Persistent Identifier: urn:nbn:de:0294-sp-2013-ppphe-2

DPG Spectrum Phytomedizin

C. Schneider, C. Leifert, F. Feldmann (eds.)

Endophytes for plant protection: the state of the art

Proceedings of the
5th International Symposium on Plant Protection and Plant Health in Europe

held at the Faculty of Agriculture and Horticulture (LGF), Humboldt University Berlin, Berlin-Dahlem, Germany, 26-29 May 2013

jointly organised by

the Deutsche Phytomedizinische Gesellschaft - German Society for Plant Protection and Plant Health (DPG) and
the COST Action FA 1103

in co-operation with the Faculty of Agriculture and Horticulture (LGF), Humboldt University Berlin, and the Julius Kühn-Institut (JKI), Berlin, Germany

Publisher

Persistent Identifier: urn:nbn:de:0294-sp-2013-ppphe-2

Bibliografische Information der Deutschen Bibliothek

Die Deutsche Bibliothek verzeichnet diese Publikation in der Deutschen Nationalbibliografie;

Detaillierte bibliografische Daten sind im Internet über http://dnb.ddb.de abrufbar.

ISBN: 978-3-941261-11-2

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Messeweg 11-12, 38104 Braunschweig

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Internet: www.phytomedizin.org

Lectorate: Dr. Carolin Schneider, Dr. Carlo Leifert, Dr. Falko Feldmann

Production: Dr. C. Carstensen, InterKulturIntern, Edenkoben

Design (cover): C. Senftleben, Braunschweig

Foto (cover): Dr. G. M. Kovács, D. G. Knapp (Eötvös Loránd Univ., Budapest, Hungary), Dr. G. Bills (Fundación MEDINA, Granada, Spain)

Printed in Germany by Lebenshilfe Braunschweig gemeinnützige GmbH

Persistent Identifier: urn:nbn:de:0294-sp-2013-ppphe-2

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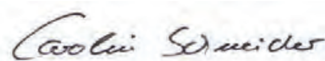
Preface

Dear colleagues,

in recent decades a new area of research has opened up in the field of microbiology, as the scientific community has cottoned on to the importance of endophytic microorganisms (EMOs) in the life cycles of the Earth's plant species. Endophytes, such as the myriad strains of bacteria and fungi extant in all known plants, are organisms which find their ecological niche within the bodies of other species without causing damage. The significance of the intimate relationship EMOs form with their host plants is just being uncovered; some of these ubiquitous life forms are now known to bring numerous benefits to the plants they colonise. This fact has generated a huge amount of interest from scientists keen to find sustainable alternatives to the chemical fertilisers and pesticides currently underpinning much of modern agriculture. With an expanding world population making ever greater demands on natural resources, ensuring food security in the coming century will mean learning to grow much more food whilst greatly reducing the environmental impact of intensive farming methods. Research groups from all over Europe are currently engaged in the large-scale European Cooperation in Science and Technology (COST) Action FA1103 ‚Endophytes in biotechnology and agriculture', built around the idea that the key to solving this conundrum may well be found in the world of endophytic microorganisms. To date, isolation, cultivation and characterisation of these so-called competent endophytes has found them to be associated with a range of properties. Bolstering systemic resistance and adaptability, improving stress tolerance, nutrient uptake and pathogenic resistance are all important functions in which EMOs can play a part as they interact genetically and biochemically with their hosts. It is thought that a greater understanding of these mechanisms will bring great potential for practical exploitation. Their capacity for new, sustainable biocontrol strategies for fighting pathogenic bacteria and diseases has generated lots of interest - on that note:

Endophytes for plant protection - the state of the art

On behalf of the editors



Persistent Identifier: urn:nbn:de:0294-sp-2013-ppphe-2

Persistent Identifier: urn:nbn:de:0294-sp-2013-ppphe-2

KEYNOTE

Persistent Identifier: urn:nbn:de:0294-sp-2013-ppphe-2

In: Schneider C, Leifert C, Feldmann F (Eds), Endophytes for plant protection: the state of the art. © 2013 Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-11-2

Challenges for endophyte research: the need to focus on food security

Leifert C

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ABSTRACT

The increasing reliance on non-renewable and/or scarce resources (e.g. mineral fertilisers, energy, water) in agricultural crop production is increasingly recognised as the greatest thread to future agricultural productivity and food security. In addition, there are increasing concerns about the environmental and human health impacts of agrochemicals (mineral fertilisers, pesticides, plant growth regulators). The rapid depletion of P-reserves, P-pollution of aquatic systems and the high energy use, nitrate leaching and greenhouse gas emissions associated with N-fertiliser manufacture and use are particular and very immediate concerns.

Endophytes can be defined as microorganisms that complete their live cycle within or in close proximity of plant tissues. While some groups of endophytes (e.g. AM-fungi, rhizobia) and their interactions with plants have been studied in detail, the ecology, functions and impacts on host plants of most other endopytes are still poorly understood. However, the potential for endophytes to address many of the sustainability issues related to both crop nutrition and protection is widely recognised.

The need to carry out more detailed studies to understand the ecology, physiology, environmental activity profiles and modes of action of endophytes and complex interactions between endophytes in and on root and above ground plant tissues is discussed in the context of utilising endophytes as alternative, more sustainable approaches to increase nutrient use efficiency and improve pest, disease and weed control in agriculture.

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SESSION 1

Occurrence and Diversity of Endophytes

Persistent Identifier: urn:nbn:de:0294-sp-2013-ppphe-2

Valverde R A, Navas-Castillo J, Are persistent plant viruses novel endophytes? In: Schneider C, Leifert C, Feldmann F (Eds), Endophytes for plant protection: the state of the art, pp. 4-9. © 2013 Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-11-2

1-1 Are persistent plant viruses novel endophytes?

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ABSTRACT

Based on the type of relationship with the host, plant viruses can be grouped as acute or persistent. Most acute viruses cause disease and have been well studied. Due to the apparent lack of effect on the host phenotype, limited information is available on persistent plant viruses. Persistent plant viruses are dsRNA virus members of the families *Endornaviridae*, *Chrysoviridae*, *Partitiviridae*, and *Totiviridae*. They have been reported to infect several economically important crops such as avocado, alfalfa, beets, cherry, common bean, fava bean, melon, pepper, rice, and tomato, among others. With the exception of *Vicia faba endornavirus*, which has been associated with male sterility, there have been no reports of effects of these persistent viruses to their hosts. We have identified persistent virus-free and persistent virus-infected lines of bell pepper and common bean jalapeño pepper, and tomato. The overall, appearance of virus-free and virus-infected lines seems similar. Nevertheless, preliminary comparative experiments using these lines suggest that in some cases, these viruses may have an effect on the phenotype, seed/fruit yield, seed emergence, and host reaction to some acute plant viruses. More focused comparative experiments with newly identified virus-free and virus-infected lines are in progress.

INTRODUCTION

Many plant species exist in a symbiotic (mutualistic) relationship with fungi and bacteria such as mycorrhizal fungi of most plants and rhizobia of legumes. In the case of plant viruses, the nature of the virus/host relationship can range from mutualistic to parasitic (Roossinck 2011). This relationship depends on the type of virus, host genotype, and the environment. Based on the type of relationship with the host, plant viruses can be grouped as acute or persistent (Roossinck 2010). Some acute plant viruses can render the host unfit for certain environments, including viruses that cause severe negative effects to the plant such as leaf malformation, mosaic, stunting, and necrosis. Nevertheless, some viruses can have beneficial effects. In a recent study, it was shown that infections of *Cucumber mosaic virus* (CMV) improved drought tolerance in several plant species and also enhanced freezing tolerance of beets (Xu *et al*. 2008). In the same study, *Nicotiana benthamiana* plants inoculated with CMV, *Tobacco mosaic virus*, or *Brome mosaic virus* were significantly more resistant to drought stress than mock inoculated plants.

Due to the apparent lack of effect on the host phenotype, limited information is available on persistent plant viruses. Five viral families contain dsRNA viruses that infect plants and fungi. They include *Partitiviridae, Chrysoviridae, Totiviridae, Reoviridae,* and *Endornaviridae* (King *et al*. 2012). With the exception of the family *Reoviridae,* all members of the other four families known to infect plants are persistent viruses. In the case of many fungal dsRNA viruses, it has been well documented that they often affect the fungi-plant interactions by altering the physiology of the fungus, and this has been studied in detail for viruses of plant pathogenic fungi (Ghabrial & Suzuki 2009). Although persistent plant viruses are very common and have been reported in many economically important plant species, little is known about their effect on their hosts. This is mainly due to the lack of an inoculation method and the difficulty of generating virus-free lines of the infected plant cultivars. Because these viruses lack cell-to-cell movement, they can only spread during cell replication; therefore, the use of conventional virus-inoculation methods, such as mechanical or graft inoculations, which deliver viruses into cells, do not work.

Comparative studies between virus-infected and virus-free plants have not been reported for persistent viruses. There have been only a few reports about the effect of these persistent viruses on their hosts. In the case of fava bean, the presence of *Vicia faba endornavirus* is correlated with male sterility, although the mechanism is not known (Pfeiffer 1998). In a transcriptome analysis of *Rhizobium* sp. colonization in white clover, a transcript that was significantly decreased was later found to be the coat protein of *White clover cryptic virus* (*Partitiviridae*). When this gene was expressed in *Lotus* sp., another legume species, it suppressed nodulation (Nakatsukasa-Akune *et al*. 2005).

Over the past several years, we have identified persistent virus-free lines of cultivars of four crops reported to be infected with five persistent virus species from three viral families: *Bell pepper endornavirus* (BPEV) (Okada *et al*. 2011), *Phaseolus vulgaris* endornavirus 1 (PvEV-1), and *Phaseolus vulgaris* endornavirus 2 (PvEV-2) (Okada *et al*. 2013)

(*Endornaviridae*); Pepper cryptic virus 1 (PCV-1) (Sabanadzovic & Valverde 2011) (*Partitiviridae*); and Southern tomato virus (STV) (Sabanadzovic *et al*. 2009) (*Totiviridae*). The objective of this investigation was to conduct preliminary comparative studies using these persistent virus-free lines with their virus-infected counterpart lines of these four crops and attempt to correlate the presence or absence of persistent viruses with phenotypic or genotypic characteristics of the host plant.

MATERIALS AND METHODS

Four persistent virus-infected crops have been identified. Hot pepper cv Jalapeño M infected with PCV-1 (Arancibia & Valverde 1995), tomato cv Celebrity with STV (Sabanadzovic *et al*. 2009), bell pepper cv Marengo with BPEV (Okada *et al*. 2011), and common bean cv Black Turtle Soup with PvEV-1 and PvEV-2 (Okada *et al*. 2013). Persistent virus-free lines of these four crop cultivars were identified from virus-infected seed lots. Because all of these four crop plants are self-pollinated, seeds were increased from single plants, and eight lines (two for each crop) were obtained after several generations of self-pollination. Seed was increased and used in comparative experiments. The investigations with PCV-1, PvEV-1, and PvEV-2 were conducted at Louisiana State University Agricultural Center, Louisiana, USA, whereas investigations with BPEV and STV were conducted at Estación Experimental La Mayora, Malaga, Spain.

Various numbers of seed from the eight lines were planted in steam-sterilized soil and kept in greenhouses throughout their life cycle. All plants were visually evaluated until maturity for seed emergence and phenotype. Seed germination experiments were conducted in the laboratory. Ten one-week-old Black Turtle Soup plants double-infected with the endornaviruses PvEV-1 and PvEV-2 and 10 endornavirus-free plants were mechanically inoculated with *Tobacco ringspot virus* (TRSV). Another set of 10 plants were inoculated with *Soybean mosaic virus* (SMV). Similarly, four set of 15 one-month-old Jalapeño M pepper plants infected with PCV-1 and 15 PCV-1-free plants were each mechanically inoculated with CMV, *Pepper mild mottle virus* (PMMoV), and *Physalis mottle virus* (PhyMV). Ten one-month old Marengo bell pepper plants infected with the endornavirus BPEV and 10 BPEV-free plants were mechanically inoculated with CMV. This experiment was repeated with three sets of 10 plants each using *Potato virus* Y (PVY), *Tomato spotted wilt virus* (TSWV), and PMMoV. Ten one-month-old Celebrity tomato plants infected with the totivirus STV and 10 STV-free tomato plants were mechanically inoculated with CMV. Mock inoculated plants were used as controls. In all cases, single viruses were inoculated and inoculations were conducted using inoculum that consisted of 2 g of infected tissue and 5 ml of 0.02 M potassium phosphate buffer pH 7.2. Virus strains used in each of the two locations (USA and Spain) were local isolates. All persistent virus-free and persistent virus-infected plants were regularly monitored for phenotypic variations and virus symptom expression throughout the life cycle.

RESULTS

Effect of persistent viruses on seed emergence/germination

In the case of Black Turtle Soup bean and Jalapeño M, seeds from persistent virus-infected lines germinated and emerged faster than seeds from virus-free lines. A difference in seed germination and emergence was not noticed with the bell pepper and tomato lines.

Effect of persistent viruses on plant phenotype

Black Turtle Soup bean plants of the line double-infected with PvEV-1 and PvEV-2 showed purple coloration on the stem and petioles. In contrast, plants of the virus-free line did not have purple coloration and seeds were slightly larger than virus-infected plants. In the case of Marengo bell pepper, BPEV-infected plants were smaller than virus-free plants. Moreover, virus-infected plants yielded bell shaped fruits while virus-free plants yielded conical shaped fruits: Tomato Celebrity plants infected with STV were slightly larger than virus-free plants. Phenotypic differences were not observed in the case of Jalapeño M pepper.

Effect of persistent viruses on yield

Endornavirus-infected Black Turtle Soup bean plants yielded lower seed weight than virus-free plants. An effect on fruit yield was not detected in the case of Marengo or Jalapeño M plants. Tomato lines were not evaluated.

Interactions of persistent viruses with acute plant viruses

When mechanically inoculated with any of the four viruses (CMV, PVY, TSWV and PMMoV), lines of bell pepper infected with BPEV and BPEV-free lines did not show obvious differences in their symptoms. Similar results were obtained with inoculations of CMV and PMMoV to Jalapeño M pepper and CMV to Celebrity tomato. In contrast, the endornavirus-infected Black Turtle Soup consistently showed more severe symptoms when inoculated with TRSV or SMV than plants from the endornavirus-free line.

DISCUSSION

We hypothesize that like many acute viruses, persistent viruses could affect the host response to biotic and abiotic agents. Although, we have not been able to inoculate these viruses to virus-free plants, we were able to generate virus-free lines for these preliminary studies.

The results obtained on seed germination are interesting because an increase in the speed of seed germination and plant emergence was observed with two distinct plant species (common bean and pepper) infected with persistent viruses. In the case of common bean, this could be related to the virus concentration in the seed. Previous research by Okada *et al.*

(2013) showed that two persistent viruses of common bean (PvEV-1 and PvEV-2) occurred in higher copy number in the seed of common bean than in other plant tissues. Faster seed germination and plant emergence could provide the plant with the capability of avoiding some biotic or abiotic stresses. Variations in the plant phenotype observed between persistent virus-infected and persistent virus-free plants could be related to the presence or absence of the virus. Under certain environmental conditions, plant size could be beneficial.

Results of the inoculation of acute viruses to common bean lines suggest that endornaviruses of common bean could interact synergistically with some acute viruses. In contrast, it is also possible that the activation of the plant immune system by persistent viruses could result in less severe diseases such as in the case of cross protection between acute viruses. More tests of acute and persistent virus combinations need to be done to determine the effect of persistent viruses in mixed infections with acute viruses.

It is possible that the plant lines used in the comparative experiments are not genetically identical and some of the differences we obtained in these preliminary studies may be due to genetic differences rather than the presence or absence of persistent viruses. It is important to emphasize that some effects that these viruses may cause to their host may be subtle and difficult to detect. Nevertheless, these effects could be significant for specific traits or the survival of the crop under certain environmental conditions.

The results of testing for the presence of persistent viruses in various crop cultivars, including analyses of their pedigree (Valverde & Sabanadzovic 2011; Zabalgogeazcoa *et al*. 1993), suggest that plant breeders and possibly people involved in earlier domestication of these crops (unknowingly) introduced these viruses into some cultivars of some crops but may have eliminated them from other crops. For example, in the case of *Bell pepper endornavirus* infecting bell pepper, all tested cultivars (18 of 18) were infected whereas only 2 of 50 rice cultivars were infected with *Oryza sativa endornavirus* (Okada *et al*. 2011; Valverde & Sabanadzovic 2011). It needs to be determined whether the presence of these viruses in newly developed crop cultivars provides them with a beneficial effect. As pointed out earlier, whether positive or negative, the effect may be subtle but nevertheless it could have a significant impact on crop yields or crop quality.

The development of a transmission method for these viruses and the generation or identification of virus-free lines of virus-infected cultivars are necessary steps that must be accomplished before we can conduct further studies on the interactions that these viruses may have with their hosts.

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Persistent Identifier: urn:nbn:de:0294-sp-2013-ppphe-2

Brinkmann N et al., Investigation of latent bacterial infections in callus cultures reveal new *Paenibacillus* species. In: Schneider C, Leifert C, Feldmann F (Eds), Endophytes for plant protection: the state of the art, pp. 10-11. © 2013 Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-11-2.

1-2 Investigation of latent bacterial infections in callus cultures reveals new *Paenibacillus* species

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ABSTRACT

Since more than 20 years the DSMZ collection of plant cell cultures maintains about 700 undifferentiated plant cell lines with a main focus on secondary metabolite producing cultures. These calli are widely used for fundamental and applied research. Twelve calli potentially carried latent bacterial infections, which seemed to be of symbiotic or mutualistic nature, as they stayed invisible without symptoms of callus damage for a long time of subcultivation. Within the framework of a Cost Action FA1103 research project these calli were investigated for endophytes using culturing methods as well as DNA- and RNA-based cloning approaches. The major objective of this study is to identify hidden endophytes and to uncover potential interactions between a callus and its microbial community. Scanning electron microscopy (SEM) and FISH analysis will clarify the spatial distribution of endophytes within the calli. Although endophytic bacteria can be found in almost all plant organs less information about endophytes in plant calli or effects on calli is available. Potential physiological and molecular interactions have not been investigated so far. Population densities of endophytes are highly variable and seem to depend on the bacterial species or host genotype as well as on environmental conditions (Hartmann *et al*. 2009). First results show that three of the investigated calli, i.e. *Ludwigia octavalvis* (Jacq.) P. H. Raven (Onagraceae), *Melanoselinum decipiens* (Schrad *et* H. L. Wendl.) Hoffm. (Umbelliferae) and *Stephania wightii* Dunn (Menispermaceae) harbor bacterial species of the genus *Paenibacillus* with less than 98 % sequence similarity (16S rDNA) to known species. The presence of these *Paenibacillus* species was confirmed by clone library analysis generated directly from callus material. In nature, plants select their specific endophytes out of a large pool of soil or rhizospheric species (Hartmann *et al*. 2009). Investigated calli are

just derivatives of plants, i.e. undifferentiated cells that are maintained for more than two decades in long-term culturing processes separated from any natural habitat. In this study, it will be tested, if detected bacteria are “true endophytes” with capacity to infect non-infected calli bringing benefits to the host. First efforts have been made to screen isolated bacteria in serial dilution tests for their biological activity. If the isolates originate from a rhizospheric bacterial community they may possibly exudate antimicrobial compounds. Preliminary results indicate that extracts of the *Paenibacillus* species show activity against Gram-positive bacteria and fungi providing first evidence of a rhizospheric origin.

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Persistent Identifier: urn:nbn:de:0294-sp-2013-ppphe-2

Nigris S et al., Is the bacterial endophyte community, living in Glera (*Vitis vinifera*) plants, active in biocontrol? In: Schneider C, Leifert C, Feldmann F (Eds), Endophytes for plant protection: the state of the art, pp. 12-16. © 2013 Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-11-2.

1-3 Is the bacterial endophyte community, living in Glera (*Vitis vinifera*) plants, active in biocontrol?

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ABSTRACT

This project aims at characterizing the molecular and functional properties of endophytic bacteria that colonize *Vitis vinifera* L. cv. *Glera* (Prosecco) in order to select plant growth-promoting bacteria as biocontrol agents to stimulate plant growth and improve soil and plant health. This work is focused on two antimicrobial abilities of endophytes: cyclic lipopeptides (LPs) and siderophores production. LPs are small cyclic peptides, belonging to fengycin, surfactin and mycosubtilin families, with known antimicrobial activities. The analysis of genes coding for LP synthetase, the enzyme which synthesize LPs, has demonstrated that 20% of the analyzed strains carry the gene encoding for at least one of the LP synthetases investigated. In addition, CAS-agar microbiological assay indicated that some of the isolated endophytic strains were able to produce siderophores. Future work will be required to verify the antimicrobial/antifungal activity *in vivo* and *in vitro* of selected strains and to determine their ability to re-colonize and move within plant tissues, in order to develop biocontrol growth-enhancing inoculants for *Glera* grapevine cultivations.

INTRODUCTION

Bacterial endophytes are bacteria that live inside plants colonising inner tissues of hosts without causing signs of plant diseases. Endophytes move into plants from soil through cracks due to emerging lateral root; from roots bacteria spread in leaves, flowers and fruits via vascular plant system (Hardoim *et al.* 2008; Compant *et al.* 2011). Bacteria inside the plant take advantage of a major availability of nutrients, and plants receive from bacteria

both protection against pathogens and enhanced growth. Plant growth stimulation by endophytic bacteria is largely due to phytohormone production, nitrogen fixation, phosphate solubilization and ammonium ion production. Endophytes improve plant health acting as biocontrol agents; bacteria protect host plants by the synthesis of a large spectrum of antimicrobial molecules and the production of siderophores for iron uptake, by nutrient competition with pathogens and by producing other molecules which are able to elicit systemic resistance (ISR, Induced Systemic Resistance) in colonized plant (Compant *et al.* 2010). One class of these molecules that plays an important role in biocontrol is that of cyclic lipopeptides (LPs). These amphiphilic compounds are formed by a short cyclic oligopeptide linked to a lipid tail (Pérez-Garcìa *et al.* 2011). Surfactin, mycosubtilin and fengycin are three families of LPs with different aminoacid sequence which show antimicrobial properties. Recently surfactins and fengycins have been identified as bacterial elicitors of ISR (Jourdan *et al.* 2009). Bacteria growing in iron starvation conditions produce a large variety of molecules to bind and uptake free ferric ions. These molecules, called siderophores, are secreted by bacteria in the environment and are classified as catecholates, hydroxamates, and α-carboxylates, depending on the chemical nature of their coordination sites (Pérez-Miranda *et al.* 2007). The CAS agar assay is often used to detect siderophores producers (Schwyn & Neilands 1987). In this test, siderophores secreted by bacteria sequester iron from CAS medium producing a colour change from blue to orange or purple. In this work 380 bacterial endophytes isolated from *Vitis vinifera cv. Glera* were screened for the presence of fengycin, surfactin and mycosubtilin synthetase genes and were assayed for siderophores production to identify and select strains that could be used for biocontrol in *Vitis vinifera* cultivation.

MATERIALS AND METHODS

Detection of LPs synthetase via PCR

Bacterial strains

Bacteria used in this work were endophytes isolated from surface sterilized leaves, stems and roots of *Vitis vinifera cv. Glera* sampled in 6 different vineyards located in Veneto, in the North-East of Italy. Endophytes were grown in Nutrient agar for 24 h, at 28 °C. *Escherichia coli*, grown in Luria-Bertani (LB) agar for 24 h, at 37 °C, was used as negative control for PCR screening.

PCR conditions

In this work, endophytes were investigated by PCR for the presence of three families of LPs synthetase genes: surfactin synthetase, fengycin synthetase and mycosubtilin synthetase. DNA from endophytes was extracted using an alkaline lysis protocol (0.05 M NaOH and 0.25% SDS) in a sterile tube. Extracted DNA was used as template for PCR analysis.

Persistent Identifier: urn:nbn:de:0294-sp-2013-ppphe-2

Degenerated primers for surfactin synthetase (As1-F and Ts2-R), fengycin synthetase (Af2-F and Tf1-R) and mycosubtilin synthetase (Am1-F and Tm1-R) were used to amplify the largest number of target sequences as described by Tapi *et al.* (2010). One positive band for each amplified sequence, revealed by DNA electrophoresis, was sequenced to confirm the positive result.

Siderophores production test

CAS agar assay was performed in order to detect the ability of grapevine endophytes to produce siderophores. Bacteria were streaked onto CAS agar Petri dish and incubated for 72 h in the dark at 28 °C. Bacteria produce an orange/purple halo in the blue medium when siderophores are secreted.

DISCUSSION

In this work 380 endophytes, isolated from *Vitis vinifera* cv. *Glera,* were investigated for the presence of LP synthetase genes by PCR analysis and for siderophores production using CAS agar assay. Amplification showed that the presence of LPs synthetase genes in the endophytic community of grapevine is rather common: about 20% of strains had at least one of the LPs synthetase gene (Figure 1A); two bacterial strains showed more than one of the target genes (Table 1) suggesting the possibility that these strains could act as biocontrol agents producing LPs inside plant tissues or at rhizosphere level. CAS agar assay revealed that many strains were able to sequester iron by siderophore production; in particular strain GL83, belonging to the genus *Pantoea*, showed a strong ability to produce these molecules as after 12 h a large yellow halo appeared (Figure 1B).

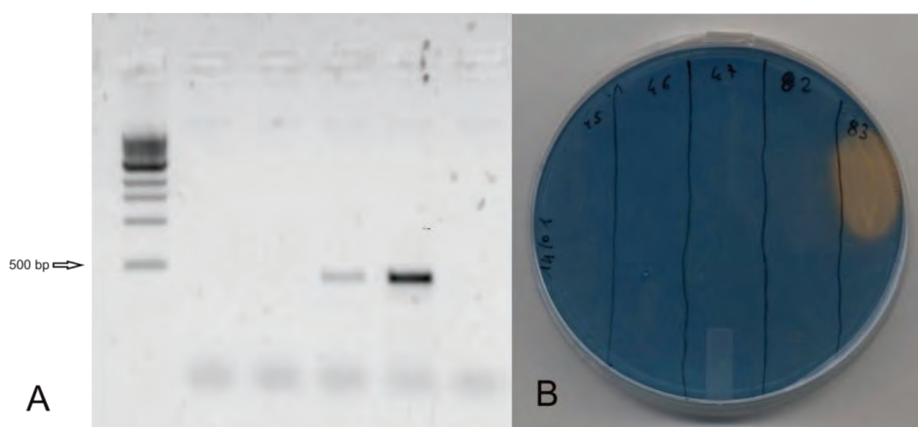

Figure 1. (A) PCR amplification by As1-F and Ts2-R with GL412; (B) CAS agar plate with positive GL83 strain.

Interestingly, both strains with all three LPs synthetase genes were positive also for siderophore production as shown in Table 1.

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Table 1. Positive strains for LPs screening and siderophore production

Strain	Surfactin synthetase	Fengycin synthetase	Mycosubtilin synthetase	Siderophore	Taxonomic group
GL412	+	+	+	+	*Bacillus*
GL412	+	+	+	+	*Bacillus*
GL83	-	-	-	++	*Pantoea*

These data showed that some strains have the necessary enzymes to produce some antimicrobial LPs and are able to produce and secrete siderophores in the environment. Biosynthesis of LPs and siderophores should be confirmed using analytical methods like HPLC or mass spectrometry. Thus this work gave first evidence, the presence of LPs synthetase genes and the siderophore production, of a possible bioncontrol action by endophytes isolated from different organs of *V. vinifera* cv. *Glera.* Further information will be needed to confirm if these strains could be used in grapevine cultivation against pathogens to reduce chemical treatments. *In vitro* e *in vivo* antagonism assays will be performed to investigate and quantify effects of endophytes on *Glera* plants infected by fungal or bacterial pathogens. Moreover, selected endophytes will be visualized, by confocal microscopy, inside tissues after re-inoculation with fluorescence-tagged strains to confirm their ability to colonize the whole plant. In conclusion this work is the first step for a deep characterization of endophytic community living in *Vitis vinifera* cv. *Glera,* towards the formulation of a bacterial consortium to improve plant growth and health. This will help to reduce chemicals used in vineyards in order to foster a sustainable grapevine cultivation in Italy .

ACKNOWLEGMENT

S.N. was recipient of a Ph.D. fellowship from MIUR, Italy. We thank COST Action FA1103 for supporting this work.

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In: Schneider C, Leifert C, Feldmann F (Eds), Endophytes for plant protection: the state of the art. © 2013 Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-11-2

1-4 Role of root-infecting fungi in plant-plant interactions

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ABSTRACT

Ascomycetous fungi were isolated from the roots of the grass *Festuca brevipila*, a dominant plant in a semi-managed grassland in northeastern Germany. The process of culturing, sequencing, and a BLAST search retrieve fifteen culture matches; most of them previously reported grass-associated root endophytes as well as pathogenic fungi. Those isolates have been used to set up greenhouse experiments to test how this fungi-root interaction may modify plant-plant interactions. One of these experiments aims to test how *F. brevipila* plants colonized with five isolates (*Periconia sp; Phomopsis sp, Drechslera sp; Microdochium sp* and *Gaeummanomyces sp*) differed in the competitive response to conspecific neighbors compared to non-colonized plants. To do that an hexagonal fan design is used in a factorial fashion, where the growth of a target individual of *F. brevipila* is monitored either when it has no con-specifics competitors or when it is challenged with 6, 18 or 36 neighbors and when plants are colonized by each of the five isolates.

Persistent Identifier: urn:nbn:de:0294-sp-2013-ppphe-2

In: Schneider C, Leifert C, Feldmann F (Eds), Endophytes for plant protection: the state of the art. © 2013 Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-11-2.

1-5 Studies into endophytes of potatoes

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ABSTRACT

Recently, a large international company producing crop protection agents released a product containing the bacterium Bacillus mycoides. Based on their studies, it is shown that B. mycoides is present in the rhizosphere and induces systemic acquired resistance in potatoes, protecting them against a variety of pathogens.

In this new PhD study, the goal is to find mechanisms by which potatoes favor " good" endophytes over "bad" pathogens. During trials and random sampling of potatoes, it became apparent that Bacillus mycoides is present in the tubers of many different cultivars of potatoes. This, together with its unique morphology and its known effect as a plant protector, makes it an ideal organism to study host-endophyte relations in potato. The poster aims to show the first results of this study (incl. experiments to be performed in early 2013).

Persistent Identifier: urn:nbn:de:0294-sp-2013-ppphe-2

Franco C; Smith S, Endophytic Bacterial Populations of Potato Plants. In: Schneider C, Leifert C, Feldmann F (Eds), Endophytes for plant protection: the state of the art, pp. 19-26. © 2013 Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-11-2.

1-6 Endophytic bacterial populations of potato plants

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ABSTRACT

The endophytic actinobacterial population of roots, leaves and rhizosphere of potato plants was examined using culture dependant methods and compared to molecular characterisation based on total Pyrosequencing analysis of bacterial 16S rRNA genes. The predominant genera isolated from surface-sterilised roots and leaves were *Streptomyces* and *Microbispora*, together with lower numbers of *Micromonospora*. When eubacterial primers were used the results from the Pyrosequencing showed at least 55 genera, the predominant ones being *Enterococcus, Pantoea, Teribacillus, Curtobacteria, Bacillus, Pseudomonas, Lactococcus, Exiguobacterium, Rhodococcus,* but very low levels of *Streptomyces*. Cluster analysis revealed that the populations in roots were similar to soil populations, but not to the leaf populations.

INTRODUCTION

Plants provide the perfect environment for colonisation by a vast array of microbes in their phyllo-, rhizo- and endospheres. Endophytic bacteria, defined as bacteria that can be isolated from visually asymptomatic, surface-sterilized plant tissues (Hallmann 1997) are extremely diverse, as each individual plant is considered to host one or more type of endophyte (Strobel & Daisy 2003; Qin Xing *et al*. 2011). Studying plant bacterial endophytes is important for understanding ecological interactions and for developing biotechnological applications (Ryan *et al*. 2008). Studies of agricultural and native plants show that endophytic bacteria have the capacity to control plant pathogens, and positively contribute to plant nutrient levels and promote plant growth (Hardoim *et al*. 2008; Compant *et al*. 2010; Reinhold-Hurek & Hurek 2011). Investigating the diversity of bacterial endophytes may lead to identification of novel natural biocontrol agents. Molecular-based approaches (e.g. rRNA cloning,

sequencing, terminal restriction fragment length polymorphism and Pyrosequencing) to the study of microbial ecology generally reveal a broader microbial diversity than can be obtained by traditional cultivation methods. In this paper, we used culture-dependant and –independent methods to identify and characterise endophytic bacteria in the roots, leaves and rhizosphere of two potato varieties grown in field trials under natural conditions.

METHODS

Collection of potato plants and soil

Nine varieties of potato plants (Table 1) were collected from sites in Virginia and Pinnaroo, South Australia. Two varieties (Bernadette and Russet Burbank) and their associated soil were collected 10-12 weeks post-planting from two areas in Australia; Penola, South Australia and Forthside, Tasmania.

Surface sterilization of potato plants

Potato plants and tubers were washed by gently scrubbing by hand under running water to remove loose soil and debris. The plants were then cut into two sections: roots and leaves/stems. The roots, leaves and stems were surface sterilized to remove surface bacteria and thus only permit detection of endophytic bacteria. Surface sterilization within a laminar flow cabinet was done by soaking the plant parts in 100% ethanol for one minute, 4% sodium hypochlorite for five minutes, 90% ethanol for 30 seconds, 5% sodium thiosulphate for five minutes and a final soak in sterile reverse osmosis (RO) water. The plant parts were then either plated onto isolation media.

