (isolate 7/3/14) transcriptome during interaction with the host plant *Lactuca sativa*

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Abstract: Phytopathogenic fungi are responsible for economic crop losses worldwide and therefore the control of these fungi is important for crop production to ensure nourishment of humans and animals. The species complex *Rhizoctonia solani* comprises several serious pathogens for different economically important plants. The focus of this study was to analyze the interaction between *R. solani* (isolate 7/3/14) (Grosch *et al*., 2004) of the anastomosis group AG1-IB and one of its host plants *Lactuca sativa* (butterhead lettuce).

To identify *R. solani* genes involved in virulence and pathogenicity during host plant interaction, the transcriptional activity of the pathogen was followed during its interaction with lettuce by means of RNA-Seq. A leaf interaction model was used for which three distinct interaction zones were determined and distinguished by means of microscopy and sampled for RNA extraction. The interaction zones can be visually described as follows: zone 3 represents the lesion zone directly at the inoculation site surrounded by zone 2 characterized by a faintly brown lesion area, whereas zone 1 is visually unaffected plant material from the same leaf. For transcriptome sequencing, the Illumina HiSeq 1500 platform was applied and the resulting reads were mapped on the previously established *R. solani* AG1-IB genome sequence (Wibberg *et al*., 2013; Wibberg *et al*., 2015). Analysis of global transcription levels and differential transcription profiles were based on RPKM values for the former and DESeq calculations for the latter. Obtained results were evaluated by enrichment analyses and functionally characterized *via* comparisons to different databases, e.g. KOG (eukaryotic proteins) (Wu *et al*., 2011) and the Pathogen Host Interaction database (PHI-base) (Urban *et al*., 2014).

Each interaction zone was characterized by specific sets of transcripts that differentiate activity of the fungus. For example, a group of previously undescribed genes and several genes representing partial YABBY plant transcription factor motifs were found to be only transcribed within the interaction zone 1. Based on the KOG category enrichment analysis, the fungus featured high metabolic activity within the interaction zone 2. Finally, in zone 3 many transcripts related to *Rhizoctonia* agglutinin and apoptosis were differentially up-regulated. To conclude, this study provided valuable insights into the *R. solani* AG1-IB (isolate 7/3/14) disease cycle in relation to the host species lettuce.

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Sense and nonsense of the data requirement for secondary metabolites of microbial biocontrol agents (mBCAs)

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Abstract: The present risk assessment of secondary metabolites of microbial biocontrol agents (mBCAs) is complicated, leading to delay in admittance of the microorganism to Annex I.

We identify reasons causing the risk assessment to have become so complicated. For example:

- Commission regulation (EU) No 283/2013 for the data requirements mBCAs gives different messages for SMs per data requirement. This is confusing.
- Limited general knowledge on secondary metabolites leading to unnecessary questions.
- There is a vast and diverse area of research on secondary metabolites. Main research objectives are given. Most endpoints deriving from these studies were not intended to be used in risk assessment. Nevertheless they seem influence the perception of secondary metabolites.

We conclude that the present risk assessment is too complicated for a number of aspects. The poster discusses the possibilities to improve the risk assessment of secondary metabolites, thus enhancing the admittance of microorganisms.

Key words: secondary metabolites, risk assessment, data requirements

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Management of southern corn leaf blight via induction of systemic resistance by *Bacillus cereus* C1L in combination with reduced use of dithiocarbamate fungicides

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Abstract: Dithiocarbamate fungicides such as maneb and mancozeb are widely used nonsystemic protectant fungicides to control various plant fungal diseases. Dithiocarbamate fungicides should be frequently applied to achieve optimal efficacy of disease control and avoid either decline in effectiveness or wash-off from leaf surface. Dithiocarbamates are of low resistance risk but have the potential to cause human neurological diseases. The objective of this study was to develop a strategy to effectively control plant disease with reduced use of dithiocarbamtes. Southern corn leaf blight was the model pathosystem for the investigation. When corn plants were drench-treated with *B. cereus* C1L, a rhizobacterium able to induce systemic resistance in corn plants against southern leaf blight, frequency of spraying dithiocarbamate fungicides could be decreased. Moreover, treatment of *B. cereus* C1L was able to protect maize from southern leaf blight while residues of dithiocarbamates on leaf surface were too low to provide sufficient protection. Our results provide the information that plant disease can be well controlled by rhizobacteria-mediated induced systemic resistance in combination with reduced but appropriate application of dithiocarbamate fungicides just before a heavy infection period.

Key words: *Bacillus cereus*, dithiocarbamate, *Cochliobolus heterostrophus*, induced systemic resistance