Culture-Dependent endophyte isolation

Using methods that have been previously used in our laboratory (Coombs & Franco 2003), endophytic actinobacteria were isolated from the potato plant sections (root, leaf/stem, rhizosphere) of all nine varieties. The isolation media consisted of: Tap Water Yeast Extract agar (TWYE), Mannitol-Soya agar (MS), Humic acid Vitamin B agar (HV), VL70 Starch agar (VL70 S), VL70 Amino acid agar (VL70 AA), and VL70 Carboxy methylcellulose (VL70 CMC). Putative characterization of the isolates was based primarily on colony morphology.

Extraction of bacterial genomic DNA

In order to obtain the bacterial pellet freeze-dried potato samples were ground to a fine powder using a mortar and pestle, and then suspended in enzyme solution (0.1% macerozyme, 1.0% cellulase, 0.7 mol/l mannitol, 5 mmol/l N-morpholinoethanesulfonic acid, 9 mmol/l $CaCl_2$, 65 µmol /l KH_2PO_4, pH5.7)(Jiao *et al*. 2006). The suspensions were incubated for 16 hours with gentle agitation at 28 ^{o}C before subjected to differential centrifugation. First, the mixture was centrifuged at 200 g for five minutes, the supernatant

was collected and the pellet (i.e. 200 g-pellet) was discarded. This step was repeated three times and the pooled supernatants were centrifuged at 3000 g for 20 minutes and the pellet was collected. Microbial DNA was extracted from the 3000 g-pellet using the MoBIO PowerFood® DNA extraction kit. At SARDI, soil was dried in a 40°C oven. Genomic DNA extraction was performed on these dried samples using a proprietary method developed at SARDI. DNA yield was determined using a PicoGreen® assay and also by using a NanoDrop spectrophotometer (ND-8000; NanoDrop, Wilmington, DE, USA). Genomic DNA samples were subjected to 0.8% agarose gel electrophoresis at 100 volts for 40 minutes and bands were detected using a LAS-4000 and ImageQuant™ software (Fujifilm Life Science, Brookvale, NSW, Australia).

Sequencing of 16S rRNA genes and Pyrosequencing analysis of soil and plant samples

For the varieties Russet Burbank and Bernadette, the genomic DNA samples were sent for Pyrosequencing to Research and Testing Laboratory (Lubbock, Texas, USA).The 16S rRNA gene was amplified from the genomic DNA by PCR using the universal eubacterial pyrosequencing primers 28F (5' GAGTTTGATCNTGGCTCAG 3') and 519R (5' GTNTTACNGCGGCKGCTG 3'). The following cycling parameters were used: 94°C for two minutes, 25 cycles of 94°C for one minute, 50°C for one minute and 72°C for two minutes, and then 72°C for 10 minutes.

RESULTS

Culture-Dependent isolation of actinobacterial endophytes

Nine varieties of potato plants grown fields in South Australia and Tasmania were used to isolate actinobacterial endophytes from surface sterilized potato plant tissue. The number of isolates from each variety (Table 1) and the parts of the plants from where they were isolated (Table 2), and the putative characterization of the isolates, based primarily on colony morphology (Table 3) is shown below.

Culture-Independent endophyte characterisation

Pyrosequencing analysis was performed on DNA samples from the different parts of the plant and rhizosphere from Russet Burbank and Bernadette potato plants. This technique produced up to 3,000-4,000 sequence readouts with BLAST results to the genus level. The percentage of each genus present is shown in Table 4.

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Table 1. Number of actinobacteria endophytes isolates from nine potato varieties collected in Australia

Potato variety	Number isolated
Bernadette	26
Coliban potter	37
Desiree	60
Kestral	50
Maranca	75
White Lady	58
Nicola	19
Red Ruby	54
Russet Burbank	73
Total	452

Table 2 Part of the potato plant from where the endophytes were isolated

Plant part	Number isolated
Flower (Desiree)	35
Leaves	91
Tubers	99
Roots	227
Total	452

Table 3. Characterisation of actinobacteria endophyte isolates based primarily on morphological features

Genus	Number isolated
Microbispora	128
Kribbella	1
Micromonospora	7
Streptomyces	233
Sphaerisporangium	2
Not yet identified	81
Total	452

Table 4 Genus assignment for clones (%) from potato varieties Russet Burbank and Bernadette on the basis of Pyrosequencing analysis of 16S rRNA genes.

	RB SA		RB TAS			Bern		
Genus	R	S	R	L	S	R	L	S
Acidobacterium		3			3			4
Agrobacterium				1			3	2
Arthrobacter					3		2	13
Bacillus		17		10	1	90	3	6
Bradyrhizobium		3			2			
Chloroflexus					1			3
Clostridium		3						
Conexibacter		4			16			3
Curtobacterium				12			3	
Enterobacter	89		55	1				
Exiguobacterium				3				
Frigoribacterium				2			3	
Gemmatimonas					2			1
Hyphomicrobium		2						
Klebsiella	4		5					
Lactococcus				5				
Marmoricola		1			1			
Massilia								2
Methylobacterium		2		4			1	2
Microbacterium							4	
Mycobacterium		2						
Nitrosovibrio		3						
Nocardioides		2			3			4
Pantoea	5		38	16				
Patulibacter		4						
Patulibacter					16			3
Pseudomonas				4			50	1
Rhizobium		3			1			
Rhodococcus							3	
Rubrobacter								2
Sanguibacter				1			8	

Table 4 continued						
Sinorhizobium			2			
Solirubrobacter	2		10			2
Sphingomonas			1			2
Sporosarcina	4					
Stenotrophomonas					3	
Streptomyces	2		2			3
Terribacillus		36		8	4	
Thermomicrobium			3			3

RB SA=Russet Burbank South Australia grown; RB TAS =Russet Burbank Tasmania grown; Bern=Bernadette; Numbers in columns represents % of clones identified

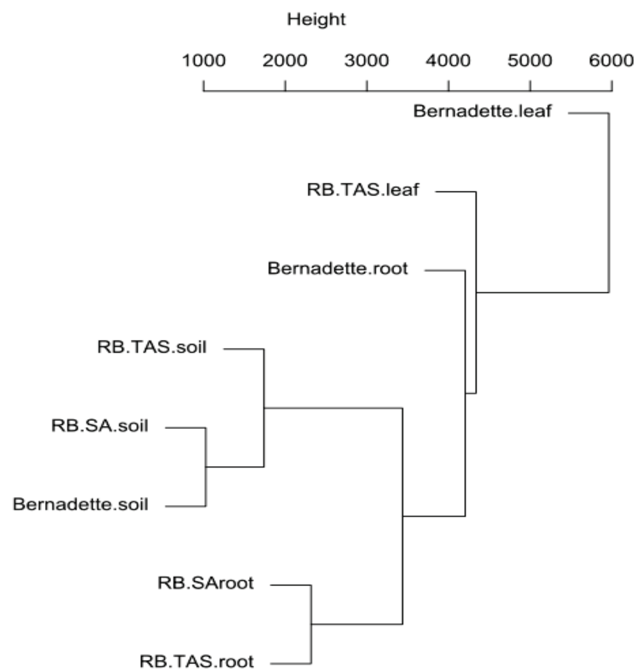

Figure 1. Cluster dendogram of soil vs. root microbial populations based on Pyrosequencing analysis

Hierarchical clustering was performed to observe the relatedness of the genera from the parts of the plants from the two varieties (Figure 1) showing Jaccard's similarity coefficient. The results indicated that the populations in the rhizosphere samples from the three locations are closely related, although those from Russet Burbank South Australia and Bernadette are more closely related to each other than from Russet Burbank Tasmania. Interestingly, the microbial populations in the two Russet Burbank root samples are closely related to each other, but not to the Bernadette root sample. The populations in the two leaf samples analysed do not appear to be very closely related to each other.

DISCUSSION

This study used a combination of culture-dependant and -independent methods to isolate and characterise endophytic and other bacterial populations from various potato plants, grown in South Australia and Tasmania, Australia. There were differences in numbers of isolates obtained between potato varieties; this is not necessarily due to a lower number of endophytes being present in the particular variety, but could also be the result of the presence of fungal or bacterial endophytes. Of the potato varieties tested, 50% of actinobacterial isolates were found in roots, which support other findings that this is an endophyte-rich organ (Manter *et al*. 2010). These results have shown that the endophytic community is diverse and that the most abundant genus recovered is *Streptomyces*, which is consistent with other reports from a variety of hosts (Qin *et al*. 2011). Next generation sequencing analysis, such as Pyrosequencing, has identified numerous new endophytic bacterial species, from many plant and rhizosphere types, including potatoes (Inceoglu *et al*. 2011). Our study aimed at identifying and characterising genera from two varieties of potato, grown at two locations, by 16S rRNA sequencing. The genera of bacteria present in plant roots grown in the two soils (South Australia and Tasmania) were unique to each location and only in a few cases did the same genera occur in both soils for all three varieties. The abundance of any one genus in any of the three rhizosphere samples did not exceed 17%, which supports other findings that this is a diverse and bacteria-enriched environment (Pisa *et al*. 2011). The roots of the two Russet Burbank consisted predominately of *Enterobacter* (89% for South Australian grown and 55% for Tasmanian grown), whereas for the Bernadette variety, *Bacillus* was 90% abundant and not present in either Russet Burbank samples. This finding suggests that there are different requirements for nutrient levels and growth between potato varieties, which has been shown previously (Inceoglu *et al*. 2011). The endophytic populations of the leaf samples were diverse, with *Terribacillus* (36%) being the most abundant genus for Russet Burbank (Tasmania), and *Pseudomonas* the most abundant for Bernadette (50%). Cluster analysis showed that, despite minimal overlap of genera, the rhizospheres of the two South Australia grown varieties (Russet Burbank and Bernadette) contained populations more closely related to each other than to Russet Burbank (Tasmania), which supports studies that found that diversity is determined by soil type and field locations (Weinert *et al*. 2010). The genera in the roots of the two Russet Burbank varieties were more closely related to each other than to microorganisms in Bernadette, supporting other work that investigated differences between cultivars (Inceoglu *et al*. 2011). This study has confirmed that by using selective media, endophytic actinobacteria were isolated form individual plant parts for nine potato varieties grown in Australia, with *Streptomyces* being the predominant species. The use of Pyrosequencing has provided a baseline for characterisation of endophytic bacteria in two varieties at two field locations. As the same effects were not found for both field experiments, probably because the genotype effect is strongly influenced by vastly differing environmental factors such as soil type or climate, further characterisation using increased numbers of cultivars and soil locations may be required.

Persistent Identifier: urn:nbn:de:0294-sp-2013-ppphe-2

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Persistent Identifier: urn:nbn:de:0294-sp-2013-ppphe-2

Haddad N; Krimi Z; Raio A, Endophytic bacteria from weeds promote growth of tomato plants in vitro and in greenhouse. In: Schneider C, Leifert C, Feldmann F (Eds), Endophytes for plant protection: the state of the art, pp. 27-32. © 2013 Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-11-2.

1-7 Endophytic bacteria from weeds promote growth of tomato plants in vitro and in greenhouse

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ABSTRACT

Eight endophytic bacteria belonging to *Pseudomonas* spp. and *Bacillus* spp. that were isolated from spontaneous plants were tested for their ability to promote tomato plant growth. Experiments were carried out according to two protocols: i) tomato plants grown *in vitro* on MS medium supplemented with the bacterial filtrates. ii) Tomato plants obtained from bacterized seeds grown under greenhouse conditions. The phytostimulation was assessed by the evaluation of some growth parameters (seed germination percentage, length of the stem and of the main root, fresh and dry weight of aerial parts and roots). The endophytic bacteria positively affected seed germination and stimulated plant growth both *in vitro* and in greenhouse. Tomato seedlings treated with the bacterial filtrates *in vitro* and plants from bacterized seeds showed an increase in all growth parameters compared to controls, suggesting an effect of stimulation of nutrient acquisition and hormone production.

INTRODUCTION

The exploitation of microbial flora activities in agriculture is due to the involvement of microrganisms in many fundamental processes related to soil fertility. Endophytic bacteria living inside plant tissues are an important source of natural products responsible of many beneficial effects on plants (Rosenblueth & Martínez-Romero, 2006) and potentially exploitable in medicine and industry too. Several endophytic bacteria stimulate plant growth and increase nutrient availability by providing phytohormones (Jacobson *et al.* 1994),

enzymes, antimicrobial and antibiotics (Bangera & Thomashow 1996) and siderophores (O'Sullivan & Gara 1992). Bacterial members of the genera *Pseudomonas* spp. and *Bacillus* spp. have been applied to a wide range of cultivated species to increase plant growth and biomass and to control diseases (Subramaniam & Balabaskar, 2012). Moreover, it has been reported that endophytes may increase resistance to plant pathogens and this ability makes them of particular interest (O'Sullivan & Gara 1992). The objective of this study was to evaluate the plant growth promotion ability of eight endophytic bacteria isolated from different weeds harvested from a fallow soil in Blida, Algeria.

MATERIAL AND METHODS

Preparation of bacterial suspensions and filtrates

Six strains of *Bacillus* spp. and two *Pseudomonas* spp. strains (Krimi *et al.* 2012) were screened for plant growth promotion activity. Strain species and origin are reported in Table 1. The eight bacterial strains were grown onto the culture medium LPGA at 28°C for 72h. A colony from each culture was suspended in LPG broth and incubated at 28°C for 24h. The concentration of each bacterial culture was determined by a spectrophotometer at λ 580 nm and bacterial concentration was adjusted to 10^8 CFU / ml. These suspensions were used directly for seed bacterization in greenhouse experiments. For the *in vitro* test, sterile filtrates containing the bacterial metabolites were obtained from the suspensions by filtration through 0.2 µm Millipore filters.

Table 1 Origin of bacterial strains

Bacterial strains	**Plant host**
Bacillus amyloliquefaciens OS4	*Urtica dioica*
Bacillus cereus EHR1	*Euphorbia helioscopia*
Bacillus pumilus OS2	*Urtica dioica*
Bacillus subtilis EHF5	*Euphorbia helioscopia*
Bacillus spp. CR1	*Calendula arvensis*
Bacillus spp. PF3	*Plantago lanceolata*
Pseudomonas spp. EPR3	*Euphorbia peplus*
Pseudomonas spp. PS1	*Plantago lanceolata*

In vitro experiments

Glass tubes containing 15 ml of sterile MS medium (Murashigue & Skoog 1965) were supplemented at the moment of pouring with a volume of 0.5 ml of each bacterial filtrate. Tomato seeds (*Lycopersicon esculentum* cv Saint-Pierre) were washed under running tap water and disinfected with a solution of sodium hypochloride (2%) for 5 min. Seeds were then dipped in 70% ethanol for 5 min and washed thoroughly three times with sterile distilled water (SDW). Tomato seeds were then placed onto the surface of the MS medium supplemented with the bacterial filtrates. The tubes were kept at room temperature (23 ± 2°C) under a 3000 lux light and a 12 hours photoperiod. For each bacterial strain twelve tubes containing one seed each, were used. Growth parameters were analyzed four weeks after sowing.

Greenhouse experiments

Seeds of tomato were surface-sterilized with 2% sodium hypochlorite for 2 min, rinsed thoroughly in SDW and then placed on sterile discs of Whattman paper inside Petri dishes. Tomato seeds were bacterized by covering them with the suspension of bacteria (10^8cfu/ml) and then incubated at room temperature at 28°C for 72h before sowing. In parallel, a mixture composed by equal parts of peat, sand and field soil was prepared and autoclaved twice for 20 min at 120°C with a 24 hours interval. Sterile substrate was distributed in plastic pots (6 x 6 x 5.5) where the bacterized seeds were transferred. Seeds were again bacterized with one milliliter of bacterial suspensions and then covered with soil. For each bacterial strain fifteen pots containing three seeds each were used. Treatments were arranged in a factorial experiment based on completely randomized design. Growth parameters were recorded four weeks after sowing.

Measurement of plant growth parameters and data analysis

Standing plants were counted and percentage of seed germination was calculated at the end of *in vitro* and *in vivo* assays. To evaluate the other growth parameters, plants were uprooted and aerial parts and roots were separated. Length of the stem and of the main root, fresh and dry weight of shoot and root biomasses of each plant were then determined. Data were statistically treated by ANOVA, at $P < 0.05$. The vigor index was determined using the formula: Vigor index= (mean root length + mean shoot length) × % germination (Abdul Baki & Anderson 1973).

RESULTS

Treatment of seeds with bacterial filtrates and suspensions significantly increased germination percentage, stem height, fresh and dry weight of aerial parts and roots. However, the rate of enhancement varied with bacterial strains. *Bacillus cereus* EHR1 and

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Pseudomonas spp. PS1 were the most effective strains both *in vitro* and *in vivo*. Only *Pseudomonas* spp. strain EPR3 was not always effective in greenhouse (Table 2).

Table 2. Effect of endophytic bacteria on growth characteristics of tomato seedlings in *in vitro* and in greenhouse conditions

Treatment		Stem l. (cm)	Aerial p. f. w. (g)	Aerial p. d. w. (g)	Root l. (cm)	Root f.w. (g)	Root d.w. (g)
CR1	*in vitro*	5,86*	0,093*	0,0056*	9,91*	0,0087*	0,00094*
***Bacillus* spp.**	*greenhouse*	23,20*	17,287*	0,3086*	10,44*	2,2596*	0,03151*
EHF5	*in vitro*	6,12*	0,084*	0,0053*	10,07*	0,0066*	0,00068*
B. subtilis	*greenhouse*	23,44*	17,307*	0,2880*	11,68*	2,1730*	0,02952*
EHR1	*in vitro*	6,33*	0,112*	0,0058*	10,62*	0,0070*	0,00080*
B. cereus	*greenhouse*	27,74*	18,413*	0,4146*	12,80*	2,1750*	0,04404*
EPR3	*in vitro*	5,71*	0,077*	0,0044*	9,91*	0,0061*	0,00078*
***Pseudom.* spp.**	*greenhouse*	22,43*	15,040	0,2661*	7,66	1,9318	0,02200
OS2	*in vitro*	5,62*	0,0730*	0,0040*	8,44*	0,0057*	0,00058
B. pumilus	*greenhouse*	24,85*	17,023*	0,2666*	10,63*	2,1331*	0,02541*
OS4	*in vitro*	06,41*	0,101*	0,0051*	9,48*	0,0122*	0,00109*
B. amyloliquef.	*greenhouse*	22,49*	16,610*	0,2590*	10,63*	2,0542*	0,02398*
PF3	*in vitro*	5,92*	0,105*	0,0055*	9,21*	0,0113*	0,00084*
***Bacillus* spp.**	*greenhouse*	23,17*	17,509*	0,3213*	9,95*	2,3285*	0,03364*
PS1	*in vitro*	6,51*	0,116*	0,0070*	9,55*	0,0129*	0,00134*
***Pseudom.* spp.**	*greenhouse*	25,66*	17,590*	0,3296*	9,96*	2,5679*	0,06898*
Control	*in vitro*	5,10	0,066	0,0035	6,79	0,0042	0,00050
	greenhouse	19,03	13,901	0,1850	7,21	1,6393	0,01932

*p<0,05 (significant) . Numbers represent the average value of single plant measurements; l = length; p = part; f. w. = fresh weight; d. w. = dry weight

All bacterial strains increased seed germination *in vitro* up to 83%, *Bacillus* spp. strain PF3 was the most stimulating (Fig.1a). The highest enhancement of vigor indexes was determined by *Bacillus* spp. PF3 and *B. amyloliquefaciens* strain OS4, which recorded 1515 and 1456 vigor index respectively (Fig.1a).

In pot trial, the percentage of germination increased up to 84% and *B. amyloliquefaciens* strain OS4, promoted the germination of all seeds (100%) (Fig.1b). *Bacillus cereus* EHR1, *Pseudomonas* spp. PS1, and *B. amyloliquefaciens* OS4 showed the highest vigor index with respective values of 3603, 3247 and 3213 (Fig. 1b).

DISCUSSION

In general, all tested endophytic bacterial strains were able to improve seed germination and to enhance tomato plant growth both *in vitro* and *in vivo*. Nevertheless, it appears that some strains were more effective than others. Similar improvement of seed germination parameters due to PGPR has been reported in other plants such as maize (Jarak *et al.* 2012).

Some beneficial bacteria may increase synthesis of hormones like gibberellins, which trigger the activity of specific enzymes that promote early germination, such as α amylase, which increase starch assimilation (Ma *et al.* 2011). The eight filtrates, such as the whole bacterial cells, induced a significant increase of root growth that may have positively influenced the development of the aerial part. This vegetative biostimulation can be due to secondary metabolites secreted by the bacteria that were present in the filtrates. It would be interesting to identify these molecules in the next future analyses.

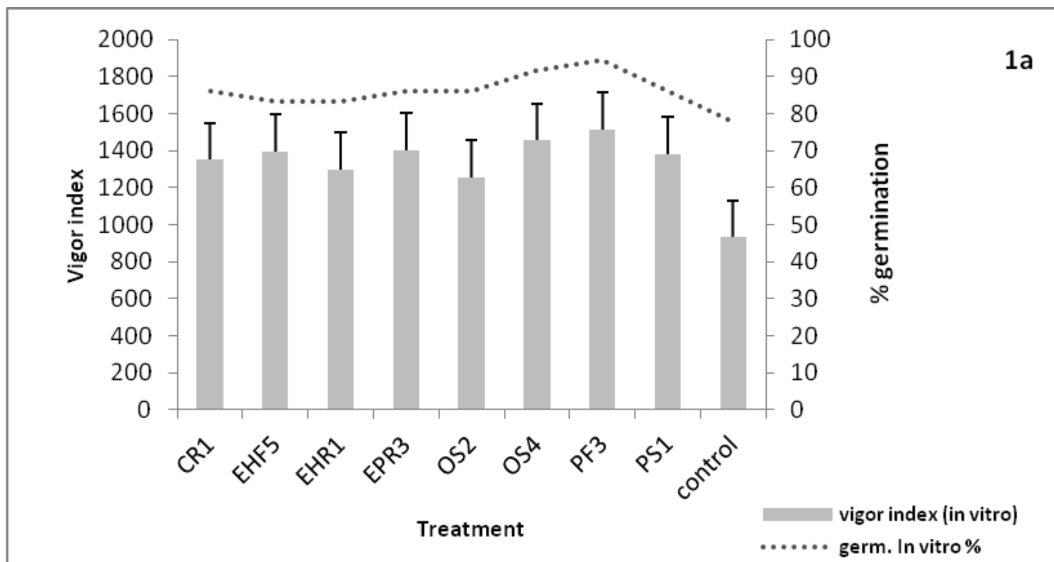

Figure 1a

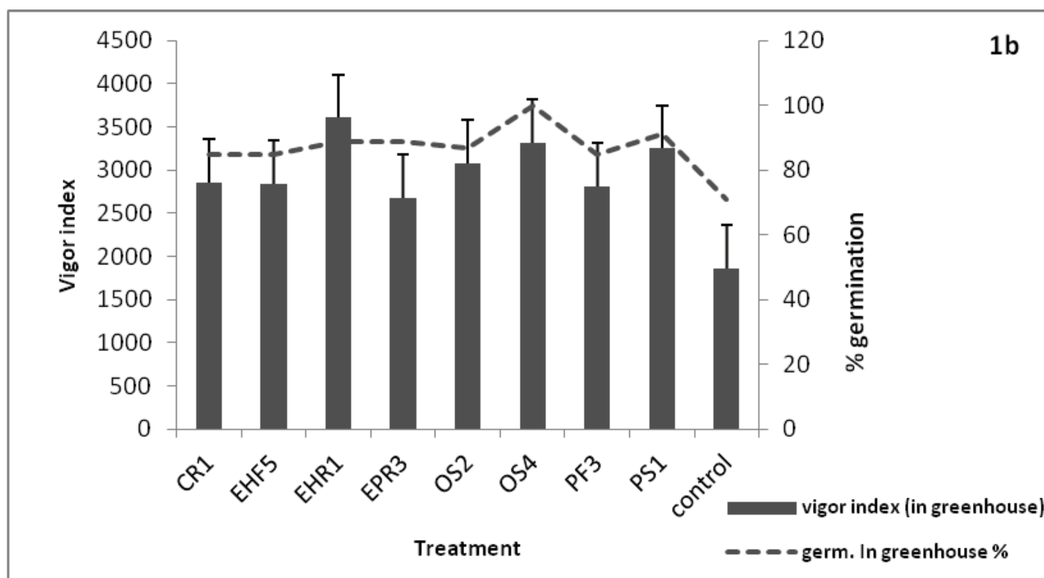

Figure 1b Effects of seed bacterization on germination and vigor of tomato plants in *in vitro* (1a) and in greenhouse conditions (1b)

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Persistent Identifier: urn:nbn:de:0294-sp-2013-ppphe-2

Soto-Barajas M C et al., Incidence and identification of endophytes *Epichloë/Neotyphodium* in wild populations of *Lolium perenne*. In: Schneider C, Leifert C, Feldmann F (Eds), Endophytes for plant protection: the state of the art, pp. 33-38. © 2013 Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-11-2.

1-8 Incidence and identification of endophytes *Epichloë/Neotyphodium* in wild populations of *Lolium perenne*

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ABSTRACT

Ryegrass (*Lolium perenne*) is one of the most important forage and turf grasses in temperate zones of the world and a host of fungal endophytes belonging to the *Epichloë* and *Neotyphodium* genera. These fungi produce several types of alkaloids toxic to herbivores (mammals or insects). We present here results of a study whose purpose is to survey genetic, taxonomic, and alkaloid variation of epichloid endophytes associated to wild populations of *L. perenne*, in order to identify fungal strains useful for the improvement of turf and forage cultivars. The results of a preliminary survey of 378 plants sampled at 8 different locations in western Spain indicated that 38% of the plants were infected by epichloid endophytes, and population infection rates ranged from 12% to 60%. A morphological classification allowed to classify the fungal strains in at least four morphotypes. Members of each morphotype were confirmed to belong to *Epichloë* or *Neotyphodium* genera by means of a partial nucleotide sequence of the β-tubulin gene and were classified in nine genotypes. These results indicate that there is considerable genetic variation among the epichloid endophytes present in wild populations of *L. perenne* in Spain.

INTRODUCTION

Perennial ryegrass (*Lolium perenne)* as the major turfgrass sown in Europe and other parts of the world (North America, Australia and New Zealand). Significant improvements have been achieved in turf quality, resistance to biotic and abiotic stresses, as well as in wear tolerance. However, some improvements such as insect resistance are yet to be performed This goal could be accomplished with the use of innovative methods and valuable native

genetic variability found in wild population of ryegrass (Sampoux *et al.* 2012). One of these methods is the use of fungal endophytes.

Some of the best known interactions between plants and fungi are those of grasses and clavicipitaceous endophytes referred as systemic, clavicipitaceous, balansiaceous, type 1, or epichloid endophytes (Clay & Schardl 2002, Schardl *et al.* 2004, Kuldau & Bacon, 2008, Rodriguez *et al.* 2009). In interactions between *Epichloë* and *Neotyphodium* endophytes (E/N) and grasses, the endophyte is provided with nutrition and a means of propagation within the host, and in return, endophyte-infected grasses contain several toxic alkaloids produced by the fungus which are toxic to herbivores (Clay & Schardl 2002). As variation on the type of alkaloid was observed among fungal strains and plants infected by endophytes, selection of fungal strains with suitable alkaloid profiles is an objective of interest for the forage and turfgrass seed industry (Vazquez de Aldana *et al.* 2010).

In order to select useful strains of endophytes for ryegrass improvement, it is desirable to have a wide fungal germplasm collection. In grasslands of western Spain, endophyte genotypic variability has been detected in *Festuca rubra* (Arroyo Garcia *et al.* 2002). The objective of this work was to determine the prevalence and to classify *Epichloë*/*Neotyphodium* endophytes from wild populations of *Lolium perenne* in Spain.

METHODS

Plant sampling

We sampled 378 plants of *Lolium perenne* at eight different locations in Spain (Table 1). The plant sampling was conducted in the late spring and early summer of 2012. Some plants presented in their flowering stems fungal stromata characteristic of *Epichloë typhina*. Plants were dug out and transported to Salamanca, where they were transplanted to pots and maintained outdoors in a greenhouse.

Endophyte isolation

The procedure used to isolate *Lolium perenne* endophytes was as follows: leaf sheaths from each plant were longitudinally cut in pieces of 2-5 mm, surface sterilized by washing for 10 min in a 20% commercial bleach solution (1.0% active chlorine), and rinsed in sterile water. The tissue pieces were then placed on plates of potato dextrose agar (PDA) containing 200 mg l^{-1} of chloramphenicol. The plates were incubated in the dark at 22 ºC and examined daily for the presence of endophytic mycelium emerging from leaf pieces. The *Epichloë/Neotyphodium* endophytes grew up after five or more days; other endophytes with faster emergence rates were discarded. To obtain cultures of epichloid endophytes, a small amount of the mycelium emerging from leaf pieces was picked and transferred to new PDA plates.

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Endophyte classification

The endophyte strains were grouped into morphotypes according to macroscopic characteristics of their cultures, taking into account characters such as color, texture, and growth rate. In a second phase, a genotypic classification of the strains was made based on the nucleotide sequence of a 5' region of the β-tubulin gene (*tub2*). This region was amplified by PCR using primers tub2-exon1d-1 and tub2-exon4u-2 (Moon *et al.* 2004). Both strands of each PCR product were sequenced, and sequence chromatograms were checked for the presence of ambiguous nucleotides, which could diagnose the hybrid condition of an epichloid endophyte (Moon *et al.* 2004). Finally, all sequences were aligned and clustered in a neighbor joining tree in order to detect distinct genotypes.

Table 1 Locations and other characteristics of the *Lolium perenne* populations analyzed.

Location	Province	Altitude (masl)	Number of plants	Habitat
Cedeira	Coruña	62	51	Coastal forest of Eucalyptus
Ciudad Rodrigo	Salamanca	625	25	Riverbank
La Vecilla	León	879	49	Agricultural land
Los Valles	Salamanca	813	50	Dehesa grassland
Montemayor	Salamanca	628	50	Chestnut forest
Porqueriza	Salamanca	807	50	Dehesa grassland
Tábara	Zamora	766	50	Oak forest
Valle Fuentes	León	1133	53	Low woodland

RESULTS

Incidence of epichloid endophytes in *Lolium* populations

The mean incidence of infections by *Epichloë/Neotyphodium endophytes* in the *Lolium perenne* populations studied was 37.7% (137 of 378 plants), ranging from 12.0% at Montemayor to 60.0% at Ciudad Rodrigo (Fig. 1).

Morphological classification of strains

The delay in the isolate emergence ranged from one week to six months. The 144 strains obtained were classified into four morphological groups: M1, white cultures with slow-growth and strongly aggregated 'brain-like' mycelium (Fig. 2a); M2, white, fast-growth with cottony aerial mycelium (Fig. 2b); M3, tan, with flat and smooth mycelium (Fig. 2c); the remaining group, NM, was composed of several strains with morphotypes different from those above described (Fig. 2d).

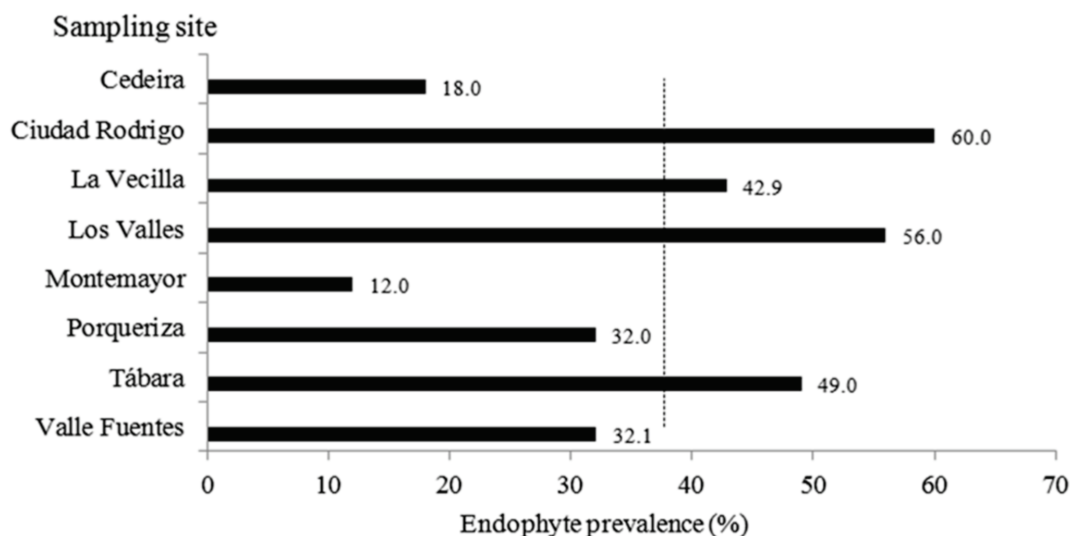

Figure 1 Prevalence of epichloid endophyte infections at eight populations of *Lolium perenne*. Average infection rate (---).

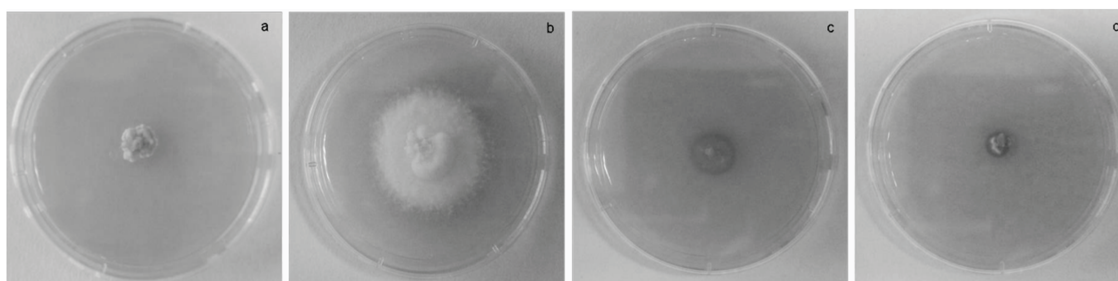

Figure 2 Four representative strains of *Epichloë/Neotyphodium* endophytes from *Lolium perenne:* (a) M1:'brain-like form' and slow growth; (b) M2:faster growing rate with cottony aerial mycelium; (c) M3: slow-growing strain with few or no aerial mycelium; (d) NM: show a mixture of characteristics from the other three groups already described above.

Strains belonging to different morphotypes occurred at all locations. Table 2 shows the morphotype distribution at each location. The most common morphotype was as M1 (44.4% of strains), followed by M3 (20.8%). The rest of the isolates were allocated with the following distribution: 18.7% for the NM group and 15.9% in group M2. All the endophytes isolated from plants of *L. perenne* which had fungal stromata were classified as M2 morphotypes.

There were seven cases where two morphologically different endophyte strains (belonging mainly to the groups M1 and M2) were isolated from the same plant. Mixed infections of epichloid endophytes have been previously reported in plants of *L. perenne* (Bony *et al.* 2001; Moon *et al.* 2004).

Table 2 Morphological classification of endophyte strains obtained at each location.

Location	Morphotype M1	M2	M3	NM	TOTAL
	Number of strains				
Cedeira	5	0	0	4	9
Ciudad Rodrigo	3	6	6	0	15
Los Valles	12	12	2	5	31
La Vecilla	17	1	0	4	22
Montemayor	3	0	0	4	7
Porqueriza	4	4	4	4	16
Tábara	12	0	14	1	27
Valle Fuentes	8	0	4	5	17
Total	64	23	30	27	144

Molecular classification

Sequence data of the β-tubulin gene (*tub2*) has been obtained from 51 strains of different morphotypes. The sequences could be classified in nine groups (G1 to G9), based in their nucleotide differences detected in alignments. Almost one third of strains (66.7%) shared the G1 genotype, while only two other groups had more than one strain, group G4 with 7 strains (13.7%) and group G3 with 4 strains (7.8%). The remaining six groups, (G2, G5, G6, G7, G8, G9), were conformed by single strains which together sum the remaining 12% of the strains.

A neighbor joining dendrogram was made up with each genotype and reference sequences of *Epichloë* and *Neotyphodium* species. The phylogenetic analysis produced a tree with two main clades, one including 6 genotypes (39 strains, 76.5%) with sequences similar to *Epichloë festucae and Neotyphodium lolii*, and another with 3 genotypes (12 strains, 23.5%) with sequences similar to that of *E. typhina* (Fig. 3). All strains obtained from plants having stromata were in the latter clade.

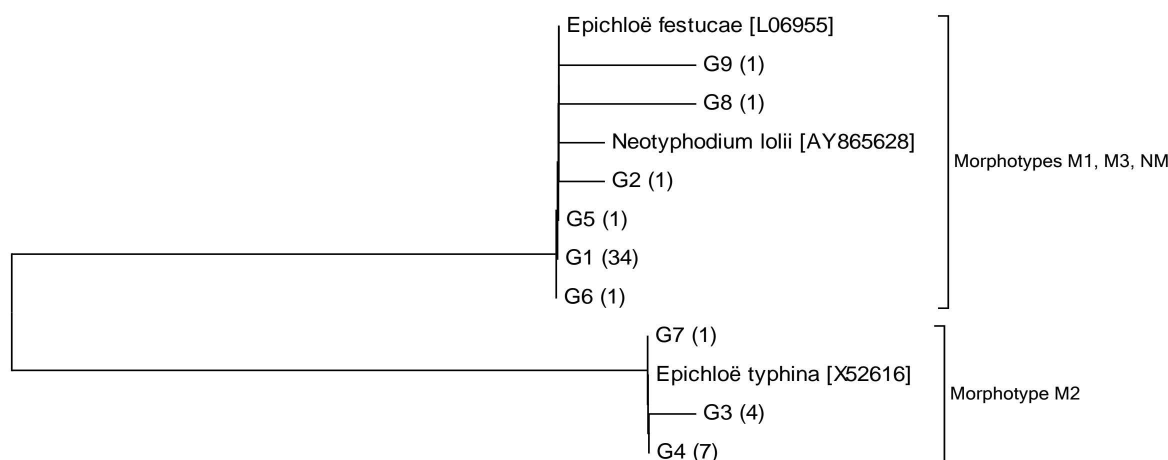

Figure 3 Tubulin based tree of endophyte genotypes (G1 to G9) detected in populations of *Lolium perenne.* Sequences of reference strains of *Epichloë festucae*, *Neotyphodium lolii* and *E. typhina* are included. (In parentheses the numbers of strains per group, in brackets the GenBank accession numbers).

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CONCLUSION

The incidence of epichloid endophytes in natural populations of *Lolium perenne* from Spain is variable, but relatively high, 37.7% on average. In view of the results obtained from the morphological classification of the strains in four or more groups, and more significantly, from the genotypic data, with 9 genetically different groups, we consider that the strains of epichloid endophytes isolated from wild populations of *L. perenne* are sufficiently variable to be used as fungal germplasm for a future research aimed at identifying strains with adequate alkaloid profiles needed for the improvement of *L. perenne* cultivars used for pastures or lawns.

ACKNOWLEDGEMENTS

This research was financed with project AGL2011-22783 granted by the Spanish Ministry of Economy and Competiveness. M.C. Soto was supported by a CONACyT (Mexican Council of Sciences and Technology) doctoral fellowship.

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Persistent Identifier: urn:nbn:de:0294-sp-2013-ppphe-2

In: Schneider C, Leifert C, Feldmann F (Eds), Endophytes for plant protection: the state of the art. © 2013 Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-11-2.

1-09 Diversity of endophytic fungi in irrigated and upland rice ecosystems in Kenya

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ABSTRACT

Irrigated and upland ecosystems are the only ecosystems of rice production in Kenya. Over 95% of the 920,000 tonnes of rice is produced under irrigation in Mwea, Ahero, west Kano and Bunyala paddy schemes. The remaining 5% is rain-fed. In these agro-ecosystems, rice interacts with various organisms including fungal endophytes. Such associations are mostly symbiotic (mutual), where the endophyte may improve host resistance against pests and diseases in return for carbon sources from their hosts. In this study, we isolated, characterised and compared novel fungal endophytes from rice roots in both irrigated and upland ecosystems through culture-dependent approaches. The results show *Epicoccum nigrum* as the dominant species in the irrigated ecosystem, which is now assessed alongside a few other selected isolates for their capacity to induce local and systemic resistance against plant-parasitic nematodes. Since endophytes may directly produce hormones or trigger plants to synthesize these compounds, the role of hormone signalling pathways is being analysed by comparing the level of nematode infestation in endophyte colonised wild-type, mutant or transgenic rice lines. Finally, building from previous results from our lab, which have shown direct roles of hormones in induction of host resistance, we aim to analyse by Q-PCR the expression of some genes known to be involved in defence against plant-parasitic nematodes in rice.

Persistent Identifier: urn:nbn:de:0294-sp-2013-ppphe-2

In: Schneider C, Leifert C, Feldmann F (Eds), Endophytes for plant protection: the state of the art. © 2013 Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-11-2.

1-10 Endophytic fungi in higher plants in Kyrgyzstan

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ABSTRACT

For the first time in Kyrgyzstan, studies were conducted to identify the species diversity of endophytic fungi - symbionts of higher plants found in different climatic conditions on the vertical zoning districts. The biological properties, distribution and ecological characteristics of the obtained isolates were studied. The dependence of the symbiotic existence of endophytic fungi in the body of higher plants in relation to environmental factors: temperature, humidity, density of grass was established.

Persistent Identifier: urn:nbn:de:0294-sp-2013-ppphe-2

SESSION 2

Ecology of Endophytes

Persistent Identifier: urn:nbn:de:0294-sp-2013-ppphe-2

In: Schneider C, Leifert C, Feldmann F (Eds), Endophytes for plant protection: the state of the art. © 2013 Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-11-2.

2-1 Metagenomics and genomics to reveal the ecology and functional potential of bacterial endophyte communities

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ABSTRACT

Endophytes are microorganisms colonizing plants internally without conferring pathogenicity to their host. Many of them have beneficial effects on plants as they may provide nutrients, antagonize pathogens and reduce plant stress symptoms. Plants are usually colonized by complex bacterial endophyte communities, which to a large extent are not easily to cultivate. We therefore have studied how endophyte communities are structured and affected by various parameters including soil, vegetation stage, plant genotype and plant stress. To obtain better understanding on the functional potential of (uncultured) endophyte communities we have applied function- as well as sequence-based metagenomic approaches. Furthermore, genomics of individual, cultured endophytes enables a better understanding of endophytic life-style strategies, interaction with the plant and functional characteristics.

Persistent Identifier: urn:nbn:de:0294-sp-2013-ppphe-2

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2-2 Halotolerant microorganisms associated with the plants in saline soil

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ABSTRACT

Soil salinization increased significantly during last decades and causes major problems for plant productivity. A promising strategy to improve crop salt tolerance can be the application of salt-tolerant (halotolerant) plant growth promoting microbes. However, high salinity constitutes an environmental stress also for microorganisms.

The research has been done in the industry and natural saline areas in Central Poland: salty meadow in the vicinity of soda factory (S1) and area affected with natural brine in the health resort (S2). Investigated plants belong to Chenopodiaceae - Salicornia herbacea L. (glasswort) and Asteraceae – Aster tripolium L. (sea aster). Bacterial and fungal strains were isolated and quantified from three zones with different influence of analysed plant: interior of plant roots - endophytes (E), rhizosphere - part of the soil closely adjacent to the plant roots (R) and soil not influenced by root exudates (S). Bacteria and fungi were obtained by plating the dilutions on appropriate agar mediums with different concentartions of NaCl (0, 100, 200, 400, 600 mM). In the studies on the metabolic biodiversity - Biolog®EcoPlateTM-microplates were used. For isolated strains a preliminary genetic analysis were carried out.

Significant differences between density and metabolic activity of halotolerant microorganisms associated with analysed zones of isolation: (E) – endophytes, (R) - rhizosphre and (S) – soil were observed. We have revealed changes in analysed parameters between two plant species (S. herbacea, A tripolium) and two analysed test sites (with industrial - S1 and natural - S2 salinity).

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2-3 Leaf-inhabiting endophytic fungi of European Beech (*Fagus sylvatica* L.) are rare in leaf litter and decaying wood of the same host.

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ABSTRACT

Many microfungi are able to live for a prolonged period of time in living plant tissues. In contrast to plant pathogens and parasites these so-called endophytic fungi do not cause obvious disease symptoms in their hosts. Nevertheless, they constitute an ubiquitous active component in direct and multi-trophic interactions. The present study was conducted to assess the level of overlap of cultivable fungal assemblages in living and decaying tissues of European Beech (*Fagus sylvatica* L.) from a forest stand in North-Eastern Germany. Fungal cultures were isolated from living leaves, leaf litter and dead wood still attached to the tree by dilution-to-extinction cultivation in the years 2007–2010. Analyses of species identity, species richness and species composition were predominantly based on the fungal DNA 'barcode' ITS (internal transcribed spacer). Species richness of microfungi isolated from litter equaled that of wood-inhabiting fungi and exceeded that of leaf endophytes. Although the most distinctive species assemblage was observed on wood, fungal species composition in living leaves and leaf litter were also significantly different from each other. However, a considerable compositional overlap between leaf and litter fungi was concertedly revealed by phylogenetic reconstructions, cluster analysis and non-metric multidimensional scaling. The taxa accounting most to the similarity between living and decaying leaves were assignable to Capnodiales, Xylariales, Diaporthales and Pleosporales. The exclusive but frequent isolation of Polyporales, clavicipitaceous strains, and genotypes related to the Xylariaceae *Hypoxylon rubiginosum* and *Nemania diffusa* from wood separated the wood-inhabiting fungal community from that in living and decaying foliage. Finally, a comparison of cultivated beech endophytes from sterilized leaves with a fungal 454 sequence dataset from unsterile beech phyllosphere might help in separating future 454 datasets into active fungal endophytes and "epiphytes" and non-functional fungal propagules.

Persistent Identifier: urn:nbn:de:0294-sp-2013-ppphe-2

Grosch R et al., Factors affecting disease suppression of plant associated bacteria towards bottom rot on lettuce and the indigenous microbial rhizosphere community. In: Schneider C, Leifert C, Feldmann F (Eds), Endophytes for plant protection: the state of the art, pp. 45-46. © 2013 Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-11-2.

2-4 Factors affecting disease suppression of plant associated bacteria towards bottom rot on lettuce and the indigenous microbial rhizosphere community

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ABSTRACT

Bottom rot on lettuce is caused by *Rhizoctonia solani* AG1-IB a soil-borne pathogen which affects various economically important crops worldwide. The pathogen can be responsible for significant crop losses where ever lettuce grow. The use of chemicals for control of the bottom rot pathogen is critical under consideration of pesticide residues in the final food product. Hence, the application of beneficial bacteria living in association with plants is promising among the control alternatives to the use of chemicals. The interesting candidates *Pseudomonas jessenii* RU47 and *Serratia plymuthica* 3Re4-18 with efficient disease suppression effects against *R. solani* in lettuce were selected in previous experiments. However, the efficiency of such inoculants varied in the field and the reason for this variability is largely unknown. Therefore, a better understanding of the complex interaction between bacterial inoculants and the plant rhizosphere microbial community under consideration of the soil type is required for a successful exploitation of the disease suppression potential. Hence, a field experiment has been set up with a unique plot system at the experimental station of the IGZ comparing the impact of three soil types on lettuce growth, disease suppression effects of the two inoculants and especially on the complex microbial interactions. This experimental approach made it possible to analyze the influence of the soil type independently from other factors such as climate and cropping history. First results showed that both inoculants colonized effectively the lettuce rhizosphere and

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decreased significantly the disease severity of bottom rot independent from the soil type. However, the disease severity of bottom rot seems affected by the soil type.

In contrast, the analysis of the indigeneous microbial community by denaturing gradient gel electrophoresis (DGGE) revealed highly significant differences in bacterial and fungal community pattern between the soil types. But the indigenous microbial community was not affected by the treatment of plants with the two inoculants. Hence, a negative ecological effect is unlikely by application of the bacterial inoculants. In conclusion the strains are promising biocontrol candidates and to propose for use in disease management.

Persistent Identifier: urn:nbn:de:0294-sp-2013-ppphe-2

Kolařík M et al., New endophytic species from the phloem of broadleaf trees. In: Schneider C, Leifert C, Feldmann F (Eds), Endophytes for plant protection: the state of the art, pp. 47-52. © 2013 Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-11-2.

2-5 New endophytic species from the phloem of broadleaf trees

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INTRODUCTION

Our research focuses on endophytes of the broadleaf trees as a potential source of fungi involved in the interactions with subcortical insects. Diversity of endophytic species (Koukol et al. 2012), their secondary metabolite production (Stodůlková et al. 2009), and life strategies of insect-associated fungi are studied (Kolařík et al. 2007; Pažoutová et al. 2010). A remarkable coelomycete with conidiomata dehiscing by a prominent rupture has been found among the most abundant taxa in *Salix alba* but only exceptionally in *Quercus*, *Ulmus*, and *Alnus* spp. It was described as a new genus *Liberomyces* phylogenetically close to the order Xylariales, with two species (Pažoutová et al. 2010). In the study of *Salix* and *Quercus* endophytes, Petrini and Fisher (1990) did not report any similar fungi.

In the phloem of *Ulmus laevis* another fungus was found, that was identified as an *Alternaria* species from *A. infectoria* species group. On the primary isolation plates this species showed unique antibiotic effects. *Alternaria* is a genus comprising filamentous fungi living as plant endophytes, pathogens or saprophytes, common in soil and dust and also causing animal mycoses (Thomma 2003). *Alternaria* toxicity to plant and animal cells stimulated study of their secondary metabolites, which are reported as mycotoxins and antibiotics with numerous biological functions (Aly et al. 2008; Feng & Yangmin 2010; Gu 2009).

METHODS

The endophytic and saprotrophic fungi in healthy, decaying and dead broad–leaved trees growing on alluvial floodplains were isolated using traditional surface sterilization and sliver cultivation techniques (Sieber & Hugentobler 1987). The fungi were isolated and maintained on malt extract agar. According to appearance, they were grouped to morphotypes. From representative isolates of each group, nrDNA sequences were obtained as described

previously (Pažoutová et al. 2012) and compared to the databases to confirm taxonomical identification. The phylogeny of *Liberomyces* and related species was inferred by using the Maximum Likelihood method based on the K 2+G model. The ITS1-5.8S-ITS2 dataset contained 555 positions, 102 of them were parsimony-informative. The phylogeny of *Alternaria* and related species was computed using the Maximum Likelihood method and default settings on a concatenated ITS1-5.8S-ITS2 and EF1-α sequence dataset. All positions with less than 30% site coverage were eliminated so that the final dataset amounted to 418 positions. *Brachycladium papaveris* was used as an outgroup. Both evolutionary analyses were conducted in MEGA5 (Tamura et al. 2011). Support values from 500x bootstrap higher than 60% are shown on the branches.

The secondary metabolite production was tested in shaken cultures on malt extract medium (malt extract, 20 g/l; glucose, 20 g/l; and peptone 1 g/l). The submerged cultivations were done in 250-ml Erlenmeyer flasks: The active metabolites from the culture were extracted with ethyl acetate, then partitioned between n-hexane and 90% MeOH. The 90% MeOH fraction was purified by chromatography over silica gel F254 (Merck, Darmstadt, Germany) with gradient elution (hexane/EtOAc/CH2Cl2/MeOH). The resulting fractions were tested for biological effects and further purified by a semipreparative HPLC. The structure of pure chemicals was assessed using MS, NMR and X-ray crystallography.

RESULTS AND DISCUSSION

Comparison of *Liberomyces* nrDNA sequences to databases has shown that several undescribed and/or uncultivated plant endophytes worldwide may belong to the same genus. The nrDNA sequence phylogenetic analysis (Fig. 1) has shown four possible groupings inside the genus. Two species of *Liberomyces*, *L. saliciphilus* and *L. macrosporus*, were described previously (Pažoutová et al. 2010) and to each of them related sequences have been found originating from broadleaf trees of temperate regions. Two species denoted as Asteromella formed the second clade. There are two species denoted as *Asteromella*. This coelomycetous polyphyletic genus resembles superficially *Liberomyces*. However, the species with very small bacilloid conidia (spermatia) belong to *Mycosphaerella* (Capnodiales) (Ramaley 1991; Sutton & Cole 1983) and *Asteromella tiliae* were placed among Pleosporales (de Gruyter et al. 2009), therefore a revision of *A. pistaciarum* order placement is necessary. The fourth clade contained closely related endophytes of plants from warm regions.

Recently, we obtained from the original authors the representatives of the latter two groups, namely *Asteromella pistaciarum*, a leaf pathogen of *Pistacia vera*, and an endophyte from *Coffea arabica* (Vega et al. 2010). The Italian isolate of *A. pistaciarum* formed pycnidia in culture and its bacillary conidia were remarkably small (Fig. 2a).The conidia size (3.4±0.3 × 1.0±0.1 μm) corresponded to that in the original description based on pycnidia from a host plant from Turkey (Bremer & Petrak 1947).

Persistent Identifier: urn:nbn:de:0294-sp-2013-ppphe-2

On the other hand, the conidia of the *Coffea* endophyte (Fig. 2b) were narrowly fusiform and much longer than any of the spores observed in *Liberomyces* so far (21.3±1.5 × 1.7±0.2 µm); therefore the clade of related endophytes was labeled "Megasporus" in Fig. 1. However, all observed species shared the mean conidia width under 2 µm. In *A. pistaciarum* and the *Coffea* endophyte the conidia were formed sympodially as in both *Liberomyces* species.

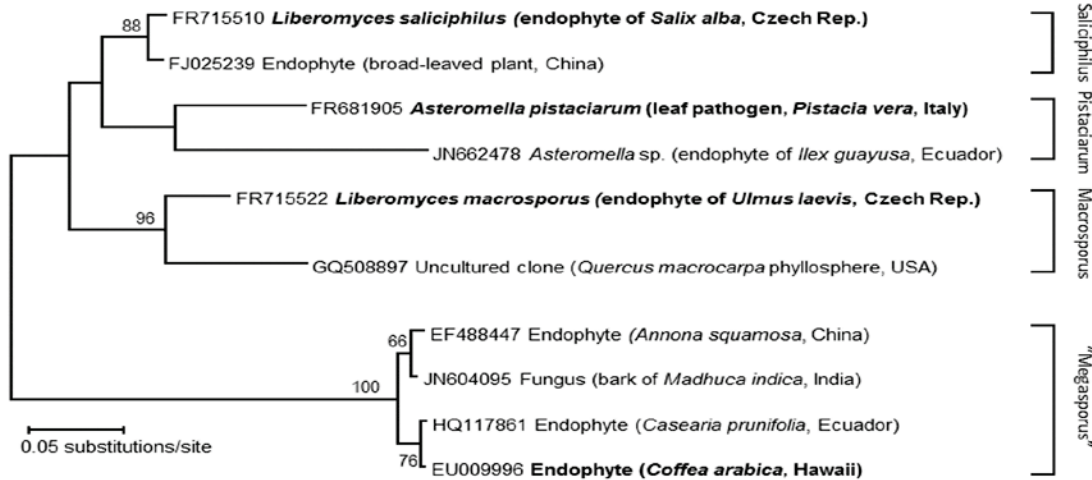

Figure 1. Phylogram of the *Liberomyces* – related fungi. Species labeled in bold were morphologically characterized.

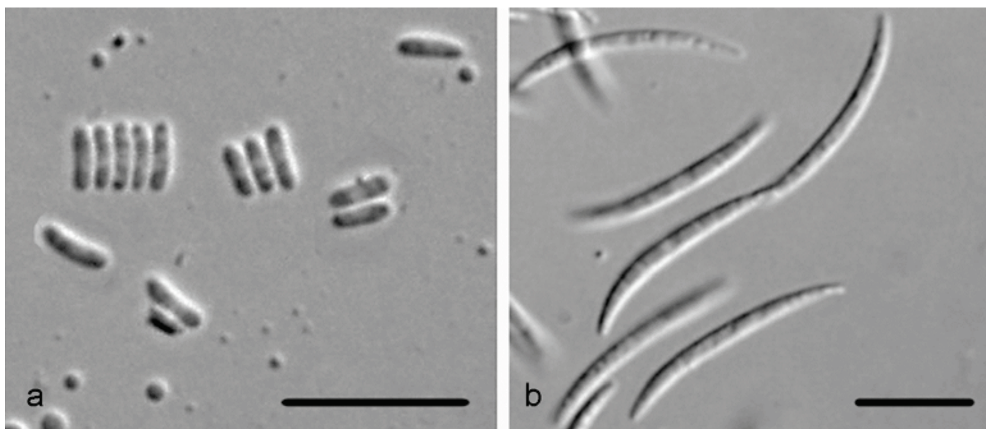

Figure 2. Conidia of *Asteromella pistaciarum* (a) and an endophyte of *Coffea arabica*. Bar – 10 µm

Phylogenetic analysis of *Alternaria* sp. AK165/08 placed the isolate to a separate clade (sister to *Alternaria infectoria*) consisting of endophytic fungi from various habitats and geographical locations (Fig. 3). The only strain belonging to the same phylogenetic group, identified to the species level, was *Alternaria rosae* JQ693639. The whole *A. infectoria* group, together with *A. rosae*, is curently under the polyphasis taxonomic study (Lawrence D.P. et al., University of Arizona) that could resolve taxonomic position of our isolate.

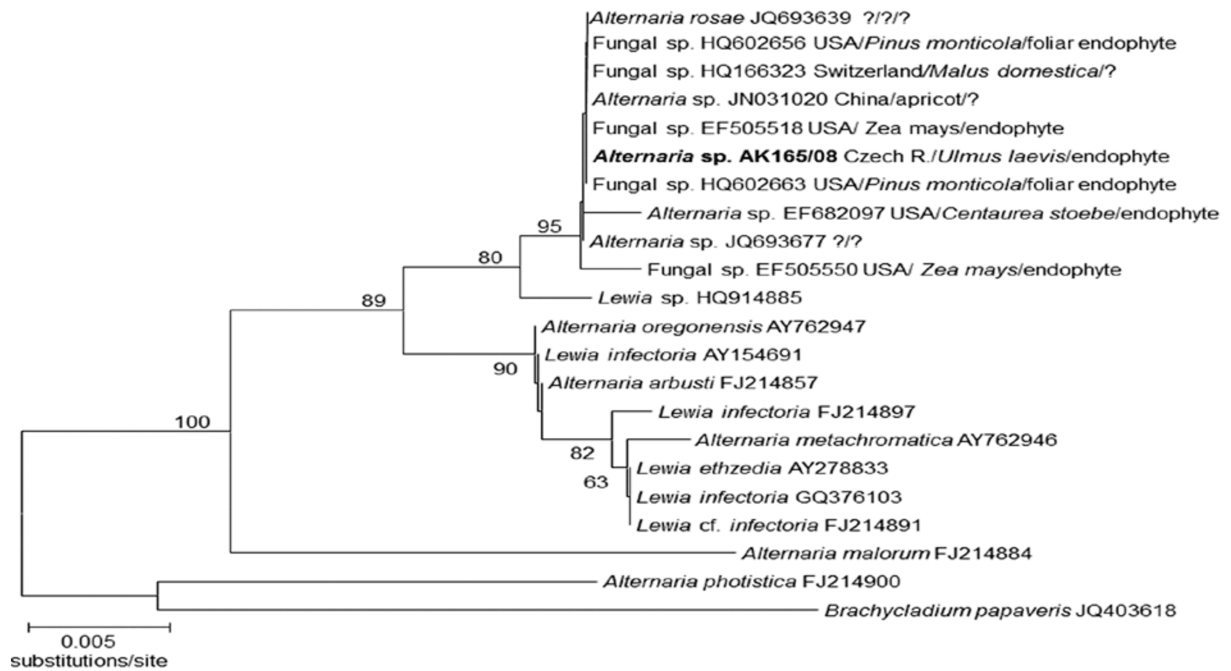

Figure 3. Phylogenetic analysis of *Alternaria infectoria* group and related isolates

On the primary isolation plates this species showed unique antibiotic effect against a highly aggressive fungus *Pyronema domesticum* (Fig. 4). A comprehensive analysis of natural products produced by *Alternaria* sp. AK165/08 revealed the production of diverse pyranonaphthoquinone antibiotics, which are known by a variety of biological activities (Sperry et al. 2008). Twelve chemical compunds were idenfied so far, together with new structures or chemicals firstly reported in natural producers (Stodůlková et al., unpublished). From the known compounds, Ascomycone B, discovered from unidentified ascomycete is reported as active against phytophathogenic fungi (Opatz et al. 2008). 6-Deoxyfusarubin, 6-deoxyanhydrofusarubin and their derivates that are known for antimicrobial activites (Opatz et al. 2008; Parisot et al. 1989, 1992) have been found. Due to its endophytic and mycostatic capacity, the new species has a potential as a biocontrol agent.

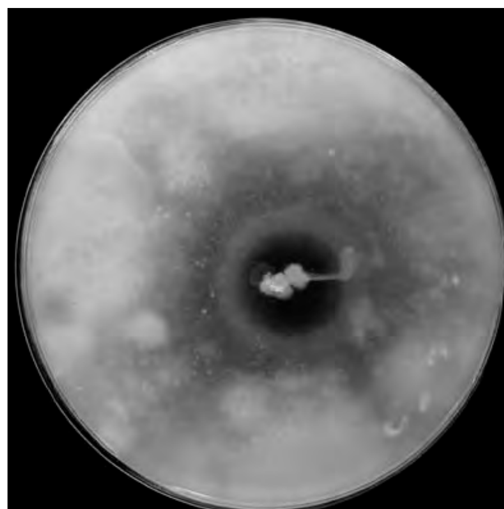

Figure 4 Antifungal action of *Alternaria* sp. AK165/08 against *Pyronema domesticum*.

Persistent Identifier: urn:nbn:de:0294-sp-2013-ppphe-2

ACKNOWLEDGEMENTS

Thanks are due to Dr Alessandra Belisario and Dr Fernando E. Vega for sending us the cultures of *Asteromella pistaciarum* and *Coffea* endophyte. The study was partially supported by a grant of Czech Ministry of Education (LD13039) related to a COST Action FA 1103.

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Persistent Identifier: urn:nbn:de:0294-sp-2013-ppphe-2

Koch E et al., Association of the loose smuts of wheat and barley with fusaria and *Acremonium*-like fungi. In: Schneider C, Leifert C, Feldmann F (Eds), Endophytes for plant protection: the state of the art, pp. 53-54. © 2013 Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-11-2.

2-6 Association of the loose smuts of wheat and barley with fusaria and *Acremonium*-like fungi

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INTRODUCTION

Ustilago nuda and *U. tritici*, the causal agents of the loose smuts of barley and wheat, respectively, grow endophytically during most of their life time. Following floret infection by smut spores, both fungi invade the scutellum where they remain in a state of dormancy until the seed starts to germinate. Within a few days after the onset of germination *U. nuda* and *U. tritici* start to colonize the apical meristem and leaf initials. The plants remain symptomless until emergence of the smutted ears. It has been noted that hyperparasites, usually species of *Fusarium*, may cover and permeate the sori of *U. tritici* with whitish to pinkish mycelium (Wilcoxson and Saari 1996). However, to our knowledge more detailled information on these hyperparasites and especially on the origin of the hyperparasitic inoculum is not available.

MATERIALS AND METHODS

Loose smut ears carrying fungal mycelium were observed on plants growing in the field and in the greenhouse. Spores of these superficially growing fungi were aseptically removed under a dissecting microscope and placed on potato dextrose agar (PDA) plates. Fungal colonies that developed were transferred to synthetic nutrient agar (SNA), and their taxonomic identity was determined based on morphology. In one preliminary experiment, suspensions containing spores of *F. proliferatum,* alone or in mixture with smut spores of *U. tritici*, were used for floret inoculation (Poehlmann 1945) of Apogee wheat (Bugbee et al. 1997). The resulting kernels were harvested, sown in pots, and after emergence of loose smut ears the latter were insepcted for the presence of hyperparasites.

RESULTS AND DISCUSSION

In most of the cases the fungi were present only on parts of the smutted ears, but occasionally the latter were fully covered with mycelium (Fig. 1). The fungi isolated from the loose smuts were identified as *Simplicillium lamellicola* (= *Verticillium lamellicola*) (from *U. tritici, U. nuda, U. avenae*), *Acremonium strictum* (from *U. nuda*), *Fusarium* sp. (from *U. tritici*) and *F. proliferatum* (from *U. nuda*). *Sarocladium bactrocephalum* (= *A. bactrocephalum*), a relative to *S. lamellicola* and *A. strictum*, has previosly been isolated from smuts (Gams 1971).

Figure 1: Wheat loose smut (*U. tritici*) covered with mycelium of *Fusarium* sp.. Note the chlorotic lesion on the sheath of the flag leaf (arrow).

The origin of the infections is not clear. Contamination by air-borne inoculum cannot be totally ruled out. However, at least in the case of *Fusarium* sp. on wheat, where the smutted ears were already substantially colonised when they emerged it appears likely that they became infected inside the plant. This would also explain the chlorotic spots on the flag leaf sheaths (Fig. 1) that may have resulted from the close contact with the *Fusarium*-colonized smutted ear. After floret inoculation with *F. proliferatum*, many of the kernels showed the “Black Point” symptom, a discoloration at the germ-end typical for infection with different fungal and bacterial pathogens. From such kernels *F. proliferatum* could be isolated, and some of the smutted ears on plants growing from these kernels carried mycelium of *F. proliferatum*. This indicates that entrance via the florets is a route for systemic infection of wheat with fusaria and may also lead to colonization of the developing smutted ear.

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Persistent Identifier: urn:nbn:de:0294-sp-2013-ppphe-2

SESSION 3

Host-Endophyte Interactions

Persistent Identifier: urn:nbn:de:0294-sp-2013-ppphe-2

Kloepper JW, McInroy JA, and Hu C-H, Association of plant damage with increased populations of deleterious endophytes following use of Benlate systemic fungicide. In: Schneider C, Leifert C, Feldmann F (Eds), Endophytes for plant protection: the state of the art, pp. 56-69. © 2013 Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-11-2.

3-1 Association of plant damage with increased populations of deleterious endophytes following use of Benlate systemic fungicide

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ABSTRACT

According to classical concepts of plant microbial ecology, microbes living inside plants are either pathogens or nonpathogenic beneficial or neutral symbionts. Hence, a common concept is that endophytic bacteria do not cause damage to the host plant, partly because they colonize at population densities lower than those of pathogenic bacteria. Evidence is growing, however, that a clear demarcation between pathogens and neutral or beneficial endophytes is not always present. A model is presented wherein a pertubation in rhizosphere and endorhizal microbial ecology, resulting from use of systemic fungicides, leads to increased populations of deleterious endophytic bacteria. In vegetatively propagated perennial plants, the deleterious endophytes consitute latent infections which can result in distorted growth or restricted plant development and can be passed to daughter plants produced via cuttings or rhizomes of the damaged plants. Examples will be presented in three plant systems: banana, citrus, and leatherleaf fern. In each example, applications of the systemic fungicide Benlate resulted in increased populations of endophytic fluorescent pseudomonads and total culturable bacteria. With citrus and leatherleaf fern, Benlate treatment also resulted in higher percentages of virulent endophytic bacteria as shown in the hypersensitive reaction in tobacco and the production of pectinolytic enzymes in the potato slice assay. Collectively the results and the emerging microbial ecology model indicate that the development of systemically active agrichemicals should include assessment of effects on endophytic bacteria.

Persistent Identifier: urn:nbn:de:0294-sp-2013-ppphe-2

INTRODUCTION

The term "endophytes" refers to microorganisms living inside plants, and the term has evolved often to include the connotation of microorganisms that are not harmful. For example, in the background material for this symposium (Anonymos 2013), it was stated that endophytes are "microbes that colonize living, internal tissues of plants without causing any immediate, apparent negative effects". This definition was then immediately followed by the following qualifying comments. "Growing without symptoms here means 'growing without visible damages'. While such a symptomless nature of endophyte occupation in plant tissue has prompted focus on symbiotic or mutualistic relationships between endophytes and their hosts, the observed biodiversity of endophytes suggests they can also be aggressive saprophytes or opportunistic pathogens" (Anonymous 2013). Opportunistic pathogens are microorganisms that typically exist in the plant environment without causing damage, but when there is a change in the system, such as reduced competition from indigenous microbes or stress on the plant host, the population of the opportunists increase and plant damage is seen.

In this article, the effects of Benlate systemic fungicide on endophytes and the corresponding effect on plant growth are presented and discussed. A review of previous work on leatherleaf fern (*Rumohra* adiantiformis) is presented first. Then selected results of new studies on banana and citrus are presented. A model is presented whereby Benlate is the trigger for a series of microbial changes that result in increased populations of deleterious endophytes and damage to plants.

REVIEW OF PUBLICATIONS RELATED TO FERN DISTORTION SYNDROME (FDS) AND THE ROLE OF BENLATE AND DELETERIOUS ENDOPHYTIC BACTERIA IN INCITING FDS

Leatherleaf fern (*Rumohra adiantiformis*) is a tropical ornamental plant of high economic value due to production of highly symmetrical, pyramidal-shaped, dark-green fronds used in flower arrangements. Costa Rica is a major producer of leatherleaf fern and exports the product to markets in North America and Europe. Growers began reporting an increase in irregularly shaped fronds in the mid-1990s (Kloepper *et al.* 2010), and production decreased substantially as a result. In 2010, the problem was described for the first time in the literature (Kloepper *et al.* 2010) and was termed fern distortion syndrome (FDS). The main diagnostic symptom of FDS is distorted growth of fronds, which can range from a slight bending of the frond tip to severe twisting and loss of the symmetrical shape of the frond. Other symptoms associated with FDS that make fronds unmarketable include thickening of mature fronds, uneven patterns of sporulation, and the presence of yellow, red, or bronze streaking on the pinnae of fronds. Below-ground symptoms of FDS may include reduced root growth, small-diameter rhizomes, and internal discoloration of the rhizomes.

Prior to the description of FDS in 2010, distortions of leatherleaf fern had been noted in Florida, and their appearance was reported to coincide with the use of Benlate DF systemic fungicide (Mills *et al.* 1996). Kremer *et al*. (1996) compared fluorescent pseudomonads from the rhizosphere of Benlate-treated or nontreated ferns for their capacity to be “allelopathic” (deleterious) in a lettuce seed bioassay. Their results showed that 80% of the pseudomonad strains from Benlate-treated plants were deleterious compared to 6% of strains from control plants. These two studies suggested that rhizosphere communities of deleterious pseudomonads triggered by Benlate DF might be the cause of distorted ferns. A modification of this suggestion was investigated in the 2010 study (Kloepper *et al.* 2010) by examining the association between FDS and endophytic fluorescent pseudomonads and total culturable bacteria. Paired samplings of rhizomes from symptomatic and healthy-appearing ferns in six commercial ferneries in Costa Rica revealed that endophytic populations of fluorescent pseudomonads were significantly greater at all six locations and populations of total culturable bacteria were greater at five of the six locations in symptomatic than in healthy-appearing plants.

In 2012, results of two-year greenhouse studies on the effects of Benlate on leatherleaf fern were published (Kloepper *et al.* 2012). The trials were conducted to test the hypotheses that 1) Benlate treatment of leatherleaf fern leads to long-term increases in populations of deleterious fluorescent pseudomonads endophytically colonizing rhizomes; and 2) endophytic colonization of rhizomes following treatment with Benlate is associated with FDS symptoms. To avoid the possibility of latent infections inside rhizomes of field-collected ferns, commercial tissue culture plants were used in this study. Treatment with Benlate WP and Benlate DF resulted in increased populations of pseudomonads inside rhizomes compared to controls after 24 months. At this time, ferns treated with Benlate had reduced frond weight per plant. Benlate treatments also resulted in significant increases in the severity of FDS using a rating scale that assessed the degree of frond deformations. Benlate also led to increases in the number of newest fronds with a twisted rachis and decreased diameter of rhizomes, which are two additional symptoms of FDS. Dwarfing of plants (severe stunting) was exhibited by half of the plants treated with Benlate 50 DF but by none of the control plants.

In the same study (Kloepper *et al.* 2012), the development of FDS symptoms by Benlate treatments corresponded with significant increases in the endophytic populations both of fluorescent pseudomonads and total aerobic culturable bacteria in both studies. Also, assessing the incidence of rhizomes containing detectable populations of endophytic pseudomonads showed that 75-90% of rhizomes on plants treated with Benlate contained fluorescent pseudomonads compared to 20% of control rhizomes. There was also a marked increase in pectinolytic enzyme activity of endophytic pseudomonads inside rhizomes of Benlate-treated plants compared to control plants. DNA sequencing of fluorescent pseudomonads isolated from inside rhizomes or petioles indicated that Benlate treatments resulted in pronounced shifts in phylogenetic clusters of endophytes. Overall, the two-year study showed that treatment of leatherleaf fern with Benlate 50 WP and Benlate 50 DF

caused a sequence of long-term deleterious effects that were associated with increased populations of fluorescent pseudomonads that were functionally and phylogenetically distinct from bacteria inside control plants.

Confirmation that the main symptoms of FDS are caused by endophytic fluorescent pseudomonads was presented in 2013 (Kloepper *et al*. 2013). A collection of 47 strains of fluorescent pseudomonads was used in this study: 17 strains isolated from inside rhizomes of healthy-appearing ferns grown in Florida in a fernery with no history of Benlate use, 14 strains from the surface of roots and rhizomes of symptomatic plants in Costa Rica, and 16 strains from inside rhizomes of symptomatic plants in Costa Rica. Phylogenetic analysis of the strains revealed that they fit into five clusters and that there were clear differences between strains from healthy ferns and strains from symptomatic ferns. The same strains were characterized for three traits that have been reported to be related to virulence: elicitation of the hypersensitive reaction (HR) in tobacco leaves (Mathesium *et al.* 2003), production of pectinolytic enzymes (Berg *et al.* 2005), and production of indole acetic acid (IAA) (Preston 2004). Differences in the frequency of HR and potato slice, but not IAA, were noted between strains from symptomatic and healthy ferns. None of the pseudomonads isolated from inside rhizomes of healthy plants without a history of Benlate use elicited HR. In contrast, HR was elicited by 77% of the strains isolated from inside rhizomes or from the rhizosphere of diseases symptomatic ferns. Pectinolytic enzyme production was exhibited by 46% of the strains from symptomatic plants but from none of the pseudomonads from healthy plants.

In the same study (Kloepper *et al.* 2013), micropropagated ferns from tissue culture were grown for one year to produce rhizomes free of possible latent infections of endophytic bacteria from fields. The rhizomes were inoculated by dipping into 9 different treatments: water (control) and two concentrations of four mixtures of bacteria. Bacterial mixtures included the group of endophytic bacteria from rhizomes of healthy plants and three groups from the plants exhibiting FDS: two groups of bacteria from inside rhizomes and one group from the rhizosphere. At 12 months after inoculation, all the main symptoms of FDS were recreated with the strains of fluorescent pseudomonads isolated from diseased but not from healthy plants. Surprisingly, many of the secondary symptoms of FDS were also recreated by the pseudomonads from diseased plants, including thickening of older fronds, reduced overall growth sometimes resulting in dwarfing of plants, the presence of red or yellow streaks on the pinnae of fronds, an irregular pattern of sporulation, reduced size of new rhizomes, and internal discoloration of rhizomes. Ferns inoculated with pseudomonads from healthy plants had none of these symptoms. In fact, inoculation with bacteria from healthy plants significantly increased one parameter of plant growth, frond width, compared to the water control. This finding shows that some endophytic bacteria in rhizomes of healthy plants are beneficial to plant development, which is consistent with studies on plant growth-promoting rhizobacteria.

Persistent Identifier: urn:nbn:de:0294-sp-2013-ppphe-2

EXPERIMENTAL WORK ON BANANA

Methods

An experiment was conducted to determine the effects of Benlate on plant growth and populations of endophytic bacteria in banana. Banana (*Musa acuminata*) 'Dwarf Cavendish' plantlets from tissue culture were grown for 3 months in a 1:1 mixture of field soil and sand in a greenhouse prior to applying treatments. The experiment was a randomized complete block with 6 treatments, each with 10 replicate plants. Treatments were foliar sprays or drenches of water (controls) and two formulations of the commercial fungicide Benlate from DuPont—Benlate 50 DF and Benlate 50 WP. Benlate applications were made at 1.9 g/L, which is equivalent to the label rate of 2 lbs per acre in 125 gallons of water. Foliar sprays were applied by spraying leaves to the point of run-off. Drench applications were made by applying 50 ml per pot. Treatments were applied three times at monthly intervals. The experiment was conducted twice: once for destructive sampling 6 months after the first application of treatments and once for destructive sampling 15 months after applications.

Destructive sampling was done by removing each plant from the pot and washing roots. In experiment one, treatment effects on plant growth were determined by measuring plant height, shoot fresh weight (stems + leaves), and root dry weight. In experiment two, effects on plant growth were determined by measuring the stem diameter, stem height, stem weight, weight of the main rhizome, and weight of the new (daughter) rhizomes. In both experiments, populations of endophytes inside stems were determined by surface disinfesting stem tissue by soaking in 80% ethanol for 2 min and then in 20% bleach for 2 min followed by rinses in sterile distilled water. After trituration in mortars, dilution plating was performed on 50% King's medium B for fluorescent pseuduomonads and on 10% tryptic soy agar for total culturable bacteria. After incubation for 48-72 hours at room temperature, colonies were counted to determine mean log cfu/g.

Results

Both formulations of Benlate, applied as drenches and foliar sprays, resulted in significant reductions in all of the measured parameters of plant growth at 6 and 15 months after the first application (Tables 1A and 1B). At 15 months, significant growth reductions were noted in the diameter, height, and weight of the stem as well as the weight of the original rhizome and new (daughter) rhizomes (Table 1B and Figure 1). These decreases in plant growth after Benlate applications were associated with increases in the endophytic populations of fluorescent pseudomonads and total aerobic bacteria (Tables 1A and 1B).

Persistent Identifier: urn:nbn:de:0294-sp-2013-ppphe-2

Table 1A. Effect of Benlate on endophytic bacteria and growth of Banana 6 months after first application of Benlate

Treatment	Plant height (cm)	Shoot fresh weight (g)	Root dry weight (g)	Populations of endophytic bacteria inside stems (log CFU/g)	
				Fluorescent pseuduomonads	Total aerobic bacteria
1. Benlate 50 WP foliar spray	31.8*	341.4*	13.05*	1.56	4.43*
2. Benlate 50 DF foliar spray	32.7*	364.6*	11.13*	2.78*	4.54*
3. Water control foliar spray	42.0	636.9	24.82	0.53	3.47
4. Benlate 50 DF drench	32.1*	386.4*	11.54*	2.37*	4.62*
5. Benlate 50 WP drench	32.2*	380.2*	12.90*	2.48*	4.70*
6. Water control drench	42.0	650.1	22.92	0.59	3.36
$LSD_{0.01}$	2.2	48.1	4.35	1.53	0.51

Values shown are means of 10 plants per treatment.
* Indicates significantly lower value than the corresponding water control at $P = 0.01$

Table 1B. Effect of Benlate on endophytic bacteria and growth of Banana 15 months after first application of Benlate

Treatment	Stem diameter (cm)	Stem height (cm)	Stem weight (g)	Main rhizome weight (g)	Weight of new rhizomes (g)	Populations of endophytic bacteria inside stems (log CFU/g)	
						Fluorescent pseudo-monads	Total aerobic bacteria
1. Benlate 50 WP foliar spray	6.13*	50.5*	956*	386*	207*	2.58*	4.95*
2. Benlate 50 DF foliar spray	5.80*	48.5*	850*	324*	178*	2.47*	4.89*
3. Water control foliar spray	8.19	58.9	1375	0680	253	0.79	3.56
4. Benlate 50 DF drench	6.06*	49.5*	813*	373*	171*	2.45*	4.86*
5. Benlate 50 WP drench	5.81*	47.4*	775*	306*	178*	2.56*	4.97*
6. Water control drench	8.25	59.8	14563	669	283	0.81	3.63
$LSD_{0.01}$	0.64	2.72	169	88	75	0.93	0.60

Values shown are means of 10 plants per treatment.
* Indicates significantly lower value than the corresponding water control at $P = 0.01$

Figure 1. Effect of Benlate on growth of banana plants 6 months after first spray with Benlate DF (left) and water (right); lower photo is close-up of stem, showing smaller diameter on Benlate-treated banana.

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EXPERIEMNTAL WORK ON CITRUS

Methods

An experiment was conducted to determine the effects of Benlate on plant growth and populations of endophytic bacteria in citrus. Citrus plants used were orange variety Valencia budded onto Swingle rootstock. Plants were obtained from a commercial citrus nursery in Florida and were transplanted into citrus pots (25 cm square X 35 cm deep) containing a 1:1 mixture of field soil and sand. The experiment was a randomized complete block with 6 treatments, each with 8 replicate plants. Treatments were foliar sprays or drenches of water (controls) and two formulations of the commercial fungicide Benlate from DuPont—Benlate 50 DF and Benlate 50 WP. Benlate WP and DF were applied at the same rate and the same monthly frequency as in the banana test. The experiment was conducted twice, once for destructive sampling 7 months after the first application of treatments and once for destructive sampling 16 months after applications.

In experiment one, treatment effects on plant growth were assessed at 5.5 months after the first application of treatments by measuring the cumulative length of new shoot growth and stem caliper. At 7 months after treatment, plants were removed from pots and roots were washed. Roots were scanned with a WinRhizo root analyzer, and three aspects of root architecture were measured for each plant: total root length, surface area, and total number of root tips. At the same time, the endophytic population densities of fluorescent pseudomonads and total aerobic bacteria inside stems were determined as described for the banana experiment.

In experiment two, some plants began blooming about one year after the first application of treatments. The number of blooms and small fruits per plant were counted 15.5 months after the first application of treatments. The experiment was destructively sampled at 16 months after the first application of treatments. Roots were washed, and fresh weights of shoots and roots were measured. In addition, treatment effects on root architecture and on populations of endophytic bacteria in stems were determined as in experiment one.

Results

In experiment 1 (Table 2A), both formulations of Benlate applied as foliar sprays and as drenches resulted in significant reductions in growth of shoots, as determined with length of new shoots, stem caliper, and reductions in growth of roots as measured by reduced total root length, root surface area, and total number of root tips per plant. Examples of reduced growth of stems and roots following applications of Benlate are shown in Figures 2 and 3. Mean populations of endophytic bacteria, both fluorescent pseudomonads and total aerobic bacteria, were significantly greater in all treatments with Benlate than in the water-treated control plants.

In experiment 2 (Table 2B), at 15.5 months after the first applications of Benlate, control plants were blooming and setting fruit. Blooming was significantly reduced by all treatments

with Benlate, and no fruits were present on the Benlate-treated plants. At 16 months (Table 2C), weights of shoots and roots were significantly lower for all Benlate treatments than for controls. Analysis of root system architecture revealed the same results as in experiment 1, that Benlate treatments had reduced total root length, root surface area, and total number of root tips, compared to controls. Also at 16 months after treatment, population densities of endophytic bacteria inside stems of Benlate-treated plants were significantly greater from those of control plants.

Table 2A. Citrus test 1, effect of Benlate on endophytic bacteria and growth of Valencia orange at 5.5 and 7 months after first application of Benlate

Treatment	Measurements of plant growth at 5.5 months		Measurements of root architecture at 7 months			Populations of endophytic bacteria inside stems (log CFU/g) at 7 months	
	Length of new shoot growth (cm)	Stem caliper (mm)	Total root length (cm)	Root surface area (cm^2)	Total no. of root tips	Fluorescent pseuduomonads	Total aerobic bacteria
1. Benlate 50 WP foliar spray	57.7*	5.4*	411*	415*	547*	1.56	4.43*
2. Benlate 50 DF foliar spray	61.4*	5.6*	383*	394*	499*	2.78*	4.54*
3. Water control foliar spray	98.7	6.6	651	649	771	0.53	3.47
4. Benlate 50 DF drench	60.2*	5.1*	387*	404*	494*	2.37*	4.62*
5. Benlate 50 WP drench	51.6*	5.0*	353*	381*	485*	2.48*	4.70*
6. Water control drench	98.0	6.5	634	660	787	0.59	3.36
$LSD_{0.01}$	15.1	0.6	48.5	57.0	61.1	1.53	0.51

Values shown are means of 8 plants per treatment.
* Indicates significantly lower value than the corresponding water control at $P = 0.01$

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Table 2B. Citrus test 2, effect of Benlate on number of blooms and fruit on Valencia orange 15.5 months after first application of Benlate

Treatment	Number of blooms and buds per plant	Number of fruit per plant
1. Benlate 50 WP foliar spray	2.00*	0*
2. Benlate 50 DF foliar spray	1.25*	0*
3. Water control foliar spray	19.88	9.25
4. Benlate 50 DF drench	0.38*	0*
5. Benlate 50 WP drench	0.38*	0*
6. Water control drench	26.38	9.0
$LSD_{0.01}$	16.1	6.47

Values shown are means of 8 plants per treatment.
* Indicates significantly lower value than the corresponding water control at $P = 0.01$

Table 2C. Citrus test 2, effect of Benlate on endophytic bacteria and growth of Valencia orange 16 months after first application of Benlate

Treatment	Shoot fresh weight (g)	Root fresh weight (g)	Measurements of root architecture: Total root length (cm)	Root surface area (cm^2)	Total no. of root tips	Populations of endophytic bacteria inside stems (log CFU/g): Fluorescent pseudo-monads	Total aerobic bacteria
1. Benlate 50 WP foliar spray	94.5*	81.2*	480*	885*	503*	4.43*	4.93*
2. Benlate 50 DF foliar spray	96.2*	74.6*	515*	871*	549*	4.62*	5.08*
3. Water control foliar spray	175.4	133.3	985	1531	1008	0.70	2.94
4. Benlate 50 DF drench	88.3*	90.2*	432*	962*	515*	4.77*	4.92*
5. Benlate 50 WP drench	95.4*	83.0*	482*	975*	506*	4.78*	5.05*
6. Water control drench	183.0	150.3	993	1600	1040	0.50	3.84
$LSD_{0.01}$	20.7	17.3	137	262	94	0.93	0.60

Values shown are means of 8 plants per treatment.
* Indicates significantly lower value than the corresponding water control at $P = 0.01$

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Figure 2. Valencia orange 5.5 months after first drench with Benlate DF (left) and water (right)

Figure 3. Effect of Benlate on root growth of Valencia citrus 7 months after first application of Benlate. Right = drench with Benlate DF; left = water control.

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Figure 4 Effect of Benlate on formation of citrus fruit 15.5 months after first application of Benlate. Upper photo is a water control plant; lower photo was treated with Benlate

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DISCUSSION

The genus *Pseudomonas* contains a large diversity of species and strains within species (Preston 2004). Within the fluorescent pseudomonads there are many biological control strains, some pathogenic strains, and some strains reported to be deleterious to plants. Preston (2004) pointed out that the distinction between saprophytes and pathogens is not always clear-cut because both live on and inside plant tissues where there are frequent opportunities for genetic recombination. Such recombination can occur after horizontal gene transfer, thereby conferring new phenotypic traits (or suites of traits) to bacteria sharing an ecological habitat (Berg *et al.* 2005). Therefore, in the rhizosphere or inside plants, bacterial genes for production of virulence factors may move among different phylogenetic groups. The expression of virulence factors can relate to population density of the bacterial strains. Examples of virulence factors that are regulated by plant cell density via quorum sensing are elicitation of the hypersensitive response (Mathesius *et al.* 2003), production of IAA (Preston 2004), and production of cell wall degrading enzymes including pectinase (Berg *et al.* 2005; Laasik *et al.* 2006). Quorum sensing also has been reported to regulate horizontal gene transfer and bacterial colonization of hosts (Berg *et al.* 2005), and some virulence factors can also help endophytes colonize plants. For example, pectinolytic enzymes have been reported to facilitate entry of rhizosphere bacteria inside plants by hydrolyzing pectic substances located between plant cell walls (Okon and Vanderleyden 1997).

As discussed above, in three different perennial plant systems, leatherleaf fern, banana, and citrus, applications of Benlate systemic fungicide led to increased populations of endophytic bacteria, including fluorescent pseudomonads, and to decreased growth or distortions in growth of the host plant. We propose the following as an emerging model to account for these observations. In healthy plants, the native microflora in the rhizosphere and inside roots or rhizomes includes groups of fluorescent pseudomonads, and likely other bacterial genera that contain genes for production of virulence and colonization factors. Under normal growth conditions, populations of these bacteria are kept below the level necessary for widespread production of the virulence factors because of competition with the native bacterial and fungal microflora of the rhizosphere and endorhiza. Changes in the balance of the native microflora of the rhizosphere and inside roots or rhizomes result from perturbations in the growing conditions, such as the application of the systemic fungicide Benlate. These microbial changes trigger population increases of bacteria containing the virulence and colonization traits. As their populations increase, quorum sensing activates expression of the colonization and virulence factors as well as horizontal gene transfer of virulence genes to other closely related phylogenetic groups of bacteria. Over time in perennial plant systems, there is a shift in the bacterial community inside roots, rhizomes, or stems with a concomitant development of symptoms such as stunting of root and shoot growth or deformed growth of plants. This model is consistent with reports that the rhizosphere is a reservoir of opportunistic pathogens that cause human infections, reviewed by Berg *et al.* (1005).

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Worldwide, the theme of sustainable agriculture is emphasized for crop production. The broader agricultural implication of our proposed model is that some agricultural practices are unsustainable, causing unexpected long-term shifts in microbial ecology that reduce production. With annual plants, such microbial shifts may not result in visible damage to plants or loss in crop production. However, in perennial cropping systems, the microbial shifts may be exacerbated with concomitant crop damage, and if the perennial plant is vegetatively propagated, latent infections of the deleterious microflora can be passed to the progeny plants.

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In: Schneider C, Leifert C, Feldmann F (Eds), Endophytes for plant protection: the state of the art. © 2013 Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-11-2.

3-2 Structural and functional genomics of plant-associated *Bacillus* strains used for biocontrol of plant diseases

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ABSTRACT

The whole genome sequence of the type strain of plant associated *Bacillus amyloliquefaciens* ssp. *plantarum*, FZB42T, has been determined in 2007, as the first representative of gram-positive, plant growth promoting bacteria. Its 3,918-kb genome, containing an estimated 3,695 protein-coding-sequences (CDS), lacks extended phage insertions, which occur ubiquitously in the related *Bacillus subtilis* 168 genome. The *B. amyloliquefaciens* genome reveals a huge potential to produce secondary metabolites, including the polyketides bacillaene and difficidin. More than 8.5 % of the genome is devoted to synthesizing antibiotics and siderophores by pathways not involving ribosomes. A first comparison of its genomic sequence with that of the *B. amyloliquefaciens* type strain DSM7T revealed significant differences in the genomic sequences of both strains. The strains have in common 3345 CDS residing in their core genomes; whilst 547 and 344 CDS were found to be unique in FZB42T and DSM7T, respectively. Notably, the gene clusters encoding non-ribosomal synthesis of antibacterial polyketides difficidin and macrolactin are absent in DSM7T. For comparison, *B. subtilis* 168T has a similar number of CDS in common with *B. amyloliquefaciens* strains DSM7T and FZB42T (3,222 and 3,182 CDS, respectively). Recently, besides FZB42T, the genomes of three other *B. amyloliquefaciens* ssp. *plantarum* strains have become available. In addition, except DSM7T, the genomes of three other representatives of the *B. amyloliquefaciens* ssp. *amyloliquefaciens* have been published enabling a comparative genome analysis of plant root-associated and free-living soil *B. amyloliquefaciens* strains. 130 genes were identified as being unique for subspecies plantarum and did not occur in the soil bacterium *B. amyloliquefaciens* ssp. *amyloliquefaciens*. We conclude that most of them might be involved plant-bacteria interactions. In order to support this idea we have performed transpososon mutagenesis, transcriptome and proteome analysis of FZB42 exposed directly to plants or plant root exudates, respectively. The results of this analysis will be presented.

Persistent Identifier: urn:nbn:de:0294-sp-2013-ppphe-2

García E et al., *Arabidopsis thaliana* and its natural endophytes: a model system for the study of the ecological and molecular factors involved in endophytic interactions. In: Schneider C, Leifert C, Feldmann F (Eds), Endophytes for plant protection: the state of the art, pp. 71-77. © 2013 Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-11-2.

3-3 *Arabidopsis thaliana* and its natural endophytes: a model system for the study of the ecological and molecular factors involved in endophytic interactions

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ABSTRACT

We have analyzed the culturable endophytic mycobiota associated, in the wild, with leaves and siliques of the model plant *A. thaliana*. We have studied the effect of biotic and abiotic factors in the frequency of fungal endophytes in plant specimens, and in the species composition of the endophytic community. Our results indicate that the frequency of *Arabidopsis* plants hosting endophytes depends on the time of the year and the phenological stage of the plant, and that the probability of endophyte colonization increases as the life cycle of the plant progresses. The diversity of the endophytic assemblages of natural *A. thaliana* populations was high, and precipitation and temperature were the two main factors determining the diversity and species composition of the communities. Isolates of the most abundant genera were chosen in order to set into a point their inoculation to *A. thaliana* plants under controlled conditions and investigate the outcome of the interactions.

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INTRODUCTION

Arabidopsis thaliana L. (Brassicaceae) is the laboratory plant model par excellence (Koornneef & Meinke 2010). The genome of several genotypes has been sequenced and there is a large amount of available information about its functional genetics and genomics and a variety of tools to deepen in the knowledge of plant biology (Ehrhardt & Frommer 2012). For over 20 years, *A. thaliana* has been developed as the model organism for molecular plant genetics, including the analysis of the mechanisms of resistance to parasites (Nishimura & Dangle 2010). However, *A. thaliana* is not just a lab model plant: it is an annual species with a worldwide distribution which can be found in a variety of anthropic and wild habitats (Hoffmann 2002). The knowledge generated in the lab is being applied to the study of its wild populations, so it has become a model, as well, for the study of plant ecology, adaptation and evolution (Bergelson & Roux 2010). The study of the bacterial communities associated with *A. thaliana* is currently gaining importance (Knief *et al*. 2010, Kniskern *et al*. 2007, Micallef *et al*. 2009), including the recent metagenomic analyses of the composition of the bacterial microbiomes of *A. thaliana* roots (Bulgarelli *et al*. 2012, Lundberg *et al*. 2012). As for fungi, there are examples of endophytes isolated from different hosts that are able to colonize *A. thaliana* roots and shoots (*Piriformospora indica*, Peskan-Berghofer *et al*. (2004) and *Acremonium alternatum*, Jaschke *et al*. (2010)). Very recently, it has been shown that some endophytic isolates from wild *Arabidopsis* plants are able to colonize the plant in axenic conditions (Junker *et al*. 2012).

We have surveyed different populations of *A. thaliana* from Central Spain, which is considered an origin and diversification centre of the species (Pico *et al*. 2008). The prospected *A. thaliana* populations are situated at different ecological environments at the Central Plateau of Spain, and have been very well characterized for their genetic variation and geographic structure. We have isolated a high diverse number of fungal species from above ground organs of asymptomatic plants (García *et al*. 2013). We have studied the effect of biotic and abiotic factors in the frequency of fungal endophytes in plant specimens, and in the species composition of the endophytic community. Isolates of the most abundant genera were chosen in order to set into a point their inoculation to A. thaliana plants under controlled conditions and investigate the outcome of the interactions. We propose *A. thaliana* and its endophytes as a model system for an integral approach to the principles governing the endophytic lifestyle, taking advantage of the molecular tools and the abundant knowledge accessible from the host plant.

MATERIALS AND METHODS

From each plant, three different organs (rosette leaves, stem leaves –ie. leaves from the inflorescence stem-, and siliques), were sampled when present. Two fragments of approximately 10 mm^2 of each sampled organ were surface disinfected by submerging them in 20% household bleach (1% active chlorine) and gently shaking during 5 minutes. After

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rinsing two times in sterile water, one fragment was placed in a sterile moist chamber and the other in a potato dextrose agar plate. Plates were kept at room temperature (20-24ºC). Fragments were inspected periodically and the fungal mycelium growing from them was isolated in potato dextrose agar (PDA) plates containing 200 mg/L of chloramphenicol. The effectiveness of the surface sterilization was controlled by making imprints of disinfected leaf fragments on PDA plates. The isolation frequency was calculated as the number of plants from which endophytes were isolated in relation to the analyzed plants. Endophytes were preliminary classified by the homology of their ITS1-5.8S rRNA-ITS2 sequence region with the sequences in the fungal EMBL/GenBank database. The identification of the isolate was limited to the genus, unless the sequences of type species were found among the better blast hits, and these unequivocally indicated the same species. The molecular identification was confirmed with the morphology when the fungal reproductive structures were present.

RESULTS

Isolation frequency of endophytes

We analyzed 209 Arabidopsis plants for the presence of endophytic isolates, obtaining a total number of 530 samples. Endophytes were isolated from 94 plants, which means an isolation frequency of 45%. The isolation frequency was significantly different depending on the month of sampling ($P < 10^{-3}$). It increased significantly from March to April and from April to May, reaching its maximum in June and decreasing significantly from June to November. This pattern coincides with the progress of Arabidopsis life cycle in the sampled populations, which attained the end of the cycle in May and June. The increase of the isolation frequency was mainly due to the plants at reproductive and shattering stages. In order to further analyze if the isolation frequency depended on the phenological stage of the plant independently of the month of sampling, we compared the isolation frequency of plants sampled at different phenological stages in the same month. Significant differences in the isolation frequency were found in February and May. In these months, most of the isolates were obtained from plants at reproductive or shattering stages ($P = 0.02$). Regarding the plant population, significant differences in the isolation frequency were also found in the populations sampled in February and in May ($P = 0.03$ and $P = 0.004$ respectively). In February, isolates were only obtained from plants from the populations Polán and Las Rozas, which were the most advanced in their cycle at that time of the year, with plants at reproductive stage. In May, endophytes were obtained less frequently in Marjaliza, which was the most retarded population, being the only one with plants at vegetative stage at that time of the year. Hence, the isolation frequency depends on both the month of sampling and the phenological stage of the plant. In order to analyze if there were differences in the presence of endophytes in the different plant organs, we compared the frequencies of samples with endophytes from rosette leaves. No significant differences in the isolation frequency from the rosette or stem leaves

were found. However, the isolation frequency was significantly lower in siliques than in rosette or stem leaves from the same plant (P = 0.004).

Diversity and composition of endophyte assemblages

We sequenced the ITS1-5.8srRNA-ITS2 region of 120 randomly chosen isolates, classifying the isolates based on sequence similarity. We obtained a total of 48 species, which belonged to 38 genera in 17 orders and 6 classes. Most of the isolates (117) were Ascomycota, two isolates belonged to Basidiomycota and one isolate belonged to subphylum Mucoromycotina (Zygomycota). Seventy three isolates (61% of the total) belonged to the Class Dothideomycetes, which was the most abundant with 4 orders, 17 genera and 20 species represented. The most abundant order was Pleosporales, with 13 genera, 14 species and 54 isolates represented. The most diverse genus was *Colletotrichum*, from which 5 different species were found that accounted for 11 isolates. Eleven species lacking taxonomically informative morphological structures could not be identified to genus and even to order rank because their sequences were less than 95% similar to any identified accession from the EMBL/GenBank fungus database. Only eight species had five or more isolates (*Alternaria* sp., *Cladosporium* sp. 1, *Colletotrichum* sp. 5, *Lewia* sp., *Embellisia* sp. 1, *Hipocrea lixii*, *Phoma* sp. and *Ulocladium* sp.), accounting for 50% of the isolates. In contrast with these dominant species, 58% of the species were represented by just one isolate (ie. singletons). The species accumulation curve was non-asymptotic. In contrast, we obtained a curve approaching an asymptote when only plural species consisting of two or more isolates were considered.

Total diversity was measured using Fisher's α (32.77) and Shannon diversity index (H' =3.48), with an evenness e = 0.73. Fisher's α in each population was positively correlated with the annual precipitation (r = 0.89; P = 0.04). As for sampled organ and vegetative stage of the plant, Fisher's α was higher in rosette leaves than in stem leaves and siliques, and it was lower in plants at vegetative stage than at reproductive or shattering stages. Assemblages from rosette and stem leaves were the most similar (JI = 0.32) and assemblages from siliques were the most differentiated, with JI = 0.28 and JI = 0.24 compared to stem and rosette leaves, respectively. Hence, the species composition was more similar between closer organs. The eight dominant species were shared between the three types of organs, with the exception of *H. lixii*, and *Ulocladium* sp., which were not found in siliques. There was no correlation between the similarity and the spatial proximity of the populations. In order to determine which variables were significant predictors of the dominant fungal species in each population, forward selection CCA of the geographical (elevation), ecological (genus of the dominant tree) and climatic variables (temperature and precipitation) was performed. Only mean annual precipitation and temperature were significant predictors (P = 0.004 and P = 0.03, respectively). Among the different temperature measures, absolute maximum temperature was the best adjusted to the data. Hence, the species composition of fungal assemblages in each population depended both on the precipitation and maximum temperature of the locations.

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CONCLUSIONS

- We have obtained isolates during the whole growing season of *A. thaliana*, from plants that were at different phenological stages at the same time of the year. The proportion of plants with endophytic isolates depended on the time of the year, but also on the phenological stage of the plant. Plants at reproductive and shattering stages had endophytes more frequently than plants at vegetative stage. Our results also show evidence of plant age as a factor that increases the probability of hosting endophytes. Also, the isolation frequency in siliques was significantly lower than in rosette or stem leaves of the same plant.
- The isolation frequency of endophytes found in our work is comparable to other host species in similar latitudes (Arnold and Lutzoni 2007). Species accumulation curve was non-asymptotic, which is typical from species rich ecosystems but, when only plural species were considered, the species accumulation curve attained an asymptote. This result indicates that most of the dominant species have been identified, and more intensive samplings will mostly find species that rarely occur.
- Many of the species isolated in this work showed high nucleotide identities with other endophytic isolates of the EMBL/GenBank in Blast searches. The vast majority of the isolates obtained pertained to Ascomycota phylum, and the most abundant and diverse orders were Pleosporales and Capnodiales, as in most of the foliar endophytic assemblages described to date (Arnold and Lutzoni 2007, Rodriguez *et al*. 2009, Sanchez Marquez *et al*. 2012).
- The diversity between populations was not due to isolation by distance, since JI similarity indexes were not correlated with the geographical proximity of the populations. Among all the variables analyzed, precipitation and temperature were the significant predictors of the dominant fungal species in each population. We have also found a significant correlation between the precipitation and the diversity of the endophytic assemblages. Humid conditions are, in general, more favorable for fungal sporulation and infection, but the correlation between precipitation and diversity can also be related to a better dispersal of endophytes by rain.
- We have obtained more than 120 endophytic isolates of 48 species. Some of these species are unknown, others are frequent components of endophytic assemblages and others are related with very well studied pathogens of *A. thaliana*, such as *C. higginsianum* or *Plectosphaerella cucumerina* (O'Connell *et al*. 2012, Ramos *et al*., 2013). These isolates can be used as model systems for molecular approaches for the study of the endophytic functional interactions with the host.

ACKNOWLEDGMENTS

We thank Drs. Carlos Alonso-Blanco and Fernando García-Arenal for showing us the localization of the wild populations of *A. thaliana* and Mª Ángeles Portal for the technical assistance. Meteorological data have been gently provided by the Spanish Metereology Agency (AEMET). This work was funded by grants CAM CCG07-UPM/GEN-1899 of DGUI of Comunidad de Madrid and UPM and AGL2008-00818 of Ministerio de Educación y Ciencia of the Spanish Government to Soledad Sacristán.

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Bernardo K T, Franco C M M, Chataway T, Proteomic Analysis in Arabidopsis thaliana inoculated with a beneficial endophytic Streptomyces sp. in response to a challenge by the pathogen *Fusarium oxysporum*. In: Schneider C, Leifert C, Feldmann F (Eds), Endophytes for plant protection: the state of the art, pp. 78-87. © 2013 Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-11-2.

3-4 Proteomic Analysis in *Arabidopsis thaliana* inoculated with a beneficial endophytic *Streptomyces sp*. in response to a challenge by the pathogen *Fusarium oxysporum*

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ABSTRACT

Two-dimensional difference gel electrophoresis (DIGE) was used to elucidate the changes occurring in the *Arabidopsis* leaf proteome in response to interactions with the beneficial endophyte *Streptomyces* sp. EN27 (*Str.* sp. EN27); the hemibiotrophic wilt pathogen, *Fusarium oxysporum* (*Fox*); as well as the three-way interaction between endophyte, pathogen and plant. Of the 1440 protein spots that were matched across all gels, a total of 28 spots significantly changed in abundance due to the various treatments, with the plant responding similarly to the endophyte and pathogen. Eight spots were identified by LC MS/MS and grouped into functions of defense-related, cell redox, and metabolism. Expression of genes encoding the identified proteins as well as genes typically involved in the defense response, such as *PDF1.2, PR-1* and *Hel* that were not identified in the DIGE analysis was analysed by quantitative PCR (qPCR), which revealed poor correlation between transcript and protein levels. qPCR analysis of the defense transcripts showed that plant defense against *Fox* employed salicylic acid (SA) and jasmonic acid (JA) pathways by upregulation of *PR-1* and *PDF1.2*, respectively. In contrast *Str.* sp. EN27-mediated defense resulted in increased expression of *LLP* and *Hel*. *Str.* sp. EN27 also appeared to strongly suppress JA signalling, known to facilitate pathogen-mediated leaf chlorosis and senescence. This may may explain the lack of wilt symptoms and survival of the *Str.* sp. EN27-treated plants seven days post-inoculation with *Fox*.

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INTRODUCTION

Endophytes are ideal biocontrol agents as they colonise an ecological niche similar to some phytopathogens (Berg *et al.*, 2005), have a close association with the plant, and consequently reduce application of pesticides. Besides competing with the pathogen for plant nutrients and colonization sites, endophytes may also exert biocontrol activity by direct antagonism towards pathogens (Ryan *et al.*, 2008). Actinobacteria are of particular interest, as they are prolific producers of secondary metabolites many with antimicrobial activity (Baltz, 2007). *Streptomyces, Micromonospora*, *Microbispora*, and *Nocardioides* species isolated from a variety of plants such as wheat (Coombs & Franco, 2003) and native Australian trees (Kaewkla & Franco, 2011) in our laboratory have potential to increase plant growth and protect against disease. Field trials using wheat and barley seed treated with selected isolates showed significant grain yield increases of up to 15% in diseased soil (Franco *et al.*, 2007).

Plants have evolved several strategies to defend against invading pathogens, with constitutive barriers and inducible defences. Trichomes, epidermal appendages on the surface of plant leaves, can exude biochemicals to fend off herbivores and pathogens, and serve as a first line of defence (Serna & Martin, 2006). In addition, papillae, containing the high molecular weight β-(1,3)-glucan polymer, callose, are deposited on the inner side of cell walls as an effective barrier to pathogen invasion (Luna *et al.*, 2011). Equipped with an innate immunity, individual plant cells have the ability to induce a non-specific immune response known as PAMP (pathogen associated molecular patterns)-triggered immunity (PTI) (Nürnberger & Kemmerling, 2009). Successful pathogens override PTI by secretion of effector proteins into the host cell, which in turn can be recognised by plant resistance (R) proteins to trigger a specific immune response known as effector triggered immunity (ETI) (Chisholm *et al.*, 2006). Described as more aggressive than PTI, ETI is associated with a hypersensitive response (HR) and programmed cell death (PCD) (Spoel & Dong, 2012). At the initial site of infection, a mobile signal is generated that travels to distal, uninfected areas of the plant and facilitates a rapid defence response to subsequent infection, known as systemic acquired resistance (SAR), which has a characteristic accumulation of salicylic acid (SA) (Conrath, 2006). Phenotypically similar to SAR is induced systemic resistance (ISR), which is triggered by non-pathogenic microbes, is mainly mediated by signalling molecules JA and ET (van Loon, 2007). Activation of SAR and ISR typically results in expression of defense genes encoding pathogenesis-related (PR) proteins and antimicrobial peptides such as thionins and defensins (Egorov & Odintsova, 2012).

Conn *et al.* (2008) quantified transcripts of key genes (*PR-1*, *PR-5*, *PDF1.2* and *Hel*) involved in the SA and JA/ET pathways, and found that certain endophytic actinobacteria were capable of priming *Arabidopsis* defence pathways against the pathogen *Fusarium oxysporum* (*Fox*). Rather than being limited to key genes known to be involved in SAR and ISR, the objectives of this research was to identify global protein changes occurring in the plant in response to colonisation with *Str.* sp. EN27; the pathogen *Fox*; and the three way

interaction between plant, endophyte and pathogen using the DIGE technique and to compare these results with corresponding transcript levels.

MATERIALS AND METHODS

Growth of *Streptomyces* sp. EN27 and *Fusarium oxysporum* cultures

The endophytic *Streptomyces* sp. EN27, previously isolated from surface-sterilized healthy wheat roots (Coombs & Franco, 2003) and *Fusarium oxysporum*, originally isolated from cucumber, were maintained on half-strength potato dextrose agar at 27ºC.

Plant cultivation and treatment

Arabidopsis thaliana ecotype Columbia 0 seeds were surface-sterilized according to the method described by Conn *et al.* (2008) and sown onto half-strength Murashige and Skoog salt medium (MS, Sigma-Aldrich, Sydney, Australia) with 0.8% phytagel. Seeds were inoculated with 10 µl of saline containing 10^8 *Str.* sp. EN27 spores/ml, with saline used for control plants. The MS plates were sealed with micropore tape and the seeds vernalized at 4ºC overnight to achieve even germination. Plants were transferred to a light box and grown in a vertical orientation with a 9 h light cycle, 1,920 lux, at 22 ± 4ºC. After 6 weeks, a 10 µl saline suspension of 10^8 *Fox* spores/ml was applied to the roots of untreated and *Str.* sp. EN27-treated plants, with saline used for control plants. The shoots were harvested 3 days post-inoculation (dpi) with *Fox*, weighed and snap frozen in liquid nitrogen. For qPCR analysis shoots were harvested at 0 h, 3 dpi and 7 dpi with *Fox*.

Growth characteristics

Five days after sowing, the percentage of untreated and *Str*. sp. EN27-treated seeds with cotyledons that had emerged from the seed coat was assessed. At five day intervals, the total number of visible leaves (≥ 0.5 mm^2) was counted, for up to 35 days. The rosette diameter of the plant was measured each week and the rosette harvested to measure the fresh weight at 6 weeks. The mean of 3 replicates of 20 plants for each growth measurement was compared using an independent Student's t –test.

Measurement of trichome density and callose deposition

The effect of *Str.* sp. EN27 colonization on trichome density and callose deposition was determined using typically the fifth leaf on the main stem of six week old *Arabidopsis* plants. Trichome density on the adaxial surface of the leaf was examined using a dissecting microscope (Motic SMC-168, Xiamen, China) for 3 replicates of 10 leaves. These leaves were stained with aniline blue following the protocol by Luna *et al*. (2011). The stained callose was observed using an Olympus IX71 fluorescence microscope (BP 340 nm-380 nm excitation, LP 425 nm).

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Preparation of protein extracts and 2D DIGE

Extraction of total soluble leaf proteins from three biological replicates of untreated, *Str.* sp. EN27-treated, *Fox*-challenged and *Str.* sp. EN27+*Fox*-challenged *Arabidopsis* was performed by the trichloroacetic acid (TCA)-acetone protocol of Damerval *et al.* (1986). The pellet was resolubilized in iso-electric focusing (IEF) buffer, consisting of 7 M urea, 2 M thiourea, 4% w/v 3;[(3-cholamidopropyl) dimethylammonio] propane sulfonate.

Fifty µg of each protein sample was differentially labelled with 400 pmol of either Cy3 or Cy5 CyDye DIGE Fluors (GE Healthcare, UK) (Table 1), with Cy2 used for the internal standard, which contained equal quantities of each experimental sample. Samples were separated in the first dimension using an Ettan IPGphor III IEF System (GE Healthcare) using pH 3-11 IPG strips (GE Healthcare) and in the second dimension using an Ettan DALT 6 (GE Healthcare) set-up with 12.5% acrylamide SDS gels (26 cm × 20 cm × 0.1 cm).

Table 1 DIGE experimental design for comparison of leaf proteins in untreated and *Str.* sp. EN27-treated *Arabidopsis* challenged with *Fox*

Gel	Cy2	Cy3	Cy5
1	Internal Standard	Untreated[1]	*Str.* sp. EN27+*Fox*[1]
2	Internal Standard	Untreated[2]	*Str.* sp. EN27+*Fox*[2]
3	Internal Standard	*Str.* sp. EN27+*Fox*[3]	*Str.* sp. EN27[1]
4	Internal Standard	*Str.* sp. EN27[2]	*Fox*[3]
5	Internal Standard	*Str.* sp. EN27[3]	*Fox*[2]
6	Internal Standard	*Fox*[3]	Untreated[3]

Image analysis and protein- identification

Gels were scanned using a Typhoon 9400 imager (GE Healthcare) and gel analysis was performed using DeCyder 2D Software, Version 7.0 (GE Healthcare). Statistical analysis of changes in protein abundance across gels was performed by using an independent Student's t-test and one way ANOVA between treatments. Protein spots showing a significant ($p \leq 0.05$) quantitative difference between samples with at least 50% increase or decrease in abundance in comparison to the untreated control were manually assigned as "proteins of interest". Selected spots of interest were tryptic digested and 1 µg of digested peptides analysed using Mass spectrometry using the methodS described in Wilson *et al.* (2011). All fragmentation spectra were searched against the Uniprot 2010-06 database.

Analysis of gene expression by qPCR

Total RNA was extracted from 100-200 mg of *Arabidopsis* leaves using TRIzol reagent (Invitrogen, CA, USA). The preparation of cDNA and protocols for qPCR are described in Conn *et al.* (2008). The housekeeping genes *Actin*, *18S rRNA* and *UBQ10* were used to normalize the experimental genes. Transcript analysis was performed for selected proteins identified in the DIGE analysis (*GAPC2*, *LLP*, *LPD1*, *MLP43*, *FNR1*, and *eIF5A*), as well as defense genes *Hel*, *PDF1.2* and *PR-1*, with primer sequences available as Supplementary

Table 1 at the end of this paper. The qPCR results are presented as means of triplicate qPCR where three independent experiments were carried out to validate the results.

RESULTS AND DISCUSSION

Str. sp. EN27 effect on seedling development

No significant difference was found between untreated and *Str.* sp. EN27-treated plants in size and seedling emergence (data not shown), though seedlings pre-treated with the endophyte appeared to have more accelerated growth. Treatment with *Str.* sp. EN27 also had no significant differences in callose deposition or trichome formation in comparison to untreated plants. The lack of effect on plant development by *Str.* sp. EN27 was beneficial for detailed examination of the defence response as both developmental processes and disease resistance involve the activity of the same phytohormones (Robert-Seilaniantz *et al.*, 2011).

2D DIGE analysis

A total of 1440 spots were matched across all gels, and in comparison to the untreated plants 17 spots for *Str.* sp. EN27-treated, 7 spots for *Fox*-challenged and 28 spots for *Str.* sp. EN27+*Fox*-challenged plants showed a significant change in expression, as outlined in Figure 1, and 8 spots that were identified by MS detailed in Table 2. Interestingly, in comparison to the untreated control, pre-treatment with *Str.* sp. EN27 appeared to cause a larger effect on protein expression with or without *Fox* challenge, with fold changes ranging from 1.1-fold to 1.25-fold more than that of the untreated plants challenged with *Fox*.

The proteins common to all treatments that significantly changed in abundance could be grouped into functions of metabolism (glyceraldehyde-3-phosphate dehydrogenase C2 [GAPC2]), defence (major latex-like protein 43 [MLP43] and eukaryotic translation initiation factor 5A-2 [eIF5A2]) and cell redox (dihydrolipoyl dehydrogenase-1 [LPD1] and ferredoxin $NADP^+$ oxidoreductase 1 [FNR1]). Intriguingly, metabolism protein, Δ-1-pyrroline-5-carboxylate synthase A (P5CSA), which is involved in proline biosynthesis and protection against osmotic stress (Hong *et al.*, 2000), only increased in abundance due to *Str.* sp. EN27 treatment with or without *Fox* challenge. Along with upregulation of proteins involved in oxidative stress (LPD1 and FNR1) this suggests that *Str.* sp. EN27 is causing stress within the seedlings, although these plants appeared to be robust and healthy. To confirm that the endophytic actinobacterium was not causing these responses, direct physiological measurements would be necessary. Conversely, an advantage of the endophyte inducing production of these proteins would be for preparing the plant to respond more rapidly if such stresses were encountered.

Relative to the untreated control, *Str.* sp. EN27 treatment caused a 3-fold decrease in defence protein, lectin-like protein, which in *Str.* sp. EN27+*Fox*-challenged plants had only decreased by 1.78-fold. This suggests that *Str.* sp. EN27 is suppressing LLP production to possibly evade the carbohydrate binding activity of the protein. In contrast, *Str.* sp. EN27

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appeared to prime the plant for 6-phosphogluconate dehydrogenase (6PGDH) in response to *Fox*, with 1.26-fold increased levels in comparison to untreated plants challenged with *Fox*. Elevated levels of 6PGDH may have alleviated oxidative stress occurring in the plant.

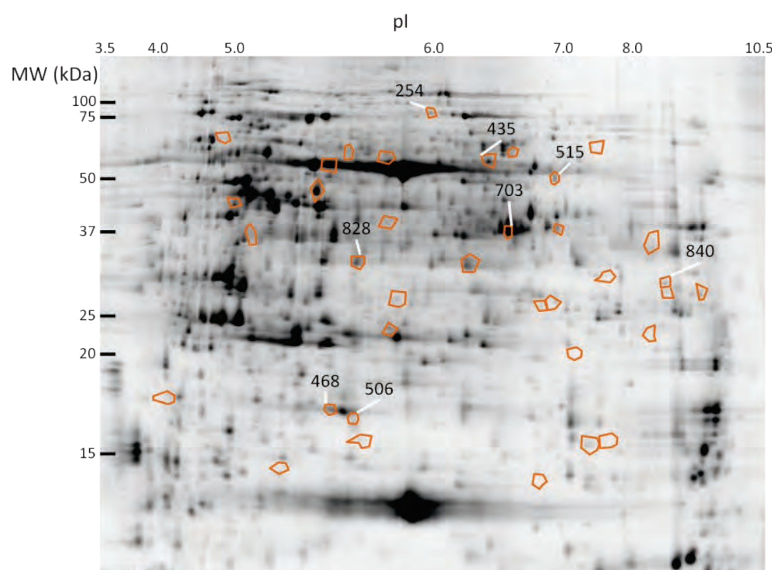

Figure 1 Representative 2D-DIGE spot map of leaf proteins extracted from *Str.* sp. EN27-treated *Arabidopsis* challenged with *Fox*.

DIGE analysis was performed at 3 dpi with *Fox* in anticipation of discovering changes at the protein level correlating to what was previously observed at the defence transcript level (Conn *et al.*, 2008). However, this study only detected a low level of changes in protein abundance in response to *Fox* challenge suggesting that at 3 dpi *Fox* colonisation in the roots was not stimulating the defence response in the systemic tissues. DIGE analysis at 7 dpi when plants showed visible signs of disease may have revealed a higher amount of proteins involved in defence against *Fox* and allowed better differentiation between proteome responses to *Str.* sp. EN27 treatment and pathogen challenge.

Table 2 MS identification of proteins from spots that exhibited significant changes in abundance between untreated and *Str.* sp. EN27-treated, *Fox*-challenged and *Str.* sp. EN27+*Fox*-challenged Arabidopsis

		Avg. Ratio (Treatment vs. Untreated)							
Protein	Spot #	EN27	*FOX*	EN27+ *FOX*	T-test	Sequence Coverage (%)	# of Peptides	Theoretical mol. mass (kDa)/pI	Observed mol. mass (kDa)/pI
P5CSA	254	2.02	1.39	1.92	0.0061	3.75	2	66.7/5.94	68.4/6.0
GAPC2	703	1.87	1.5	1.82	0.03	64.5	167	36.9/7.18	40.9/6.7
LLP	840	-3.28	1.17	-1.78	0.035	28.31	11	29.7/8.91	34.1/9.0
MLP43	469	1.7	1.52	1.87	0.031	20.25	5	17.9/5.53	18.5/5.5
LPD1	435	1.76	1.62	1.8	0.05	42.41	20	54/6.96	56.3/6.5
6PGDH	515	1.42	1.39	1.75	0.0086	7.82	2	53.5/7.02	53.2/7.3
FNR1	828	2.28	1.81	2.19	0.0073	22.52	5	35.2/5.54	36.6/5.6
eIF5A2	507	2.1	1.64	1.96	0.049	38.99	6	17.1/5.55	18.2/5.6

Transcript analysis using qPCR

Of the genes examined, only defense genes *LLP*, *PR-1*, *PDF1.2* and *Hel* were up-regulated by any of the treatments in comparison to the 0 h untreated control plants (Figure 2) showing poor correlation between protein and transcript abundance.

At the protein level LLP was found to decrease in abundance 3 dpi with *Fox* in *Str.* sp. EN27-treated plants with or without *Fox* challenge, whilst transcript analysis showed upregulation in *LLP* expression. At 3 dpi *Str.* sp. EN27-treated plant response to *Fox* challenge resulted in elevated expression of *LLP* and *PDF1.2* over untreated plants, which had higher expression of *PR-1*. This is in contrast with previous findings where *Str.* sp. EN27 enhanced expression of *PR-1* (Conn *et al.*, 2008). At 7 dpi expression of *PR-1* and *PDF1.2* substantially increased in untreated plants challenged with *Fox* over *Str.* sp. EN27-treated plants, whilst levels of *LLP* subsided. Similar expression levels of *Hel* were found for both treatments. Defense response 7 dpi with *Fox* in untreated plants appears to be strongly modulated by the JA pathway, which is associated with enhanced resistance to *Fox* (Epple *et al.* 1997), with some involvement of the SA and ET pathways. In contrast, LLP appears to

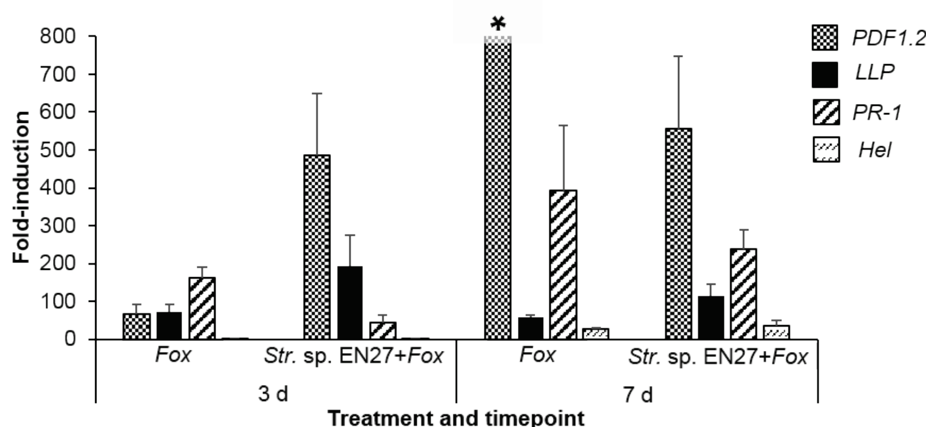

Figure 3 Fold increase relative to the untreated control at 0 h of defense gene transcripts in *Str.* sp. EN27-treated and untreated *Arabidopsis* 3 d and 7 d post-inoculation with *Fusarium oxysporum* * represents fold induction of 4,093.

play an important role in the initial stages of *Str.* sp. EN27-mediated defence against *Fox,* followed by activation of the SA and ET pathways, however, not to the extent as in untreated plants. *Str.* sp. EN27 also appeared to strongly suppress expression of JA-responsive gene, *PDF1.2*, suggesting that protection mediated by the endophyte follows a different strategy.

Contrary to elevated JA defence gene expression increasing resistance to *Fox*, Thatcher *et al.* (2009) demonstrated enhanced resistance using a *coi1* mutant known to be deficient in JA-responsive gene expression. Rather than preventing *Fox* colonisation, the *coi1* mutant disabled the ability of the pathogen to cause leaf chlorosis and senescence, which is induced by JA signalling (Wasternack, 2007). In this study, the reduced levels of *PDF1.2* in *Str.* sp. EN27-treated plants challenged with *Fox* may be due to endophyte suppression of JA signalling which in turn disrupted JA-mediated leaf senescence triggered by *Fox* effectors,

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resulting in a smaller amount of leaf chlorosis symptoms at 7 dpi in these plants. Although *Fox* colonisation is not repressed by this type of resistance, the benefit of suppressing *Fox*-mediated plant cell death is through interfering with the pathogen's hemibiotrophic lifestyle where it relies upon obtaining nutrients from diseased tissue during later stages of infection.

CONCLUDING REMARKS

Str. sp. EN27 may have a similar colonisation strategy as *Fox* to elicit the similar response seen in DIGE analysis, or that the endophyte is inducing these proteins to prepare the plant for stress. Differentiation between *Str.* sp. EN27 and *Fox* treatments may have been more obvious when the plant displayed symptoms of wilt. Poor correlation between transcript and protein found in this study may have been due to transcriptional regulation and post-translational modifications and emphasises the importance of complementing gene expression studies with that of protein abundance.

ACKNOWLEDGEMENTS

We thank Dr Mandy Walker for providing *Arabidopsis* seeds and Flinders Proteomics.

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Supplementary Table 1: Primers for quantitative real-time PCR

Target gene	Primer sequence	Product size	Ref
Actin2/ Actin 8	F: 5' GGTAACATTGTGCTCAGTGGTGG 3' R: 5' AACGACCTTAATCTTCATGCTGC 3'	108	1, 2
UBQ10	F: 5' AAACCCTAACGGGAAAGACG 3' R: 5' GAGTTCTGCCATCCTCCAAC 3'	150	3
18S rRNA	F: 5' CCTGCGGCTTAATTTGACTC 3' R: 5' AGACAAATCGCTCCACCAAC 3'	135	3
GAPC2	F: 5' TGACAAAGACAAGGCTGCTG 3' R: 5' TACTCGTGCTCGTTGACACC 3'	111	3
LLP	F: 5' CGGTTCGGTTCGGTTCAGTTTGG 3' R: 5' CAGAGTAAGCCACAGGTCGGC 3'	110	3
MLP43	F: 5' CGCACTGGCACCTGGAGTATG 3' R: 5' AAAGACCCACACACAGACGAGGAG 3'	150	3
LPD1	F: 5' CTTGTCTCAGCGGGAAGAAC 3' R: 5' ACACGCCTGGGACATTACTC 3'	124	3
FNR1	F: 5' TGTGTCAGTGAAGGCTCAGG 3' R: 5' AAGGCAGCGACCAGTGTAAG 3'	139	3
eIF5A	F: 5' CGCCCAGATGAGGCTTGGATTCG 3' R: 5' CCACCACCAACTTCCTTGACGGC 3'	105	3
Hel	F: 5' CAAGTGTTTAAGGGTGAAGA 3' R: 5' CGGTGTCTATTTGATTGAAC 3'	119	4
PR-1	F: 5' GCCTTACGGGGAAAACTTA 3' R: 5' CTTTGGCACATCCGAGTCT 3'	160	4
PR-5	F: 5' CGGAAACGGTAGATG TGTAAC 3' R: 5' GTTGAGGTCAGAGACACAGCC 3'	216	4
PDF1.2	F: 5' CTGCTCTTGTTCTCTTTGCT 3' R: 5' GTGTGCTGGGAAGACATA 3'	164	4

Reference 1: An *et al.*(1996), 2: Charrier *et al.* (2002), 3: This study, 4: Conn & Franco (2008).

Persistent Identifier: urn:nbn:de:0294-sp-2013-ppphe-2

Trognitz F, Priming of potato leaves with the endophyte *Burkholderia phytofirmans* PsJN and its lipopolysaccharides. In: Schneider C, Leifert C, Feldmann F (Eds), Endophytes for plant protection: the state of the art, pp. 88-92. © 2013 Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-11-2.

3-5 Priming of potato leaves with the endophyte *Burkholderia phytofirmans* PsJN and its lipopolysaccharides

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ABSTRACT

Endophytes are microorganisms that live within host plants for at least part of their life and do not cause apparent symptoms of diseases. *Burkholderia phytofirmans* PsJN shows plant growth-promoting effects and was reported to enhance plant vigor and resistance to biotic and abiotic stresses. We studied PsJN and its lipopolysaccharides (LPS) as possible agents to trigger induced systemic resistance. LPS are one of the most important microbe associated molecular patterns (MAMPs), which act as general elicitor of basal or innate immune response. One leaf per potato plant was infiltrated with the endophyte PsJN, its LPS or with buffer as control treatment. After 24 hours of infiltration non-infiltrated leaves were collected. Salicylic acid (SA), nitric oxide (NO), reactive oxygen species (ROS) and plant transcriptome analysis were carried out.

INTRODUCTION

In general, agricultural systems are intensive in regard to the requirement of fertilizers, pesticides and water. Sustainable agriculture is based on three main goals, i.e. environmental health, economic profitability, and social and economic equity. Microorganisms are important players in healthy production systems and can aid in the development of more sustainable agricultural systems. Like the human body also plants host millions of bacteria living inside or around the plants. Beside pathogens, which can destroy plants completely, many non-pathogenic and beneficial microorganisms are associated with plants.

Pests and disease are the major threat in plant production. It is estimated that plant diseases annually cause an 11 to 16 % loss of rice, wheat, corn, and potato harvests. For example crop

losses due to late blight in potato and its control are estimated to cost worldwide US-$ 3.25 billion annually, US-$ 750 million (25%) are being spent on fungicides (GILB, CIP, Lima, Peru, http://www.cipotato.org/gilb/). To control diseases in the year 2003 over 219.662 tones of plant protection products were used in the EU (FAO). Of these products 49% are used as fungicides and 10% as insecticides. The amounts spent on a global scale reached in 2007/2008 a value of US$405 billion (Andrews *et al.* 2012).

There is a large body of literature describing potential uses of plant-associated bacteria as agents stimulating plant growth and managing soil and plant health (see review by Compant *et al*. 2005). Bacteria are able to prevent or diminish the effect of deleterious pathogens by direct or indirect manipulation of pathogens. Certain bacteria trigger a phenomenon known as induced systemic resistance (ISR), which is phenotypically similar to systemic induced resistance. The resistance mechanisms induced by microorganisms enables the plant to mobilize pathogen defense much faster.

The plant growth-promoting *Burkholderia phytofirmans* type strain PsJN (Sessitsch *et al*. 2005) isolated from *Glomus vesiculiferum*-infected onion roots (Nowak 1998) proved to be a potent plant growth promoter. Strain PsJN is able to establish both endophytic and epiphytic populations on potato, tomato, maize, peat moss and grapevines (Compant *et al*. 2005; 2008; Frommel *et al*. 1991; Pillay & Nowak 1997). Besides stimulating plant growth in a cultivar-dependent manner (Bensalim *et al*. 1998; Pillay & Nowak 1997), PsJN can induce developmental changes in the host (Frommel *et al*. 1991) that can enhance its water management properties (Lazarovits & Nowak 1997) and also disease resistance (Ait Barka *et al*. 2002; Sharma & Nowak 1998, Stewart 1997).

Bacterial lipopolysaccharides (LPS) are important players in many plant-microbe interactions. Perception of LPS in plant cells can trigger direct defense responses or can lead to prime the plant to respond more rapidly after pathogens invasion.

The aim of this study was to investigate the effects of treatments with PsJN and its LPS on leaves of tpotato.

MATERIAL AND METHODS

For the experiment the true seed potato variety MF-II was grown in the greenhouse.

One leaf per potato plant was infiltrated with the endophyte PsJN, its LPS or with buffer as control.

Non-infiltrated leaves from primed plants were collected after 24 hours for microarray hybridization and salicylic acid measurement on a partial potato cDNA microarray.

For the measurement of ROS and NO production, leaves were harvested and inoculated with *Phytophthora infestans* suspension. ROS was measured using carboxy-H2DCFDA (Invitrogen) and NO using 4,5-Diaminofluorescein diacetate (DAF-2DA; Enzo Life Science). The fluorescence was captured using a plate reader with a wavelength of 490nm

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every 5 min over one hour. The results were compared to leaves inoculated with the growing medium.

For the transcriptome analysis RNA isolation, cDNA microarray construction and hybridization protocols are deposit at the NCBI GEO server under the following ArrayDesign name: RLP array Version I; ArrayExpress accession: GPL7326 and GSM322635 (http://www.ncbi.nlm.nih.gov/geo/)

RESULTS AND DISCUSSION

Reactive oxygen species (ROS) and nitric oxide (NO) detection after priming with PsJN and LPS

ROS are important signaling molecules that control processes such as pathogen defense, programmed cell death and stomatal functions. Accumulation of ROS in plant cells can result in the formation of the hypersensitive response (HR) and cell wall cross-linking, as well as the induction of the expression of defense-related genes.

NO serves as an important signal in plants and animals. Generation of the NO burst is a key feature of the plant defense response following pathogen recognition.

ROS and NO formation rapidly increased in leaves primed with PsJN and LPS after challenging them with the pathogen *P. infestans* in comparison to the control leaves (PBS). The boost in the production of the signal molecules was not only faster in the primed leaves but also the amount was increased. The results showed that due to PsJN and LPS plants were able to react much faster against the pathogen. Our results also showed that the bacteria had a stronger effect on the induction of NO and ROS as compared to LPS alone. It is likely that several effectors of PsJN in combination rather than LPS only induced the formation of signaling molecules.

Transcriptome analysis of leaves primed with PsJN and LPS

To obtain an overview of genes involved in the priming process a microarray experiment was conducted. The transcriptome of leaves primed with LPS and PsJN were compared to the transcriptome of leaves infiltrated with the growing medium. A partial cDNA potato microarray was used for the analysis containing about 4000 genes.

With a significance threshold of $P<0.01$, 53 genes were significantly up-regulated and 132 significantly down-regulated in LPS treated leaves. PsJN treatment resulted in 172 up-regulated and 227 down-regulated genes. By comparing the PsJN and the LPS treatments we found that both treatments shared in common 33 down-regulated genes and 10 up-regulated genes. . Among the down regulated genes are xyloglucan endotransglucosylase-hydrolase XTR3, lipid transfer protein and COP9. The regulation of these genes was confirmed by real-time PCR.

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The relative small number of genes regulated in the primed leaves may indicates, that PsJN and its LPS as endophytic bacterium have components which can prevent the expression of defense related genes. Other studies dealing with the transcriptomic changes in plants interacting with endophytes showed that in most cases only transient, weak or strictly localized responses were produced (Verhagen *et al*. 2004, Wang *et al*. 2005), which stands in contrast to the massive induction of defense responses triggered during plant–pathogen interactions.

It is hypothesized, however, that defense responses are not activated directly in primed plants, but are speeded up upon an attack, providing the plant with an enhanced capacity to respond to an invading pathogen (Conrath *et al*. 2006, Frost *et al*. 2008). Thus, defense responses are expressed only when really needed (i.e. upon pathogen attack) and only those defense responses are enabled, which are triggered by a specific pathogen.

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Persistent Identifier: urn:nbn:de:0294-sp-2013-ppphe-2

In: Schneider C, Leifert C, Feldmann F (Eds), Endophytes for plant protection: the state of the art. © 2013 Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-11-2.

3-6 Analysis of the impact of viral infection on endophytic microorganisms in potato

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ABSTRACT

Endophytic microorganisms are known to have a role in the defence response of the plant to pathogen attack. Endophytes can control pathogen invasion and spread by various mechanisms, namely by outcompeting pathogens, by production of a range of compounds active against the pathogen and by induction of plant systemic resistance (ISR) via jasmonic acid and/or ethylene signalling pathways. Nevertheless, the outcome of the harmonised activity of the plant and the endophytes depends on the structure of the endophyte population. The structure of the endophytic community depends on the plant genotype and its developmental stage. The diversity of the community is also influenced by infection by pathogens, non-pathogenic microorganisms, or by other environmental factors.

Plant-endophyte-pathogen interaction has been so far studied from different aspects. Our goal is to reveal the relationship between potato plants, Potato virus Y (PVYNTN) and endophytic microorganisms. PVYNTN is one of the most important potato viruses, causing huge losses in potato production. Potato plants infected with PVYNTN express severe symptoms on leaves, observed as chlorotic and necrotic ringspots. The interaction between the potato plants and PVYNTN has been already intensely studied in our laboratory from different aspects of the relationship. Biochemical, transcriptomic, metabolomic and proteomic analyses show fast response of potato plants of various cultivars to infection. The potato responds to infection on various levels, from alterations in phytosynthesis, carbohydrate metabolism, secondary metabolism, in hormones, and by activation of defence related genes.

Until now, the impact of infection with PVYNTN on potato endophytic microorganisms has not yet been addressed. In order to do this, endophytic population present in healthy and PVYNTN infected potato plants will be compared. The differences between the populations will be evaluated with different approaches.

Persistent Identifier: urn:nbn:de:0294-sp-2013-ppphe-2

Klocke E, Weinzierl K, Abel S, Occurrence of endophytes during *Pelargonium* protoplast culture. In: Schneider C, Leifert C, Feldmann F (Eds), Endophytes for plant protection: the state of the art, pp. 94-99. © 2013 Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-11-2.

3-7 Occurrence of endophytes during *Pelargonium* protoplast culture

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ABSTRACT

Culture of plant protoplasts is an important biotechnological tool. Up to now it is a challenge to get regenerated plants from protoplasts, especially if the protoplasts undergo fusion conditions for somatic hybridization. Isolation and fusion of protoplasts and afterwards the de novo plant regeneration is a long multistep process with very variable conditions for the plant cell. We have elaborated a protocol for *Pelargonium*, a highly appreciated ornamental plant. In *Pelargonium* protoplast cultures we observe endophytes during the regeneration process. The endophytes appear on the calluses and cause a severe browning of the tissue. However, the degree of infestation can be influenced by a strong schedule for nutrient medium exchange connected with a medium variation. Moreover, the growing *Pelargonium* protoplast callus consists of compact structures and soft areas. Under the microscope it is possible to separate the compact structures. After a cultivation on medium PPM 3 (1/4 modified Murashige and Skoog medium with phytohormones NAA 0.27 µM + BA 2.22 µM) the compact green calluses are transferred to the protoplast regeneration medium PRM 12 (1/2 modified Murashige and Skoog medium with phytohormones IAA 5.71 µM, BAP 0.44 µM, 2iP 0.49 µM). On this medium a high number of shoots develop. Regenerated plants show no endophytes and grow up well in vitro as well as in the greenhouse after hardening. The appearance of endophytes during a very special period of cell development in *Pelargonium* protoplast cultures and some of the conclusions for the general plant cell and tissue culture are being discussed.

Persistent Identifier: urn:nbn:de:0294-sp-2013-ppphe-2

INTRODUCTION

Plant protoplast culture is an important tool for crop improvement and basic research. Plant protoplasts, single cells without the plant cell wall and surrounded only with the cell membrane, are capable of giving rise to whole plants. Moreover, these totipotent cells are able to fuse under special conditions and afterwards to regenerate as well. For all techniques of plant cell tissue and organ culture including the protoplast culture it is highly demanded to work under axenic conditions. There is done a lot to avoid any contaminants at start and during the in vitro culture. These measurements include the mandatory desinfestation of all media, vessels etc., and work under the flow box to the sterilization of the plant explants (Herman 2012, Herman 2007). Nevertheless, the loss of plant cell cultures causing by sudden appearance of various bacteria or fungi is still a severe problem in plant tissue cultures and usually the reasons remain unclear.

Here, we report about *Pelargonium* protoplast culture. As a rule we observed endophytes during the regeneration process. The role of the endophytes and the possibility in spite of the endophytes to regenerate plants successfully is to be discussed.

METHODS

Greenhouse plants of *Pelargonium* x *hortorum* accessions (503, 201, AV, AP) and two wild forms 609 and 662 were kindly provided by breeder PAC® Dresden, Germany. From these plants nodal segments were sterilized as followed: vigorous shaking with NaOCl (4 % Cl) + Tween 20 (15 min) and three times washing with autoclaved water. Established in vitro plants were maintained in a climate chamber on a modified MS medium (Klocke *et al*. 2012). Leaves from these plants served as source for mesophyll protoplasts.

Other sources of protoplasts were established *Pelargonium* suspension cultures and hypocotyls from in vitro germinated seeds previously sterilized as described above (Klocke *et al*. 2012).

The isolation of protoplasts was performed in an appropriate enzyme solution (16 hours, gently shaking, darkness). After purification of the protoplasts from cell debris using sieving through a nylon mesh and several centrifugation steps the control protoplasts were cultivated in liquid PPM 1 medium [modified 1/4 MS (Murashige & Skoog 1962) , mannitol 0.52 M, NAA 5.4 µM, 6-BAP 2.2 µM]. The most protoplasts were fused, each time two partners. Chemical fusion with PEG (polyethyleneglycol) and electrofusion using BTX Electro Cell Manipulator 2001 were applied. After fusion procedure the protoplasts were cultivated like the control protoplasts in PPM 1.

After 7-10 days the mannitol content of the medium was decreased and the cytokinin content increased by adding of PPM 2 (modified ¼ MS, mannitol 0.38 M, NAA 5.4 µM, 6-BAP 4.4 µM). Microcolonies were plated on solid PPM 3 (modified ¼ MS, mannitol 0.27 M, NAA 2.7 µM, 6-BAP 4.4 µM). Green compact callus structures were separated from soft callus

using a stereo microscope. They were subcultured for shoot development on PRM 12 (modified ½ MS, no mannitol, IAA 5.71 µM, 6-BAP 0.44 µM, 2iP 0.49 µM). Plantlets grew up on medium P-MSV (modified MS, IAA 5.71 µM, Kin 4.6 µM). If not otherwise stated the media were solidified with Bacto^{TM} Agar (BD) 9 g L^{-1}.

The development of protoplasts and microcolonies was assessed regularly under the microscope.

RESULTS

Pelargonium plants in vitro were cultivated and multiplicated over more than three years. They grew well on modified MS medium supplemented with 2.85 µM IAA (indole-3-acetic acid). Over the long period of cultivation no bacteria were observed. Therefore, an application of some antibiotics or other broad acting antibiotics like PPM (plant preservation mixture) was not necessary. The medium of the suspension cultures was weekly replaced by fresh one. The maintenance of the suspension cultures is labour-intensive. From the other side vigorously growing suspension cultures were a good source for protoplasts. Also there no antibiotics were in the suspension medium.

For getting hypocotyl protoplasts seeds of *P* x *hortorum* cultivars were got from hand pollinations of plants in the greenhouse. This was a prerequisite to get sterile seedlings after a relatively soft sterilization of the seeds with sodium hypochlorite only.

In more than 60 fusion experiments with *Pelargonium* protoplasts the de novo cell wall formation was observed within a few days, in some cases already after two days of cultivation in PPM 1, showing that the PPM 1 medium is well suitable for *Pelargonium* protoplasts from various accessions and species. PPM 1 was stepwise diluted with PPM 2, diminishing the amount of mannitol, the osmotic active substance. Under these conditions after about two months microcolonies developed. Despite of light in the climate chamber the microcolonies were colorless and without visible structures. At this stage it was necessary to change from liquid to solid medium. In the other case the microcolonies stopped the growing and turned brown.

The switch from liquid PPM 1/PPM 2 to solid PPM 3 medium was very critical for the protoplast *Pelargonium* calluses. If it was chosen not the right stage (right size) the callusses arrested the development, turned brown and were frequently covered with light yellow, slimy endophytes. Otherwise, a high number of calluses proliferated on medium PPM 3 displaying in few days a differentiatiation of callus tissue with green more compact structures surrounded by soft light yellow callus usually covered with slimy endophytes (Fig. 1). The only way to get plants from these calluses was the carefully separation of the very small green structures (1-2 mm) from the remaining soft callus parts. The green small calluses were separately cultivated on medium PRM 12. This medium contained no mannitol but growth regulators which supported the shoot organogenesis (auxin IAA, 5.7 µM, and two cytokinins 6-BAP 0.44 µM, 2iP 0.49 µM). For strengthening the regenerated plantlets

were transferred to medium P-MSV. The plants looked healthy and they rooted without problems.

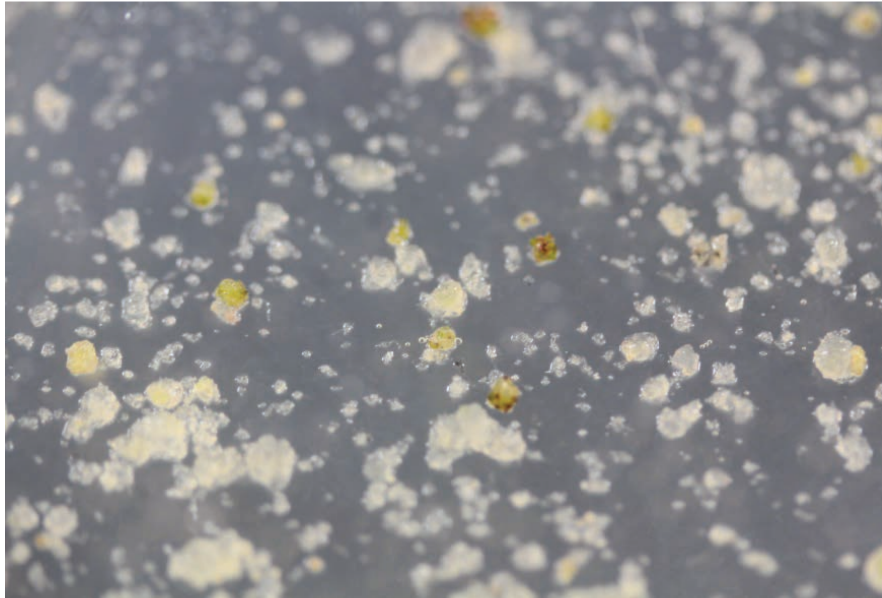

Figure 1. Protoplast fusion between *P.* x *hortorum* (+) wild type 609, soft callus with green structures on medium PPM3

If the microcolonies were to early transferred from liquid medium to solid PPM3 no green structures developed but endophytes were present. Furthermore, the transfer of soft calluses to PRM12 caused a heavy appearance of yellowish endophytes (Fig. 2).

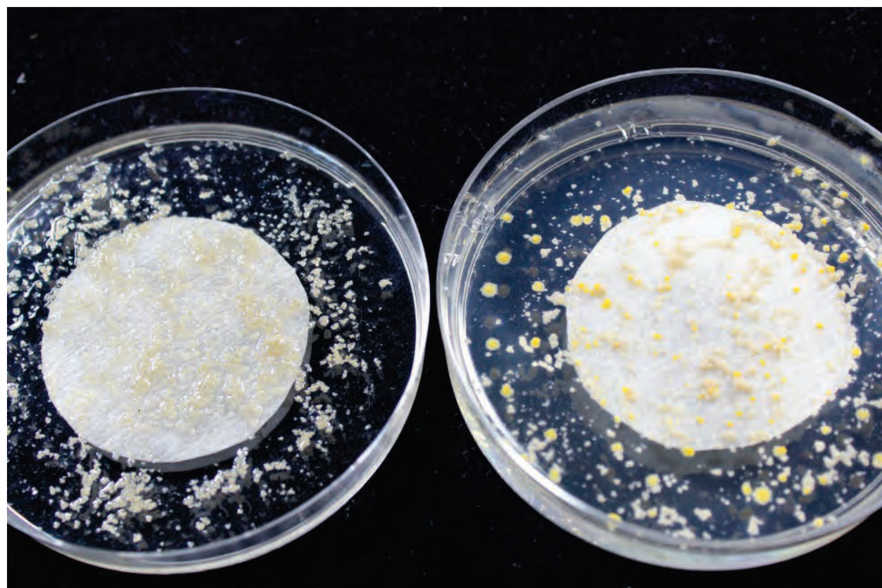

Figure 2. Protoplast fusion between *P.* x *hortorum* (+) wild type 609, typical soft callus after six weeks on medium PPM3 (left) and appearance of endophytes, if soft calluses were transferred to PRM12 (right)

In few experiments with wild accession 662 embryoid-like structures were observed. These compact structures without the soft callus grew well in liquid PPM 1 /PPM 2. Only in this

case it was possible to transfer compact calluses immediately to PRM 12 for the plant regeneration.

Unfortunately, it was impossible to cultivate the endophytes on common bacterial media like glycerin bouillon agar, tryptone soy agar or Luria Broth medium what would be a prerequisite for further characterization of these putative bacteria.

Some attempts have been made to the occurrence of the bacteria to suppress. For example, protoplast callusses were cultivated in liquid medium supplemented with 400 mg L^{-1} Timentin®. However, all calluses on medium with Timentin® turned brown fastly and died.

The addition of 0.5 g L^{-1} activated charcoal to the regeneration medium did not show an inhibitory effect on the endophytes.

Lipoic acid (LA), a metabolic antioxidant (Dan *et al.* 2009) was supplemented to P-MSV. In all concentrations investigated (5, 10, 50 µM) there was an inhibitory effect of LA on the regeneration process in comparison to the P-MSV without LA (Fig. 3). At concentration 50 µM LA no further regeneration was observed.

Figure 3 Protoplast fusion betw. *P.* x *hortorum* (+) wild type 609, influence of lipoic acid (LA) addition to medium P-MSV: left no LA, centre 10 µM LA, right 50 µM LA

DISCUSSION

During the regeneration process of *Pelargonium* protoplast cultures usually endophytes appear. Due to the high loss of microcolonies it is worth to discuss about this phenomenon. All explantats used for the protoplast isolation were cultivated under axenic conditions without antibiotics. There is the assumption that the explants were free of contaminants since the plants and suspensions grew well and bacteria in the medium especially around the rooting zone were not visible But there should be stated two points: the sudden occurrence of

visible endophytes on callusses during the regeneration and the possibility to overcome the endophytes with a special cultivation management. Because of the complexity of endophytic associations with plants we are only starting to understand these interactions (Tadych & White 2009). The knowledge of mutualists or commensals is modest. The endophytes could be contained within tissues of living plants without causing harmful effects. The plant associated endophytes are recalcitrant to cultivation on artificial media and therefore the investigation with classic microbial methods is difficult or impossible. Only now, with the development of modern molecular methods for genome identification a better view into the diversity and occurrence of endophytes is to be expected.

We assume that mutualistic symbiosis is a common prerequisite for healthy plants, also during in vitro culture. Protoplast culture conditions represent a strong stress situation for plant cells. This could be a reason for the sudden appearance of harmful endophytes. Which factor triggers endophytes to switch from mutualism to pathogenism remains unclear. Our culture management: to transfer “at the right time, the right structures to the right medium” shows that the mutualistic symbiosis could be influenced by an external impact. Therefore, it is possible to restore the mutualistic symbiosis with the benefit for both, plant and endophyt during the plant cell tissue culture.

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© 2013 Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-11-2.

3-8 Complete genome sequence of a plant growth promoting and crop protective strain of *Bacillus atrophaeus* UCMB-5137

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ABSTRACT

Endophytic and rhizosphere colonizing plant growth promoting Bacillus are widely used in biotechnology. In recent years, use of biotechnological pesticides in plant and crop protection is steadily increasing and begins to replace in part chemical pesticides as an ecologically safer approach. Vast majority of these products are based on the active strains of B. amyloliquefaciens ssp. plantarum and several other species of Bacillus [1], but the ability to colonize and protect plants have never been reported for *B. atrophaeus*. In a large scale screening experiment the strain UCMB-5137 of *B. atrophaeus* was selected, which showed outstanding capacities to colonize plant roots; inhibit fungal and bacterial phytopathogens when applied on seedlings and harvested fruits; stimulate plant growth and even burst the intrinsic resistance of plants against pest insects. Availability of the complete genome sequences of other 14 *B. atrophaeus* strains isolated from different environments made it interesting to sequence the complete genome of this plant associated bacterium for a comparative genomics study. The genome was sequenced by Macrogen using the Illumina technology. DNA reads were assembled by Velvet and CLC Bio Workbench into 20 large ordered contigs and annotated using RAST, GeneMarkHMM and by blusting of predicted ORF against the sequences of genes of reference genomes of Bacillus. Horizontally acquired genomic islands were identified by SeqWord Sniffer program. A phylogenetic tree based on concatenated alignments of 2,517 orthologous genes found in reference *B. atrophaeus*, B. amyloliquefaciens and B. subtilis genomes demonstrated belonging of the active strain to the *B. atrophaeus* lineage but a clear separation from other sequenced 14 strains of this species. Interestingly, that UCMB-5137 shared 8 genes with other plant associated B. amyloliquefaciens and B. subtilis, but these genes were absent in the genomes of free living Bacillus including those of other sequenced *B. atrophaeus*. UCMB-5137 possessed double more horizontally acquired genomic islands than it was found in its closest relatives and

Persistent Identifier: urn:nbn:de:0294-sp-2013-ppphe-2

many genes of these genomic islands looked relevant to the plant colonization behaviour. The most interesting acquisition was an operon of large surface adhesion proteins showing no sequence similarity to known genes from Bacillus but partly similar to algal genes. This operon may be responsible for the biofilm formation on plant roots that was reported by the fluorescent microscopy to be characteristic for this strain. Thus, it was concluded that the outstanding plant protective ability of this strain was associated with a horizontal acquisition of several important operons of genes that endowed the ancestral strain with a capacity to colonize plan roots. Adaptation of many other housekeeping genes to plant associated lifestyle had followed.

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3-9 Fungal Infection in Barley Roots – Friend and Foe

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ABSTRACT

This review presents the evidence for regarding fungal root infections in barley as friends as well as foes. Despite a widespread perception that fungal root endophytes will always confer benefits on their hosts, this review and other meta-analyses reveal among-study contradictions, probably due to experimental variability. Endophyte infection can be negative, positive or neutral in effect. Prevailing circumstances dictate the nature of the plant-endophyte relationship. Even if an endophyte is never pathogenic, it is not always beneficial. The most important factors that determine beneficence are the specific combination of partner genotypes (or strains) and developmental stage, and the ecological and environmental setting. A fungal endophyte that has beneficial effects on a particular barley variety may be detrimental to another variety in a different environment or at a different developmental stage. The fungal infection may give the plant a greater ability to cope with the stress if the partners are in a balanced relationship. Neither the nutrient status of the plant nor the availability of soil nutrients affects the degree of endophyte colonisation, and, unlike mycorrhizae, there is no apparent increase in endophyte-associated phosphorous transfer. There are indications of an association between successful endophyte colonisation and soil pH and clay content. The greatest benefits to the barley plant resulting from the colonisation of root tissue by a fungal endophyte seem to be obtained in abiotically stressed environments. Each new study reveals an increasing diversity of beneficial fungal root endophytes, and the full potential of these organisms is still to be determined. It may be that there is a single ideal fungal partner for each variety of barley.

INTRODUCTION

Fungal endophytes are non-mycorrhizal associates that spend most or all of their lives within plant tissues, often with no outward sign of their presence (Stone *et al.* 2004; Schulz & Boyle 2006). Some are pathogenic, but the full effects of the infection on the host plant are sometimes not apparent until the disease is well-established. In contrast, there are other kinds of endophytes that have an equal, but beneficial, impact on plant growth and survival. The term "endophyte" as used in this review therefore refers to both pathogenic and beneficial infections (see Wang *et al.* 2009). However, others use the term to refer to symptomless infections only (Kogel *et al*. 2006; Schulz & Boyle 2006). Benefits to plants infected with endophytic fungi include an increase in yield (Achatz *et al.* 2010), enhanced resistance to pathogens and herbivores (Cheplick & Faeth 2009), and increased stress tolerance (Waller *et al.* 2005; Rodriguez *et al.* 2009). A beneficial host-endophyte association is a balanced antagonism, whereas a pathogenic association is imbalanced; the pathogenic infection results in disease (Wilson, 1995; Schulz *et al.*, 1999).

Endophytic fungal pathogens of barley (*Hordeum vulgare* L.) have been extensively studied and characterised (Mathre, 1997), but not so their beneficial counterparts. Megatons of fungicides, pesticides, and fertilisers are applied to crops every year, incurring large economic costs and contributing to ecosystem degradation (Powell & Jutsum, 1993; Underwood 2000). Further costs are incurred in testing varieties of barley to use as a genetic resource for disease resistance breeding (Statkevičiūtė & Leistrumaitė 2010; Knupfer *et al.* 2011). Beneficial fungal endophytes may have the potential to reduce these costs, and may even enable the growing of crops on previously unsuitable sites.

Fungal pathogens can infect many barley tissue types, and above ground infections are usually obvious; but the generally invisible root tissue is the least studied. Several reviews have been published on fungal endophytes (Kogel *et al*. 2006; Schulz & Boyle 2006; Mayerhofer *et al. 20*12). However, there is a lack of a detailed synthesis of knowledge for the fungal endophytes of barley roots in particular. This paper addresses that gap and suggests where future research is required. It demonstrates that fungal root infections in barley should be treated as friends as well as foes.

Endophyte colonisation can have neutral, positive or negative effects on the plant, depending on many variables: including host and endophyte identity, and the nature of available nutrients (Tellenbach *et al.* 2011; Mayerhofer *et al.* 2012; Reininger *et al.* 2012). In a meta-analysis of the effects of fungal root endophytes of plants, Mayerhofer *et al. 20*12 found that trees seem to respond negatively to endophyte colonisation, while herbaceous monocots (including grasses) are more likely to respond positively to endophyte inoculation. Barley, as a model monocot, seems to display a mostly positive response to endophyte colonisation.

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BENEFICIAL FUNGAL ROOT ENDOPHYTES OF BARLEY

Since its discovery in the Thar desert of north-west India in 1997 (Verma *et al.*, 1998), the basidiomycete *Piriformospora indica* has become the model experimental organism for the study of fungal endophyte root colonisation (Oelmüller *et al.* 2009). In barley, *P. indica* has been shown to increase yield (Achatz *et al.* 2010), enable salt tolerance (Waller *et al.* 2005; Baltruschat *et al.* 2008) and enhance pathogen resistance (Waller *et al.* 2008; Felle *et al.* 2009; Rahnamaeian *et al.* 2009). In other plant groups, such as orchids, *P. indica* may predominantly exist as a mycorrhizal partner (Schafer & Kogel 2009).

Piriformospora indica, unlike many endophytes, can be easily cultured outside of its plant host (Singh *et al.* 2003; Oelmüller *et al.* 2009; Qiang *et al.* 2011); and is thus an ideal experimental subject, especially as it readily colonises the model angiosperms *Arabidopsis thaliana* and barley (Peskan-Berghofer *et al.* 2004; Stein *et al.* 2008; Oelmüller *et al.* 2009; Achatz *et al.* 2010; Qiang *et al.* 2012). Other root endophytes have also shown promise as biocontrol and biofertilizing organisms (Soytong *et al.* 2001; Hashem & Ali 2004; Istifadah & McGee 2006; Oelmüller *et al.* 2009; Fávaro *et al.* 2012).

We will now examine in more detail the barley-endophyte relationship and the benefits conferred on barley by *P. indica* and other fungal root endophytes.

COLONISATION

The nature of the relationship between barley and *P. indica* is controlled by a subtle physical and biochemical dialogue, with the final outcome dependent on the combination of genotype and developmental stage of each partner (Kogel *et al.* 2006).

The endophyte must colonise the root cell without triggering a full-blown defence response, so the relationship develops in a finely-balanced way that does not result in pathogenicity or endophyte death. There is strong evidence for a balanced antagonism between the virulence of the colonising endophyte and the plant defence response (Maciá-Vicente *et al.* 2009).

The first hurdle to be overcome by the endophyte is to gain entry to the root cell, but the cell walls of most plant roots are effective barriers to invasion. The cellulose and lignin in the cell wall must be broken down by cellulolytic and ligninolytic enzymes, and endophytes use a variety of these enzymes to help with root cell penetration (Basiewicz *et al.* 2012). Direct endophyte contact with the barley root results in increased enzyme production, but in-vitro cultures show a much reduced enzyme activity (Basiewicz *et al.* 2012).

Nevertheless, the root cell does make some attempt to arrest fungal penetration. An increase in the secretion of building blocks for cell wall apposition and plasmodesmata blocking may retard hypersensitive cell death in neighbouring cells (An *et al.* 2006). The infected cell may still die, but cell death proliferation is contained. In fact, *P. indica* colonisation of barley roots is always associated with cell death (Deshmukh *et al.* 2006). The suppression of host defence reactions is associated with a cell death regulator protein of barley (Eichmann *et al.*

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2006), and overexpression of the associated gene diminishes colonisation by *P. indica* (Imani *et al.* 2011). Older cells are more likely to undergo cell-death, and *P. indica* preferentially colonises the oldest root hairs of barley (Waller *et al.* 2005).

Once the endophyte has fully colonised the root cell, it will then move on to infect neighbouring cells or sporulate. Unlike mycorrhizal fungi, endophyte sporulation can occur inside or outside the barley roots (Schafer & Kogel 2009), for example as chlamydospores, and this gives the fungus a long-term intra-plant residence potential, with spores able to remain dormant in plant tissue. Colonisation of further cells can then initiate from these spores in differentiated tissue (Deshmukh *et al.* 2006).

Although infection by the endophyte may be entirely symptomless, there are genes that systemically indicate root colonisation by some fungal groups (Waller *et al.* 2008), and expression of these genes may provide a simple and reliable marker of colonisation.

Outside of the endophyte-host system, infection potential from the rhizosphere associated microbial population is related to many factors, and soil type is the main determinant (Berg & Smalla 2009). Soil fungi spore density and fungal development are related to pH, clay content and barley developmental stage. The ecological interactions are complex and covered elsewhere (Black & Tinker, 1979; Macia-Vicente *et al 20*08b).

With the fungal root endophyte now firmly established in a stable relationship with the barley host, the benefits to the plant can be expressed through a number of different mechanisms: resistance to pathogens, yield enhancement, stress tolerance, and interaction with other organisms.

RESISTANCE TO PATHOGENS

Fungal root endophytes can inhibit the colonisation potential of the most damaging barley pathogens, including *Gaeumannomyces graminis* (“take-all”), *Blumeria graminis* (“powdery mildew”), *Fusarium graminearum* (“fusarium head blight”) and *Pyrenophora teres* (“net blotch”).

Although the root endophyte may only colonise the root tissue, the beneficial effects for the plant may be systemic. *G. graminis* infection is systemically reduced when the roots of barley plants are colonised by endophytic fungi (Maciá-Vicente *et al.* 2008a), and some mycorrhizal fungi also show a systemic reduction of this disease (Khaosaad *et al.* 2007).

Several different endophytic organisms have been identified as potential agents for the biocontrol of *G. graminis* disease in wheat (Ghahfarokhi & Goltapeh 2010), and may have similar potential for barley. Ghahfarokhi and Goltapeh (2010) tested *Piriformospora indica, Sebacina vermifera* and *Trichoderma* species in their study, and all of these endophytes have been shown to readily colonise barley (Waller *et al.* 2005; Baltruschat *et al.* 2008; Achatz *et al.* 2010). Macia-Vicente *et al.* (2008a) tested fifteen fungal root endophytes from natural vegetation and eight of these caused systemic reduction of *G. graminis* symptoms in barley.

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Many types of powdery mildew pathogens infect barley, and while most barley varieties are resistant to some infections (e.g. *Erysiphe graminis*), the powdery mildew causing *Blumeria graminis* f. sp. *hordei* is still a serious crop pathogen. *Piriformospora indica* and the closely-related *Sebacina vermifera* both significantly reduce *B. graminis* infection. *Piriformospora indica* confers both local and systemic resistance to *Blumeria graminis* (Deshmukh *et al.* 2006), with leaf pustule frequencies reduced by up to 70% (Schafer & Kogel 2009). *Blumeria graminis* colony numbers can be significantly reduced by *P. indica* infection, with between 48-58% colony reduction in 3-week-old barley seedlings (Waller *et al.* 2005). *Sebacina vermifera* colonised barley can reduce *B. graminis* infection by 10-80% (Schafer & Kogel 2009).

Plant protection may not always be directly conferred by an endophyte antibiosis to the pathogen, and is often mediated by an endophyte-induced plant response. This type of protective mechanism is typical when barley is infected with *Fusarium graminearum.* Root rot caused by this pathogen is significantly reduced when the barley roots are inoculated with *P. indica* (Deshmukh & Kogel 2007).

Barley varieties vary widely in *Pyrenophora teres* resistance, and selected crossings have produced reliably resistant varieties (Statkevičiūtė & Leistrumaitė 2010). Nevertheless, *P. teres* is still an important pathogen of barley. Though no specific endophytes have been tested for protective competence against *P. teres* in barley, other studies have shown endophyte mediated protection in related cereal crops (Poling *et al.* 2008). The anti-microbial metabolites in non-barley studies are similar to those found in *P. teres* infections of barley.

Barley diseases can also be caused by other types of endophytes, such as bacteria and viruses, and is covered elsewhere (Mathre, 1997). These organisms can infect other plant species, with similar detrimental effects (e.g. barley yellow dwarf virus is commonly found on other cereal and grass species, and is twice as frequent in endophyte-free grasses (Clay & Schardl 2002)).

MECHANISMS OF PATHOGEN RESISTANCE

So how do the fungal root endophytes confer these beneficial effects on the barley plant? In mycorrhizal associations, the systemic bioprotectional effect depends on the degree of root colonisation by the mycobiont (Khaosaad *et al.* 2007). In wheat, the antagonistic effect of the endophyte against a pathogen can be due to activation of host defences rather than direct antagonism (Istifadah & McGee 2006), and in the expression of herbivore resistance the specific combination of host plant species/genotype and endophyte species/strain is important (Clement *et al.*, 1997).

A number of mechanisms have been implicated in endophyte induced resistance. It is clear that *P. indica* requires host cell death to proliferate (Deshmukh *et al.* 2006), and resistance-related antioxidant capacity is enhanced in *P. indica* infected barley (Waller *et al.* 2005). An

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increase in the growth hormone indoleacetic acid (IAA) is involved in the subsequent establishment of a biotrophic symbiosis (Hilbert *et al.* 2012), and might represent a compatibility factor in endophyte infection. Changes in the root surface pH are a feature of *P. indica* colonisation, resulting in a longer-term response of a decrease in leaf surface pH upon *B. graminis* infection (Felle *et al.* 2009).

Changes in gene expression are to be expected in response to endophyte and/or pathogen infection, and the expression of pathogenesis-related genes strongly increases in response to *F. graminearum* infections, but in contrast, diminishes in the presence of *P. indica* (Deshmukh & Kogel 2007). Genes related to root cell skeletal (actin) reorganisation are activated in barley attacked by powdery mildew, resulting in actin focussing at the point of infection; presumably strengthening the defence against pathogen ingress (Opalski *et al.* 2005). Endophyte contribution to this process in barley cells has not yet been demonstrated.

Hormones and other metabolites are important signalling molecules in the plant system, and these too show marked changes in expression and activity in response to fungal infection. Fungal-host interactions involve constant mutual antagonisms, often based on a coordinated response to the secondary metabolites the partners produce (Schulz *et al.*, 1999). Colonisation success may ultimately depend on the nature of plant hormone signalling activity. In barley, the evasion and suppression of the host defences during early colonisation may be related to the perturbance of plant hormone balance and the secretion of fungal effectors (Lahrmann & Zuccaro 2012). Plant hormones are important factors for compatibility in plant root-*P. indica* associations, and might provide a first explanation for colonisation success in a wide range of higher plants. *Piriformospora indica* induces ethylene synthesis in barley, which suggests that ethylene signalling is required for symbiotic root colonisation (Molitor & Kogel 2009; Khatabi *et al.* 2012). The hormones gibberellin, auxin and abscisic acid are up-regulated, and these changes are significant factors of compatibility in the mutualistic association (Schäfer *et al.* 2009). On the other hand, in *Gaeumannomyces graminis* infected barley, salicylic acid-associated gene expression is suppressed (Khaosaad *et al.* 2007). Systemically induced resistance and protection is also strongly associated with an increase in the expression of the signalling protein jasmonate (Waller *et al.* 2005; Stein *et al.* 2008; Molitor & Kogel 2009). The changes in hormone homoeostasis are accompanied with a general suppression of the plant innate immune system. Compatibility mechanisms in host–microbe interactions are complex, and an increase in phytohormone signalling may be necessary for successful fungal colonisation (Schäfer *et al.* 2009).

Barley does produce antifungal and herbicidal substances of its own, and these are enhanced by the presence of the endophyte (Schulz *et al.*, 1999). Related substances from different kingdoms of life may also act as anti-fungal agents, and an insect peptide has been shown to confer on barley a selective capacity for resistance to fungal pathogens (Rahnamaeian *et al.* 2009).

Although research has focussed on a few well-characterised fungal root endophytes, there may be many others that have the potential to inhibit barley pathogens. Macia-Vicente *et al.* (2008a) tested 73 endophyte isolates belonging to diverse genera, and all showed some inhibition of the pathogen *G. Graminis* in barley roots.

YIELD ENHANCEMENT

In most plants, grain yield improvement and an increase in biomass often go hand-in-hand, and barley is no exception. *Piriformospora indica* infected barley can increase shoot fresh weight by up to 65% after only 4 weeks of mutualistic symbiosis (Waller *et al.* 2005). In field colonised barley, plant biomass increase may be only 10% (Schafer *et al.* 2009), probably due to other stresses not encountered in the glasshouse.

Grain yield effects due to fungal root endophyte infection also vary depending on the experimental environment. *Piriformospora indica* field colonised barley grain yield increases can be up to 10% (Schafer *et al.* 2009), whereas glasshouse colonised barley grain yield increases can be up to three times more (Waller *et al.* 2005).

The increases in barley grain yield due to root colonisation by *P. indica* are apparent even under very different nutrient regimes (Achatz *et al.* 2010), due to accelerated growth of barley plants early in development and earlier maturation of ears. Achatz *et al.* (2010) showed that higher grain yield was induced by the fungus independent of markedly different phosphate and nitrogen fertilisation levels. Unlike mycorrhizal symbiosis, improved phosphate supply was not observed in the *P. indica*-barley experiments.

An endophyte-induced relative increase in root biomass over shoot biomass has been demonstrated in grass species (Czarnoleski *et al.* 2012) and rice (Redman *et al.* 2011). No equivalent pattern has been reported in barley studies, though nearly all yield related experiments have shown an overall increase in plant biomass due to endophyte infection.

ABIOTIC STRESS TOLERANCE

Although some endophyte research has studied a wide variety of abiotic stress tolerance types (Rodriguez *et al.* 2008; Rodriguez *et al.* 2009; Redman *et al.* 2011; Singh *et al.* 2011), studies using barley and fungal root endophytes have mainly focussed on salt stress.

In general, *P. indica* is beneficial in protecting the barley plant against many different biotic and abiotic stresses (Schafer *et al.* 2009). Barley is a globally important crop adapted to marginal and stress-prone environments (Willcox 2005), and these environments are often characterised by gradually increasing salinity. Therefore, salt tolerance is an important factor for the grower considering planting barley as a risk aversion crop. Saline soils can inhibit the activity of most soil fungi (Dixon *et al.*, 1993) and may lead to reduced competition for the beneficial root endophyte.

Metabolic heat efflux, salt-induced lipid changes, and fatty acid desaturation are all associated with salt stress in plants (Criddle *et al.*, 1989; Ahmad *et al.* 2013). Barley root colonisation by *P. indica* attenuated all of these factors in the leaves of a salt-sensitive cultivar, and still increased plant growth (Baltruschat *et al.* 2008). Several studies have found that endophyte-mediated salt tolerance was associated with a strong increase in antioxidants (Criddle *et al.*, 1989; Baltruschat *et al.* 2008).

The vacuoles of plant cells can act as salinity buffers, sequestering the salts for later processing (Blumwald & Poole, 1987; Horie & Schroeder 2004). This membrane-mediated transport can be enhanced in barley seedlings by the application of exogenous fatty acids (Zhao & Qin 2005), resulting in protective effects against salt stress. Mycorrhizal fungi can increase sodium neutralisation in plants grown in highly saline soils (Mohammad *et al.* 2011), and endophyte-induced salt stress tolerance may involve a similar mechanism.

INTERACTIONS WITH BACTERIA

Fungal root endophytes are often intimately associated with particular bacterial companions (Sharma *et al.* 2008), and both *P. indica* and *S. vermifera* have species-specific associations with bacteria. In fact, it is nearly always the case that both partners are found together (Sharma *et al.* 2008; Schafer & Kogel 2009). This raises the question as to whether it is the association or the individual partners that confer the beneficial effects on plants. Schafer & Kogel (2009) showed that inoculating barley with the bacteria alone (*Rhizobium radiobacter*) gave similar effects as *P. indica* inoculation.

CONCLUSIONS

Despite a widespread perception that fungal root endophytes will always confer benefits on their hosts, this review and other meta-analyses (Mayerhofer *et al.* 2012) reveal among-study contradictions, probably due to experimental variability. Endopyte infection can be negative, positive or neutral in effect.

The clinching argument against regarding fungal root endophytes as always beneficial may lie in evolutionary theory. If, as some have suggested, the fungi enabled colonisation of the land by plants in the first place (Pirozynski & Malloch, 1975), and fungal endophytes confer such an advantage, then why isn't endophyte symbiosis obligatory? Why have plants and endophytes not formed a similar intimate relationship as that of animals and bacteria? The endophyte habit may even be ancestral in some fungal groups (Weiss *et al. 20*11). We cannot survive without our bacterial partners, but plants can exist perfectly well without endophyte infection (however rarely this absence may occur).

Clearly, prevailing circumstances dictate the nature of the plant-endophyte relationship. Even if an endophyte is never pathogenic, it is not always beneficial, and is often neutral in effect. Despite the potential benefits of fungal root endophyte colonisation, there will always

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be some cost to the plant, with the fungus needing to extract at least some nutrients to survive. Fungal endophyte colonisation can reduce the availability of free sugars and amino acids to the root tip (Schäfer *et al.* 2009), and even detrimentally affect all barley parts when ammonium is the only available nitrogen source (Schafer & Kogel 2009).

The most important factors that determine beneficence are the specific combination of partner genotypes (or strains) and developmental stage, and the ecological and environmental setting. A fungal endophyte that has beneficial effects on a particular barley variety may be detrimental to another variety in a different environment or at a different developmental stage. This is why I consider the term 'endophyte' to encompass both beneficial (symptomless) and detrimental (or pathogenic) organisms. In a stress-free situation **any** fungal infection will impose some cost on the plant, and it is only the protection afforded against more virulent pathogens that gives the endophyte its beneficial status. Some definitions of endophytes consider them symptomless, but they still exact an energy cost from the plant.

But, of course, there is no such thing as a stress-free environment, and in any natural ecological setting a plant will be exposed to many and fluctuating stressors. Therefore, the fungal infection may give the plant a greater ability to cope with the stress if the partners are in a balanced relationship. As we have seen, this balance can be maintained by a variety of mechanisms.

In situations where there may be many fungal organisms in the rhizosphere, the competition for root living space will inevitably result in contingent temporal outcomes. At different times, the same fungus can be either a winner or loser, depending on the mix of competing organisms and the developmental stages of both host and fungi. Likewise, the same fungus may turn out to be predominantly beneficial or pathogenic. The antibiosis displayed in-vitro by putative beneficial fungal root endophytes may be partly a result of the unnatural experimental conditions, and may not translate to a complex natural ecology.

There seems to be no significant consistent relationship between nutrient levels and colonisation. Neither the nutrient status of the plant nor the availability of soil nutrients affects the degree of endophyte colonisation, and, unlike mycorrhizae, there is no apparent increase in endophyte-associated phosphorous transfer. However, there are indications of an association between successful endophyte colonisation and soil pH and clay content.

The greatest benefits to the barley plant resulting from the colonisation of root tissue by a fungal endophyte seem to be obtained in abiotically stressed environments. In these situations, endophyte colonisation does seem to confer consistent and predictable benefits on the barley plant. An up-regulation of antioxidants and endophyte-associated lipid changes in the plant cell membrane enhance plant tolerance of the stress, and increase growth and yield over non-infected plants. Salt-stress in particular may inhibit the activity of other potentially pathogenic fungi and lead to a relaxation of competitive pressure, enabling the full effects of the beneficial endophyte to be realised.

Persistent Identifier: urn:nbn:de:0294-sp-2013-ppphe-2

The relationship between fungal root endophytes and barley is based on a finely balanced and complex dialogue between the partners, with the final benefit to the plant uncertain.

Many changes are taking place in the world of agriculture today, with global warming and land degradation bringing new stresses to crops, leading to a reduction of suitable growing locations and local food shortages. Over-use of agrochemicals in an effort to maintain yield under increasing stress is causing serious environmental damage and increasing economic costs. Beneficial fungal root endophytes have the potential to reduce chemical use, increase pathogen resistance and enhance stress tolerance while still maintaining yield. Realising this potential means that research in this field is critically important.

So the final verdict on whether fungal root infections in barley can be friends as well as foes must be – it depends! On many variables: plant and fungal species or genotype, developmental stage of both partners, inter-microbial competition, and the biotic and abiotic environmental stresses. Each new study reveals an increasing diversity of beneficial fungal root endophytes, and the full potential of these organisms is still to be determined. It may be that there is a single ideal fungal partner for each variety of barley. If we can discover these ideal fungal partners, then we may yet see a farmer harvesting a healthy crop of chemical-free salt-marsh barley in winter.

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In: Schneider C, Leifert C, Feldmann F (Eds), Endophytes for plant protection: the state of the art. © 2013 Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-11-2.

3-10 Characterization of type *B trichothecenes* as bioactive secondary metabolites of *Microcyclospora tardicrescens* (Capnodiales, Ascomycota)

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ABSTRACT

The Capnodiales (Ascomycota) comprise saprotrophs, epi- and endophytes. Fruit colonizing sooty blotch and fly speck (SBFS) fungi are not known to be plant pathogenic. Phenotypically visible demarcation lines indicate that different SBFS fungi may interfere with each other on the plant surface. We tested whether bioactive secondary metabolites could be involved in these interactions. *Microcyclospora tardicrescens* suppressed the growth of *Colletotrichum fioriniae* retrieved from anthracnose on apples in *in-vitro* dual culture tests. An extract of secondary metabolites obtained from Czapek Yeast Agar of *M. tardicrescens* was analyzed by high performance liquid chromatography coupled with diode array and mass spectrometric detection (HPLC-DAD/MS). In addition, the crude extract was tested in bioassays against fungi *Colletotrichum fioriniae* and *Mucor hiemalis*. The secondary metabolites of extracts showing highest intensity of antimicrobial activity were separated and purified in preparative (RP) HPLC. The components of the bioactive fractions were characterized according to their UV spectrum, masses and empiric molecular formulae with HPLC-DAD and electrospray time-of-flight mass spectrometry (ESI-TOF-MS) and the individual secondary metabolites were determined by one and two dimensional nuclear magnetic resonance spectroscopy (1D and 2D NMR). The biologically active fraction consisted of two congeners of the type B trichothecenes, of which one could be determined as trichothecolon acetate. The other presents a currently unknown trichothecene. Trichothecenes are cytotoxic compounds that have several inhibitory effects on eukaryotic cells. Both compounds were antifungal (for example, against *Colletotrichum fioriniae, Pichia anomala*). The inhibitory concentration (IC_{50}) against a mouse fibroblast cell line of trichothecolone acetate was 0.08 µg/ml and that of the novel trichothecene was 0.17 µg/ml.

Persistent Identifier: urn:nbn:de:0294-sp-2013-ppphe-2

In: Schneider C, Leifert C, Feldmann F (Eds), Endophytes for plant protection: the state of the art. © 2013 Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-11-2.

3-11 Actinomycetes modulate carbon metabolism to provide protection against gray mould

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ABSTRACT

Botrytis cinerea is highly pathogenic to grapevine (*Vitis vinifera* L.), producing the characteristic grey mould symptoms and causing great loss to the yield and also the final product, wine. Control of disease caused by *B. cinerea* is mainly achieved by pre and/or post-harvest pesticide applications. However, the control of this pathogen is a challenge because of its adaptability to various environmental conditions and development of fungicide resistant strains. This is why biological control methods have gained quite a lot of attention since few years. In this work we aim to put in evidence an actinomycete strain S37 as a biocontrol agent in order to reduce the application of pesticides in the viticulture. *Streptomyces anulatus* S37 is an actinomycete able to colonize grapevine *in vitro* plants and inhibit the growth of *B. cinerea*. This strain was isolated from the rhizosphere soil of *V. vinifera* L. from Moroccan areas and has a great potential to produce antifungal metabolites. The objective of this study is to understand the modifications of photosynthesis during bacterization by S37 and also following inoculation with *B. cinerea*. Measurement of chlorophyll a fluorescence, which reflects the functionality of the photosynthetic apparatus, showed that in the S37-bacterized plants inoculated with *Botrytis,* the maximum efficiency of PSII photochemistry was comparable to that of control contrary to *Botrytis* infected plants at 48 hpi. The effective quantum yield and the photochemical quenching were higher in the S37 pre-treated plants than in the *Botrytis* infected plants. These differences were significantly different and might indicate that a modulation of carbohydrate metabolism is implied in the mechanisms by which this *S. anulatus strain* operates to reduce disease incidence. Further studies are necessary to evaluate the protective effect of this potential biocontrol agent in the greenhouse and field conditions, and also to purify and characterize the secondary metabolites produced by this actinomycete.

Persistent Identifier: urn:nbn:de:0294-sp-2013-ppphe-2

In: Schneider C, Leifert C, Feldmann F (Eds), Endophytes for plant protection: the state of the art. © 2013 Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-11-2.

3-12 Interaction between *Burkholderia phytofirmans* and grapevine: link between modulation of carbohydrate metabolism and induced tolerance to low temperatures

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ABSTRACT

Burkholderia phytofirmans strain PsJN is an endophytic Plant Growth Promoting Rhizobacteria (PGPR) that enhances chilling tolerance of grapevine plants (Ait Barka *et al.* 2006). Molecular and biochemical approaches were investigated to explore several mechanisms by which this endophyte could improve cold tolerance by analyzing: (i) effect on expression of defence and cold related genes, (ii) modulation of H_2O_2 production and (iii) stimulation of plant primary metabolism.

Before chilling exposure, bacterized plants displayed higher concentrations of carbohydrates known to be involved in cryoprotection (starch, sucrose and raffinose). When exposed to chilling, bacterized plants exhibited priming of several defence related genes (*VvPAL*, *VvStSy*, *VvLOX*…) as well as priming of the key cold regulator *VvCBF*4 (Theocharis *et al.* 2012). Furthermore, bacterized plants were characterized by a faster and stronger production of H_2O_2 and subsequent faster detoxication of this oxidative species.

Overall, our results suggest that *B. phytofirmans* strain PsJN promotes grapevine chilling tolerance using several distinct pathways, whether existing or cold-induced mechanisms. Future prospects include testing whether these variations also occurred in inflorescence of bacterized inflorescence, since this organ is especially sensitive to cold damages.

Persistent Identifier: urn:nbn:de:0294-sp-2013-ppphe-2

In: Schneider C, Leifert C, Feldmann F (Eds), Endophytes for plant protection: the state of the art. © 2013 Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-11-2.

3-13 The role of short chain AHLs and structurally similar molecules in barley

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ABSTRACT

In the plant rhizosphere complex microbial interactions occur. Bacteria use quorum sensing molecules (N-Acyl-homoserine-lactones; AHLs) for inter- and intraspecific communication. The quorum sensing (QS) is involved in mediating the interaction between bacteria species, and eukaryotic organisms like plants can eavesdrop on this communication. In recent studies it was shown, that an application of N-Acyl-homoserine-lactones towards plant roots lead to changes in root formation and root architecture.

One factor to be involved in this physiological process may be the signaling molecule nitric oxide (NO). NO modulates various reactions in the plant lifecycle including growth, development and pathogen interaction.

The aim of this study was to compare the effects of short chain AHLs and structurally similar molecules on NO accumulation in barley roots and in barley cell culture.

Surface sterilized barley seeds were grown on NB-Agar, containing different AHLs and AHL-like molecules. For NO detection, 3 day old barley roots were loaded with 10µM diaminofluorescein diacetate (DAF-2 DA) and analyzed by fluorescence microscopy. For measurement of NO accumulation in culture cells, the cells were incubated in a 96 well plate, the respective trigger molecules and 10µM DAF-2 DA. The NO-burst was photometrically detected at an excitation maximum at 495 nm and an emission maximum at 515 nm.

Persistent Identifier: urn:nbn:de:0294-sp-2013-ppphe-2

Pańka D et al., Emission of volatile organic compounds by perennial ryegrass (*Lolium perenne* L.) / *Neotyphodium lolii* association as a defense reaction towards infection by *Fusarium poae* and *Rhizoctonia solani*. In: Schneider C, Leifert C, Feldmann F (Eds), Endophytes for plant protection: the state of the art, pp. 121-122. © 2013 Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-11-2.

3-14 Emission of volatile organic compounds by perennial ryegrass (*Lolium perenne* L.) / *Neotyphodium lolii* association as a defense reaction towards infection by *Fusarium poae* and *Rhizoctonia solani*

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ABSTRACT

Neotyphodium and *Epichloë* endophytes are grass symbionts with great potential as a mean of improvement of the plant's growth and resistance to biotic and abiotic stresses. These endophytic fungi are reported to be present in many grass species. They often protect the host against pathogens and pests. The beneficial effects are mainly associated with the presence of a diverse range of secondary metabolites produced under the influence of the endophyte. However, the exact mechanism of the lower susceptibility of E+ plants to infection by pathogens is still unclear. Antibiotic chemicals are reported to be responsible for inhibition of pathogens' development. Moreover, the endophytes likely induce specific physiological and biochemical defense mechanisms in the host plant. Some role in plants response to stress factors can be also associated with volatile organic compounds (VOCs). Detailed experiments in controlled conditions were conducted to answer the following questions: 1) whether E+ perennial ryegrass plants are more resistant to disease caused by *Fusarium poae* and *Rhizoctonia solani* than E- plants, 2) whether the *N. lolii* presence in perennial ryegrass affects emission of VOCs by the plants, 3) what is the extent and direction of changes in VOCs emission under infection of perennial ryegrass by *F. poae* and *R. solani* and 4) whether three perennial ryegrass genotypes differ in susceptibility to infection by pathogens and what are the differences between them in terms of VOCs induction.

Three endophyte infected (E+) and non-infected (E–) perennial ryegrass genotypes were used for research. The plants were artificially inoculated with *F. poae* and *R. solani* infection

Persistent Identifier: urn:nbn:de:0294-sp-2013-ppphe-2

material by foliar spray. Ten days after inoculation the degree of tillers infestation was evaluated based on a five-degree scale where 0° stood for a lack of infection symptoms and 4° – above 60% of the area with disease symptoms, heavy wilting. Volatile organic compounds were collected 3, 6 and 12 days (Intervals) after inoculation from Nalophan bags which enclosed perennial ryegrass plants. There were collected 48 plants for each of three perennial ryegrass genotypes and the pathogen used. Volatiles were analyzed by coupled gas chromatography-mass spectrometry (GC-MS).

All tested genotypes of perennial ryegrass were susceptible to infection by *F. poae* and *R. solani.* Statistical analysis confirmed the highly significant influence of the host plant genotype, endophyte presence and their interaction on the level of infestation by the pathogens. Perennial ryegrass genotypes differed in their susceptibility to infection. Endophytic fungi decreased infestation of the grass genotypes. Both the endophyte infected and non-infected plants of the tested perennial ryegrass genotypes emitted most abundantly two green leaf volatiles (GLVs): ((Z)-3-hexenal, (Z)-3-hexen-1-yl acetate), three terpenes (linalool, (Z)-ocimene, β-caryophyllene) and three shikimic acid pathway derivatives (benzyl acetate, indole, and methyl salicylate). There were observed no qualitative differences between E+ and E– plants and pathogens in the emission of the listed VOCs, but there were quantitative differences. The level of VOCs produced by E+ plants was greater for all detected volatiles relative to the E– plants when averaged over genotypes and intervals. Higher amounts of VOCs were emitted by perennial ryegrass under *F. poae* than *R. solani* infection. There was no effect of *L. perenne* genotype on the VOCs production.

Persistent Identifier: urn:nbn:de:0294-sp-2013-ppphe-2

In: Schneider C, Leifert C, Feldmann F (Eds), Endophytes for plant protection: the state of the art. © 2013 Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-11-2.

3-15 Effect of *Neotyphodium lolii* on production of β-1,3-glucanases and chitinases in perennial ryegrass (*Lolium perenne* L.) infected by *Fusarium poae*

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ABSTRACT

Lolium perenne is a grass of great importance in Polish farming. Diseases caused by pathogenic fungi such as *Fusarium* ssp. are substantial problem in its cultivation. Their harmfulness can be limited thanks to natural symbiotic systems between *L. perenne* and *N. lolii*. The endophyte is able to stimulate the growth of the plant and increase its resistance to biotic and abiotic stress. The exact mechanism of higher resistance of endophyte infected plant is yet not fully understood, but it can be assumed that PR (Pathogenesis-related) proteins have a significant role. Chitinases and β-1,3-glucanases are PR proteins, which are related to the plant resistance to fungi. They tend to occur together, as their integrated action can cause more damage in fungi cell wall, than any of them could cause alone. The aim of the study was to determine the impact of the endophytic fungi *N. lolii* on induction of specific defense mechanism, including production of pathogenesis-related proteins: chitinases and β-1,3-glucanases. The quantity of chitinases and glucanases in endophyte infected (E+) and uninfected (E-) plants was determined 0, 1, 2, 3 and 4 days after infection with *Fusarium poae*. Inhabited plants were much less susceptible to infection by *F. poae*. Occurrence of the endophyte has also affected the amount of chitinases in perennial ryegrass. Its amount was significantly higher in E+ plants. The amount of enzyme was also dependent on the time after infection. Upward trend was being observed since first to third day after inoculation. No significant impact of *N. lolii* on β-1,3-glucanases production and general protein content in the plant was observed. These results indicates the significant role of chitinases in increased resistance of E+ plants exposed to infection by *F. poae*. Further research is needed to define the role of chitinases and β-1,3-glucanases in resistance of E+ plant and cooperation of these two enzymes in increased resistance.

Persistent Identifier: urn:nbn:de:0294-sp-2013-ppphe-2

Pańka D et al., Production of phenolic compounds by perennial ryegrass (*Lolium perenne* L.) /*Neotyphodium lolii* association as a defense reaction towards infection by *Fusarium poae* and *Rhizoctonia solani*. In: Schneider C, Leifert C, Feldmann F (Eds), Endophytes for plant protection: the state of the art, pp. 124-125. © 2013 Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-11-2.

3-16 Production of phenolic compounds by perennial ryegrass (*Lolium perenne* L.) /*Neotyphodium lolii* association as a defense reaction towards infection by *Fusarium poae* and *Rhizoctonia solani*

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ABSTRACT

Endophytic fungi belonging to the genus *Neotyphodium* very often form symbiotic associations with grasses. The host plants usually benefits from the association with an endophyte. Presence of the symbiont may increase host resistance to drought stress, feeding of many insects, infection by harmful nematodes, infection with some pathogens and enhance protection from herbivores. Such effects are mainly conditioned by the presence of vide range of chemicals e.g. ergot alkaloids, lolitrems, peramine, and lolines. The biologically active metabolites are often produced by endophytes *in vitro*, although they may not be synthesized in sufficient quantities *in vivo*. Apart from antibiotic activity of the endophytes the indirect influence of the symbiont on the defense reaction of the host plant may play role in the protection of grass/endophyte association from pathogens. Plant responses may involve e.g. synthesis of phenolic compounds. The aim of the study was to determine i) whether the presence of endophyte in perennial ryegrass increase the production of phenolic compounds in the plants, ii) what is the extent and direction of changes in phenolics production when perennial ryegrass is infected by *Fusarium poae* and *Rhizoctonia solani*, and iii) whether perennial ryegrass genotypes differ in their ability of phenolics induction.

Three endophyte infected (E+) and non-infected (E–) perennial ryegrass genotypes were used for research. The plants were artificially inoculated with *F. poae* and *R. solani* infection material by foliar spray. Analyses of total phenolics content in above ground parts of each plant were performed in control (not inoculated) and inoculated plants after 1, 2, 3, 4, 5, and

6 days. Phenolics were extracted with ethanol and distilled water solution (50:50). Total content of phenolic compounds was determined according to Folin-Ciocalteau procedure.

There was observed a highly significant effect of the perennial ryegrass genotype, endophyte status, time of the analysis (Intervals) and their interactions on the phenolic compounds content in plants. The presence of *N. lolii* increased significantly the production of total phenolics in all genotypes, in the control combinations. The highest content of phenolics was recorded 1 or 2 days after infection by *F. poae* and 1 day after infection by *R. solani*. Decrease of phenolics content was recorded in the next 2-4 days and then an increase depending on the grass genotype and species of the pathogen.

Results suggest that phenolic compounds can play an important role in defence mechanisms of E+ perennial ryegrass against pathogens.

Persistent Identifier: urn:nbn:de:0294-sp-2013-ppphe-2

In: Schneider C, Leifert C, Feldmann F (Eds), Endophytes for plant protection: the state of the art. © 2013 Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-11-2.

3-17 Analysis of subcellular accumulation of cadmium (Cd) by metal tolerant bacteria using transmission electron microscopy (TEM) with energy-dispersive X-ray microanalysis (EDS)

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ABSTRACT

Intensive industrialization and urbanization caused the global problem with contamination of the environment with high concentrations of heavy metals. Cadmium (Cd) is a key element which affect toxicity of heavy metals in soil ecosystems due to its availability and fast transport from the soil to microorganisms and plants. Bacteria have relatively high potential for sorption of large amounts of Cd in their biomass. Distribution of heavy metals in the bacterial cells is closely related to their structuree.g., organization of their cell wall and envelope.

In our study we have used microscopic analysis EDS to analyse location and concentration of Cd absorbed in the cells of 5 heavy metal tolerant bacterial cells: *Massilia sp., Pseudomonas sp., Pseudomonas fulva, Bacillus sp., Serratia entomophila*.

Analysis of TEM images and energy-dispersive X-ray microanalysis revealed significant differences in potential for accumulation of Cd in cellular organelles of investigated bacterial strains depending on the structure of cell wall and envelope.

Persistent Identifier: urn:nbn:de:0294-sp-2013-ppphe-2

Martins F et al., Plant-mediated effects on antagonistic activity of endophytic fungi towards olive fungal diseases. In: Schneider C, Leifert C, Feldmann F (Eds), Endophytes for plant protection: the state of the art, pp. 127-128. © 2013 Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-11-2.

3-18 Plant-mediated effects on antagonistic activity of endophytic fungi towards olive fungal diseases

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ABSTRACT

In recent years, fungal endophytes have received increasing attention as a promising supplement or alternative to chemical control. Much of the research undertaken on fungal endophytes as biocontrol agents have focused on the elucidation of the mechanisms that may control diseases suppression. However, the effect of plant host on their efficacy as biocontrol agents is poorly known. The endophytic fungus *Penicillium commune* isolated from the host plant *Olea europaea* Cv. Cobrançosa, has been shown the faculty to inhibit the growth of the phytopathogen *Colletotrichum acutatum*, which caused one of the major olive diseases - the anthracnose. The aim of this study was to determine the effect of the olive leaves on the antagonism displayed by *P. commune* against *C. acutatum* using simultaneous and sequential inoculations. For this, co-cultures on Petri dishes were established with the two strains either in the presence of one endophytic-free olive leaf (+leaf), placed in the middle of fungal inoculum, or in its absence (-leaf). The phytopathogen was inoculated on agar simultaneously with the endophyte, or 8 and 10 days after endophyte. Within the first 12 days after phytopathogen inoculation, the radial growth towards (internal radius) the interacting fungus, germination and sporulation of both fungal strains were evaluated and the outcome of interaction was assessed. To determine the degree to which plant and endophyte regulate phytopathogen infection, the colonization of leaves by the endophyte and the phytopathogen were assessed during the assay. The results indicate that in the presence of leaves the *P. commune* reduced significantly the sporulation (in an average 1.9-fold) and germination (in an average 2.6-fold) of *C. acutatum* when compared to –leaf treatment. Inoculation of *P. commune* 8 and 10 days prior to pathogen resulted in a more reduced germination and sporulation of *C. acutatum* than simultaneous inoculation. No significant differences were found on *C. acutatum* growth between +leaf and –leaf treatments, when both strains were inoculated simultaneously. However, the prior inoculation of agar with the

endophyte resulted in significantly increased of *C. acutatum* growth (in an average 25-fold) in the presence of leaf when compared to –leaf treatment. The colonization of leaves by *C. acutatum* was only observed when strains were inoculated simultaneously, being its value reduced over the incubation period from 19% to 0%. In all of the treatments the colonization of leaf by the endophyte was increased over the incubation period. The greater understanding of these interactions will allow the development of more effective biological control of plant diseases.

ACKNOWLEDGMENTS

This work has been supported by FCT (reference grant PTDC/AGR-PRO/4354/2012).

Persistent Identifier: urn:nbn:de:0294-sp-2013-ppphe-2

SESSION 4

Cultivation of Endophytes

4-1 Cultivation of hitherto uncultured bacteria living is association with plants

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Persistent Identifier: urn:nbn:de:0294-sp-2013-ppphe-2

Aoi Y, New methods for cultivation of uncultured. In: Schneider C, Leifert C, Feldmann F (Eds), Endophytes for plant protection: the state of the art, pp. 130-131. © 2013 Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-11-2.

4-2 New methods for cultivation of uncultivables

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SUMMARY

Overwhelming majority of microorganisms habiting under any kinds of environments do not grow in culture, especially in pure culture. Although it is obvious that recently wide-spreading cultivation-independent approaches such as direct DNA and RNA sequencing based analysis has brought huge impact on the field of microbiology and microbial ecology, it is still not simple to estimate physiological properties of single species. It is apparent that “Growth” is the most fundamental and essential phenomenon of the environmental microorganisms, and the cultivation has been and will be the most common and effective technique to analyze variety of characteristics. Therefore, trials to widen the range of cultivable types among vast majority of unculturables are still important, not only for the purpose of adding another strain to the culture collection, but also for the application to comprehensive understandings of specific microbial eco-systems. In the presentation, development of new methods which is potentially applicable to the uncultured endophytic microorganisms and successful application of those to cultivate unculturables from various environments will be presented.

IN SITU CULTIVATION

First, development of a new high-throughput in situ cultivation method will be presented. A new high-throughput in situ cultivation method was developed and applied to various environmental samples: 1) human oral (in vivo cultivation), 2) forest soil, and 3) river water. The device is compact enough to fit in various purposes such as in vivo cultivation (potentially applicable for cultivation in plant), while $10^{5\text{-}6}$ samples can be cultivated with one device. This is achieved by combined use of hollow fiber membrane and extensive

number of (10^{6-7}) small (20 - 50 µm) agarose gel particle which initially contains 0 or 1 microbial cell per GMD. The device is As a result of application to the environmental samples, the in situ cultivation yielded higher rate of cultivability and diversity, and novelty of isolates compared with those of conventional method (agar-plate). Interestingly, the results suggested that the large proportion of microorganisms which are originally recalcitrant to cultivation in conventional methods, turned into cultivable types after the in situ cultivation.

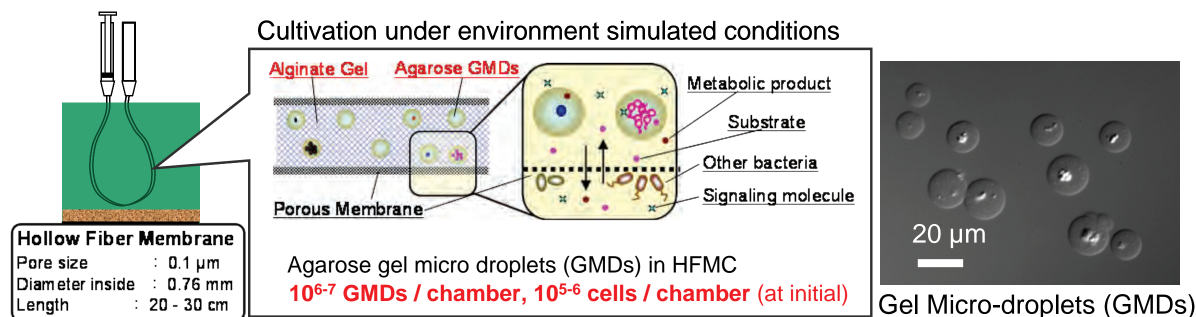

Figure 1 High through put in situ cultivation (DG-HFMC)

MICRO-COLONY SELECTIVE INOCULATION

Second, development of "micro-colony based separation" and successful application to the uncultured nitrifying bacteria will be presented. Autotrophic nitrifying microorganisms usually are recalcitrant to cultivate in spite of their environmental importance both in engineered and natural process. The new methods exploited their biological properties forming micro-colonies for the separation of target. The flow cytometric cell sorting was applied to separate target and inoculate micro-colonies one-by-one as a growth unit. This technique brought the success in the cultivation of several previously uncultivated ammonia and nitrite oxidizing microorganisms such as uncultured types of *Nitrospir*a.

PERSPECTIVES

The cultivation of uncultured is effective to obtain secure information of isolates, such as specific physiological, bio-chemical, and genomic properties. Those will be undoubtedly regarded as essential bases of any type of microbial and microbial ecological analysis including endophytes. Cultivation of previously uncultivated microorganisms through the development of new cultivation methods is effective to find a new hint for puzzling out "microbial unculturability", which is of major subject on general microbiology.

Persistent Identifier: urn:nbn:de:0294-sp-2013-ppphe-2

Aragão Börner RA et al., Microcultivation of single cell bacteria in alginate microbeas. In: Schneider C, Leifert C, Feldmann F (Eds), Endophytes for plant protection: the state of the art, pp. 132-133. © 2013 Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-11-2.

4-3 Microcultivation of single cell bacteria in alginate microbeas

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ABSTRACT

The knowledge in Microbial Diversity has increased during the past 20 years due to the use of molecular tools based on the sequencing of 16S rRNA gene. With this information it has been estimated that 99,9 % of all microorganisms are still “un-culturable”. This fact, leads to the search of new tools for isolation and cultivation of the microorganisms that until now could not be isolated. It is expected that this fact will provide a better understanding of complex communities and potential new biotechnological applications.

In order to increase the cultivation efficiency, entrapment of single cells in polymer beads and subsequent culturing to form microcolonies have been a successful strategy among different groups of aerobic microorganisms that are slow growers and difficult to cultivate using traditional techniques (Zengler *et al.* 2002). Anaerobic microorganisms have in general been less studied than the aerobes and that is also the case when it comes to the “un-culturable” organisms.

With a focus on developing a simple technique, possible to apply in different laboratory conditions, we optimized the parameters for the entrapment of single cells of anaerobic bacteria in alginate microbeads using the internal gelation system (IGS) (Poncelet *et al.* 1992) method for further micro cultivation. An anaerobic consortium which was selected for its capacity to degrade complex carbohydrates, and a pure culture of cellulose degrading bacteria were used for entrapment studies. Optimization of conditions for the formation of spherical alginate microbeads in sizes between 20-80 µm was examined. The best conditions were achieved by combining rapeseed methyl ester as oil phase and stirring at 100 rpm using a rotation impeller. Ca-alginate microbeads produced under these conditions were shown to present morphological stability, with large pores in the internal matrix that favours microcolony development. Finally, single cells were observed inside the beads after the

entrapment procedure and microcolony formation was confirmed after cultivation in cellobiose.

The results indicate that is possible to use alginate microbeads for enrichment of anaerobic cells which can be further used for isolation. The technique is suitable for cultivation of bacteria from different environments.

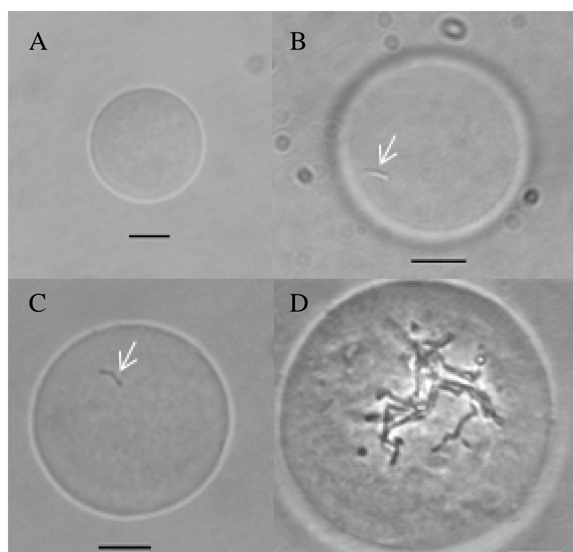

Figure 1 Microscopic image of Ca-alginate microbead. A) Empty bead B) and C) beads containing one sinlge cell, arrow indicating the single cell in the bead D) Bead containing a microcolony after 36 h cultivation. Images A to C in bright field, D in phase contrast. Bar indicates 10 µm.

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Persistent Identifier: urn:nbn:de:0294-sp-2013-ppphe-2

Lohse R, Jakobs-Schönwandt D, Patel AV, Development of a novel fermentation process for an endophytic *Beauveria bassiana* strain. In: Schneider C, Leifert C, Feldmann F (Eds), Endophytes for plant protection: the state of the art, pp. 134-139. © 2013 Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-11-2.

4-4 Development of a novel fermentation process for an endophytic *Beauveria bassiana* strain

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ABSTRACT

There is an increasing demand for alternative or complementary crop protection strategies. A novel approach could be the use of the entomopathogenic and endophytic fungus *Beauveria bassiana* isolate ATP-04. To use the endophyte as a commercial biocontrol agent, the fungus has to be mass-produced. *B. bassiana* was raised in shake flask cultures to produce submerged conidiospores (SCS) which are reported to show a higher shelf life than mycelium and blastospores (BS). It was found that in mineral media with 5% sugar beet molasses *B. bassiana* produced $0.1x10^{10}$ SCS/g sucrose in 192 h. By adding 50 g/L NaCl 48 h after inoculation the SCS yield increased to $1.4x10^{10}$ SCS/g sucrose. The scale-up to a 2 L stirred tank reactor was carried out at 25°C, 200-600 rpm and 1 vvm at pH 5.5. A total spore yield of $5.2x10^{10}$ spores/g sucrose corresponding to a SCS yield of $0.2x10^{10}$ SCS/g sucrose was obtained after 216 h. Also the yield of SCS increased to $1.1x10^{10}$ SCS/g sucrose by the addition of NaCl.

INTRODUCTION

B. bassiana is an entomopathogenic fungus and can exist asymptomatically in a variety of plants, like banana (Akello *et al.* 2009) and sorghum (Tefera *et al.* 2009). This endophytic *B. bassiana* shows efficacy against a wide range of insect pests from within the plants and has the potential of becoming a cost-effective biocontrol agent (Khachatourians 1986). Most publications on cultivation of *B. bassiana* deal with solid-state fermentation of epiphytic *B. bassiana* isolates and therefore with the mass-production of aerial conidia and mycelium (e. g. Kang *et al.* 2005). However the preferred method for large-scale production of microorganisms is submerged cultivation. The obvious advantages of a submerged

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cultivation are that the fungus produces spores in a relatively short time with high yields under controlled sterile conditions as well as a simpler scale-up in contrast to solid-state fermentation (Feng *et al.* 1994, Patel *et al.* 2010). In a submerged cultivation *B. bassiana* forms blastospores, submerged conidiospores and mycelium. Blastospores are relatively large, thin-walled and single-celled hyphal bodies (Bidochka et al. 1987). Submerged conidiospores, on the other hand, are small, spherical, more uniform in size and show a higher shelf-life than blastospores. They arise from fungal mycelia or directly from blastospores in a process known as microcycle conidiation (Thomas *et al.* 1987).

The objective of the present work was to produce submerged conidiospores in a cost-effective culture medium on lab scale, to increase SCS yield by addition of NaCl and scale-up the process to a 2 L stirred-tank reactor.

MATERIALS AND METHODS

Strain

Beauveria bassiana isolate ATP-04 was provided by the Georg-August-University, Department of Crop Sciences/Agricultural Entomology, Goettingen. The strain was raised at 25°C on ½ SDA agar containing 1% casein peptone, 2% glucose and 1.5% agar-agar at pH 5.5. Temperature optimum was determined at 25 °C and pH optimum at 5 - 6 (data not shown).

Cultivation

B. bassiana was grown in different liquid media in shake flasks with three baffles. Different culture media were distributed in 50 mL quantities in 250 mL baffled flasks. As a starter inoculum aerial conidia from agar plates (see above) were used. The aerial conidia were isolated by flooding the plates with 2 x 5 mL of sterile 0.1% Tween 80 and gently raking the plates with a sterile bristle brush. The shake flask cultures were inoculated with the spore suspension to give an initial spore density of $5.0 \cdot 10^4$ spores/mL. The flasks were incubated at 25°C, 150 rpm and pH 5.5. At seven different times after inoculation different sterile NaCl stock solutions were added to the "osmotic stress" cultures, varying the final NaCl concentration in the media. Every day, 1 mL samples were taken to check developmental stage and the concentration of the spores with a Thoma counting cell chamber under 400 x magnification (Zeiss photomicroscope). Batch fermentation was carried out in a 2 L stirred tank reactor (Sartorius Stedim System GmbH, Germany). Fermentation was started by inoculating 300 mL of a carbon source stock solution with $7.5 \cdot 10^8$ aerial conidia ($5.0 \cdot 10^4$ spores/mL). The fermentation was carried out at 25°C, 1 vvm and 200-600 rpm.

Analysis

For the determination of fungal dry biomass 15 mL samples were centrifuged for 10 min at 20000 g, washed two times with ddH_2O and centrifuged again. The pellets were diluted in 5-

7 mL of ddH$_2$O. The cell suspensions were dried at 115°C with a moisture analyzer (Sartorius, Germany).

The colony forming units (CFU) of blastospores and submerged conidiospores were determined by spreading 100 µL of diluted samples on agar plates (see above) and incubating at 25°C for 4-6 days.

RESULTS AND DISCUSSION

In total, 23 technical culture media based on different carbon sources, minerals and technical yeast extracts were screened. The most promising culture medium was a mineral medium with 5% sugar beet molasses, which consists of 50% sucrose. In this culture medium *B. bassiana* produced 1.33±0.06x10^9 total spores/mL respectively 5.32±0.24x10^{10} total spores/g sucrose 192 h after inoculation. But the concentration of submerged conidiospores was only 0.03±0.01x10^9 SCS/mL.

Sugar beet molasses is a residue of the agricultural industry and consequently it is a low-cost source. Therefore, the cost of 1 L culture medium amounts to only 0.33 € However the problem of this cultivation is the low concentration of SCS. One potential solution for this problem is the selective production of SCS by osmotic stress. To this end the influence of different times of addition and final concentrations of NaCl on the production of SCS was investigated.

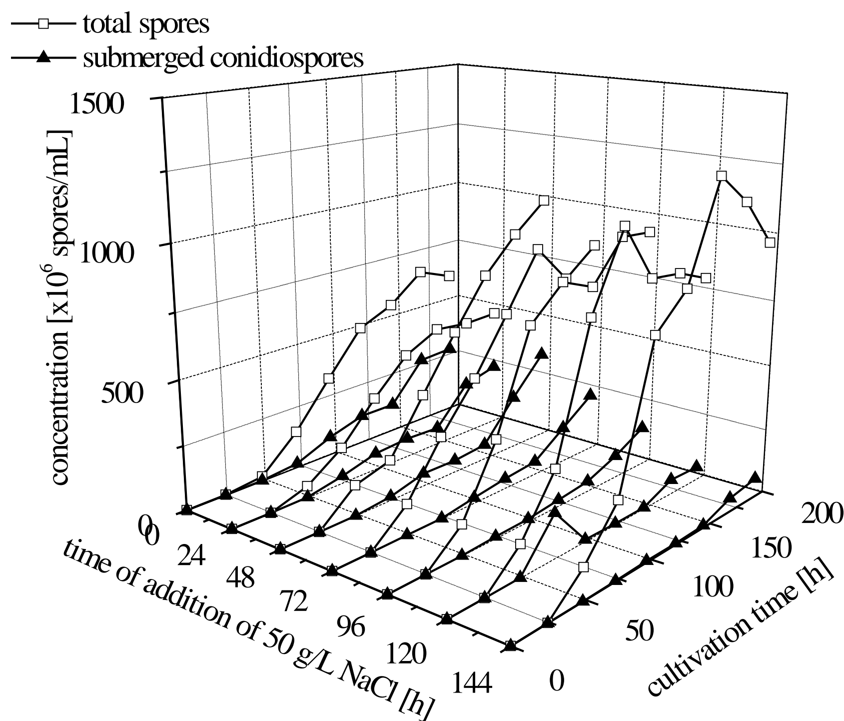
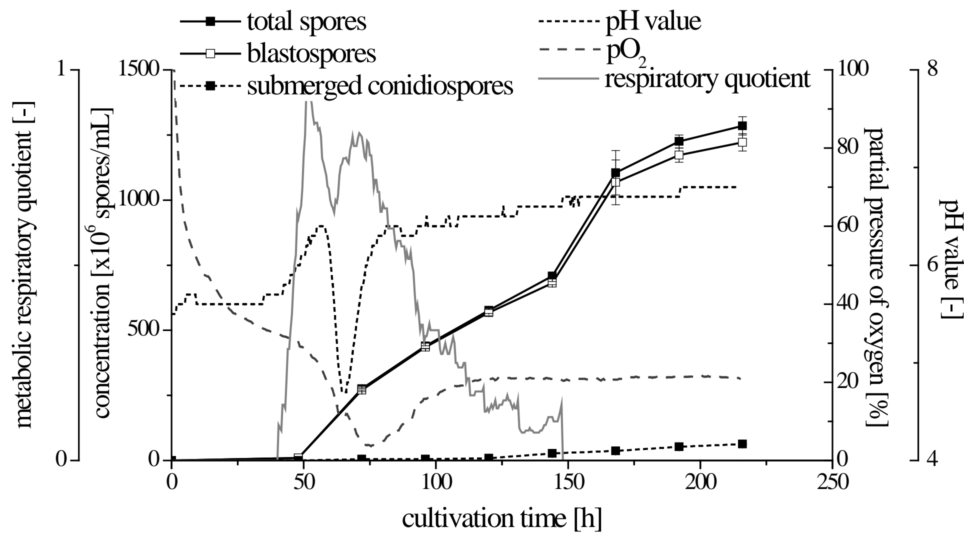


Figure 1 Influence of different times of addition of 50 g/L NaCl on the production of SCS.

In the control, where no salt was added but the same amount of water after the same time span, a concentration of 0.02±0.00x10^9 SCS/mL was obtained. In contrast, 48 h after inoculation the addition of 50 g/L NaCl led to a concentration of 0.35±0.03x10^9 SCS/mL corresponding to a yield of 1.40±0.12x10^{10} SCS/g sucrose at the end of the cultivation (Figure 1). Thus, the amount of SCS was increased 17.5-fold by the addition of NaCl at the appropriate time. It was observed that the highest yield of total spores was obtained without any addition of NaCl. When NaCl was added to the cultivation broth the yield of total spores decreased and a shift from BS to SCS was observed. The earlier the addition of NaCl, the higher the concentration of SCS and the lower the concentration of total spores.

2A



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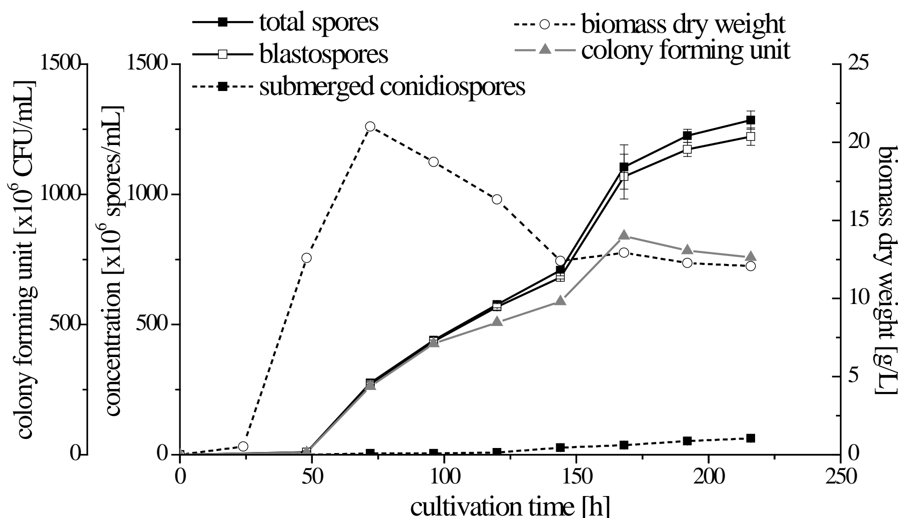

Figure 2 Cultivation of *B. bassiana* in a 2 L stirred tank reactor. The graphic shows the concentrations of total spores, BS and SCS. **A** process parameters. **B** correlation of spore counts with biomass and CFU.

Figure 2 shows a fermentation of *B. bassiana* in a 2 L stirred-tank reactor without addition of NaCl. In this fermentation *B. bassiana* produced 1.29±0.04x10^9 total spores/mL corresponding to a yield of 5.16±0.16x10^{10} total spores/g sucrose at the end of the fermentation. Therefore, the cost of 10^{12} total spores can be estimated at 0.26 € But concentration of SCS was only 0.06±0.00x10^9 SCS/mL. The amount of dry biomass increased at the beginning of the fermentation because the fungus produced mycelium. After 72 h 21 g biomass/L were obtained. Then the amount of mycelium decreased to the end of the fermentation. This could be due to the limitation of substrates. 96 h after inoculation the concentration of viable spores started to decrease to 0.78x10^9 total spores/mL corresponding to a yield of 3.12x10^{10} total spores/g sucrose at the end of the fermentation.

For the selective production of SCS 50 g/L NaCl was added to the culture broth after 48 h (Figure 3). In contrast to a cultivation without the addition of NaCl the concenration of SCS could be increased to 0.28±0.01x10^9 SCS/mL corresponding to a yield of 1.12±0.03x10^{10} SCS/g sucrose at the end of the fermentation. This represents a 5-fold increase of the SCS yield. Furthermore, *B. bassiana* did not produce mycelium during the fermentation in contrast to the fermentation without NaCl addition.

To conclude, the endophytic *B. bassiana* isolate ATP-04 was cultivated in a low-cost culture medium to high spore yields respectively to high SCS yields by addition of NaCl without oxygen limitation or pelleting of the biomass.

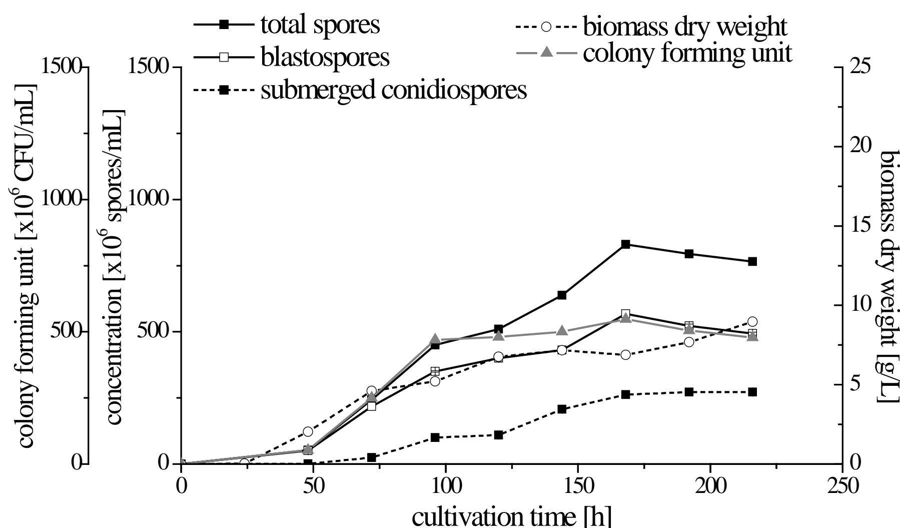

Figure 3 Cultivation of *B. bassiana* in a 2 L stirred tank reactor. The graphic shows the concentrations of total spores, BS and SCS and further analytical parameters.

ACKNOWLEDGEMENTS

This research was funded by the BMBF, Germany. (FKZ 17N1510)

Persistent Identifier: urn:nbn:de:0294-sp-2013-ppphe-2

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Persistent Identifier: urn:nbn:de:0294-sp-2013-ppphe-2

In: Schneider C, Leifert C, Feldmann F (Eds), Endophytes for plant protection: the state of the art. © 2013 Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-11-2.

4-5 Bacteria associated with plant tissue culture: differences between well and poorly growing *Prunus avium* genotypes.

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ABSTRACT

Prunus avium (wild cherry) is a hardwood cultivated for the production of high quality furniture. To optimize growth characteristics (fast growth, straight stem) single plants showing a favourable habitus are selected and propagated as in-vitro clones under the trademark silvaSELECT®. During in vitro propagation high scale losses of plantlets are observed, especially during rooting and acclimatisation. It is supposed, that these are caused by the presence or absence of endophytes in in vitro plantlets.

To test if there is a link between endogenous bacteria and growth of in vitro shoot cultures, we selected cherry genotypes with good or poor propagation success for our analysis. The bacterial communities of three *Prunus avium* genotypes were characterized by culture independent methods.

A PCR was conducted on 16S rDNA with bacteria-specific primers. Amplified fragments were cloned and 95 *E. coli*-colonies of each sample were analysed by ARDRA (amplified ribosomal DNA restriction analysis). This sample size led to more than 90% coverage of the clone library. One colony from each group was selected and identified by sequencing and comparison to NCBI database entries.

Our results show that the community structures of the well growing cherry genotype 'Neptun' and the poorly growing genotype 'Fama' differ significantly. Genera which were previously reported to have plant growth promoting effects were so far only detected in 'Neptun'. A third well growing cherry genotype is currently under investigation. Bacteria that were cultivable on growth media were isolated and purified. A closer look on their taxonomic classification by sequencing of the ribosomal internal transcribed spacer (ITS) region and development of species-specific primers for qPCR is in progress.

Persistent Identifier: urn:nbn:de:0294-sp-2013-ppphe-2

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4-6 Bacterial endophytes in wheat (*Triticum aestivum*): isolation, characterisation and bio-prospecting

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ABSTRACT

This study uses material from the Broadbalk experimental plots at Rothamsted Research to complete a comparative study of bacterial endophyte communities in winter wheat (*Triticum aestivum* cv. Hereward) under contrasting fertiliser regimes. This work aims to determine whether agricultural inputs alter the dominant community structure of bacterial endophytes in wheat roots and shoots, to explore the consequences of endophyte colonisation on the wheat plant and to identify endophyte niches within plants.

A combination of culture and molecular techniques were used for isolation and identification of endophytes. Endophyte populations were profiled using 16S rRNA gene RFLP analysis and unique profiles were selected for 16S rRNA gene sequencing. Both the roots and shoots feature low complexity bacterial endophyte communities containing operational taxonomic units from four phyla: Proteobacteria, Bacteriodetes, Actinobacteria and Firmicutes. True endophyte status will be confirmed through re-isolation and visualisation in the plant through GFP or LUX tagging. Selected endophytes will be subject to further physiological and morphological characterisation and screening for biochemical traits such as indole acetic acid production or for expression of potentially beneficial genes such as those involved in nitrogen fixation (eg. *nifH*).

The most recent results concerning endophyte identity, abundance and properties are presented.

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SESSION 5

Endophytes for Plant Protection and Production

Persistent Identifier: urn:nbn:de:0294-sp-2013-ppphe-2

Sikora R A, Biological management of plant parasitic nematodes using fungal and bacterial endophytes – past, present, and future. In: Schneider C, Leifert C, Feldmann F (Eds), Endophytes for plant protection: the state of the art, pp.144-148.
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5-1 Biological management of plant parasitic nematodes using fungal and bacterial endophytes – past, present, and future

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INTRODUCTION

Most microbial interactions in the soil that are of direct importance to the biological control of nematodes, insects or fungal pathogens are associated with the demise and ultimate death of the target pest. There are numerous fungal, bacterial and nematode antagonists that are known to be pathogenic or parasitic to pest and disease organisms that have been studied extensively for their importance in plant health and for their unique modes-of-action. However, we know little about the complex and intimate interrelationships between the fungi and bacteria in the endorhiza, the nematode pest and the host plant. Past research has shown that endophytic microbial plant interactions have positive, detrimental or neutral impact on plant pests and diseases. In this presentation a short review of the impact of endophytic bacteria and fungi on plant parasitic nematode infection will be given.

The modes of action that are involved are diverse and these unique mechanisms will be presented. These mechanisms include: the production of toxic secondary metabolites, competitive exclusion, competition for nutrients, predation, the production of repellent compounds, changes in root exudates, the induction of systemic resistance or a combination of these elements.

Endophytic bacteria and fungi have been shown to exhibit plant growth and/or health promoting effects when applied to the seed or seedling prior to exposure to plant parasitic nematodes infection. Renewed scientific interest and industrial promotion of seed health incorporation biological control agents is of enormous importance to the science of biological control of plant parasitic nematodes as well as other pests and diseases.

However, the moderate levels of control obtained when seed or seedlings are treated with endophytes verses high levels of control with systemic nematicides is a problem facing industrial sales of biological products ans acceptance by the growers. One IPM approach suggested to alleviate this discrepancy has been the use of cocktails of microorganisms with

different modes of action, designed to improve seed and seedling resiliency to nematode infection.

The question "will multiple inoculates improve biological control efficacy in the endorhiza" has not been clarified for many of the different combinations that could be used.

In this talk I will discuss recent research involving three organisms with different abilities to colonize root tissue: *Rhizobium etli* strain G12 (rhizospheric and endophytic bacteria); *Glomus intraradices* strain 510 (obligate symbiotic arbuscular mycorrhizal fungus) and *Fusarium oxysporum* 162 (mutualistic rhizospheric and endophytic fungus). The objective was to determine if cocktails of endophytic antagonists ith different modes of action can increase the level of biological control of the root-knot nematode on tomato. Similar tests have been conducted with banana an on other crops as seen in the literature cited below. The data to be presented comes from two types of experiments that use altered root systems to detect microbial interaction in the presence of the root-knot nematode and interactions between antagonists in the absence of the nematode.

Influence of single or multiple inoculation of tomato with the mututalistic endophytes *Fusarium oxysporum* strain Fo162, *Glomus intraradices* strain 510 and *Rhizobium etli* strain G12 on *Meloidogyne incognita* infection and development

The objectives were to: 1) Determine the effect of single and dual applications of *F. oxysporum* strain Fo162 with *G. intraradices* strain 510 or with *R. etli* strain G12 on the penetration of *M. incognita*. 2) Evaluate the influence of dual inoculation with *F. oxysporum* strain Fo162 and *R. etli* strain G12, in a spatially-separated plant bioassay on the early root penetration of *M.*

METHODS AND MATERIALS

Experimental design1 A: Single and dual application of Fo162 with AMF or G12 on *M. incognita* penetration

To assess whether a co-application of Fo162 with AMF or G12 lead to additive or synergistic biocontrol activity towards *M. incognita*, two bioassays were setup as follow:

Bioassay 1. Two weeks old tomato plants were inoculated with water, Fo162, AMF or Fo162+AMF.

Bioassay 2. Two weeks old tomato plants were inoculated with water, Fo162, G12 or Fo162+G12.

Fo162 was applied with 5 ml of a spore suspension at a concentration of 1 x 10^6 CFU g^{-1} of substrate dispensed over 3 holes around the selected plant base. AMF contained in expanded clay was incorporated at a rate of 5% of total substrate volume. G12 was applied with 5 ml pot^{-1} of a bacterial suspension (OD_{560} = 2) as a drench around the stem base of the selected plant. Fo162 and G12 inoculations were repeated 2 weeks later. Four weeks after the first

inoculation, all plants were inoculated with a 5 ml tap water suspension containing 1000 J2 plant^{-1}.

Eight plants per treatment were used and the experiment was conducted twice. Two weeks after nematode inoculation, the number of penetrated nematodes was determined by staining the roots with 0.1% acid fuchsin solution, followed by heating to boiling using a microwave for 1.5 to 2 min. The number of penetrated J2 was then counted under a stereomicroscope (100x magnification) and the total number of J2 per root system calculated.

Experimental design B: Spatially-separated dual inoculation of Fo162 and G12 on *M. incognita* penetration and endophyte colonization

The shoots of 6-week-old tomato plants were completely detached from their respective root system and the basal part of the shoot split longitudinally into three sections, over 7 cm in length. Each section was replanted in a separate 11 cm pot filled with 300 g of autoclaved substrate and each pot was separated approximately 1-cm to guarantee complete physical separation (Fig. 1). The lower leaves were pruned to reduce transpiration during the growth of adventitious roots. The triple-split-rooted plants were maintained in a greenhouse for 2 weeks at 27 ± 3°C with 16 h day-1 diurnal light.

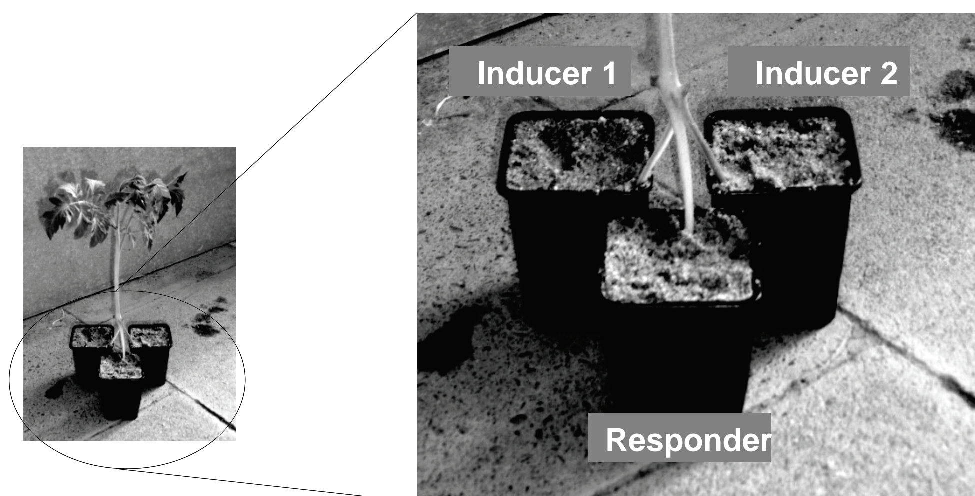

Figure 1. Triple-split-root tomato plant with labels used for inducer and responders.

Two sections of the triple-split-root plants were then labeled ‘inducer’, and the third section was labeled ‘responder’. One inducer root section was inoculated with Fo162, and the other inducer section was inoculated with G12. Three controls were included in the experiment, in which both of the inducer sections were inoculated with Fo162, G12, or water. Fo162 was applied as a spore suspension in 5 ml pot^{-1} at a concentration of 1 x 10^6 CFU g^{-1} of substrate. G12 was applied with 5 ml pot^{-1} of a bacterial suspension (OD_{560} = 2) as a drench around the inducer section of the plant and inoculation repeated 2 weeks later. Four weeks after the first inoculation, the responder section of each plant was inoculated with a 5 ml suspension containing 1000 J2.

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Treatments were replicated six times and the experiment was conducted twice. The plants were arranged in a completely randomized design in the same greenhouse at 27 ± 3°C with 16 h day^{-1} diurnal light. Two weeks after nematode inoculation, inducer roots and responder roots were collected separately. Nematode penetration was then determined by staining the responder roots with 0.1% acid fuchsin solution.

To determine Fo162 colonization in the inducer root sections, the roots were surface sterilized by submersion in a 1.5% NaOCl solution for 3 min, cut into 0.5 cm sections and 18 root segments per treatment were randomly selected and placed on two Petri dishes (150 mm diameter) containing PDA medium. After 4 to 7 days of growth at 25°C in the dark, fungi emerging from each root segment were phenotypically verified as being Fo162. These data were used for calculating the percentage of root colonization per inducer root section. The density of G12 was not determined due to the similarity of the colonies with other root inhabiting microorganisms.

RESULTS AND CONCLUSIONS

The results of these experiments and other studies conducted in the past, demonstrate the complexity of endophyte-plant interactions as they impact nematode infection processes and parasite development. The results which will be given in detail in the talk and are available in published form, also demonstrate the problems that can arise when using antagonists with similar modes of action and/or similar systemic induced resistance signalling processes to improve plant health. In summary:

1. The individual application of F. oxysporum, G. intraradices or R. etli results in a significant reduction in the number of M. incognita that penetrated into tomato roots. Concomitant enhancement with F. oxysporum together with G. intraradices or with R. etli did not lead to significant synergistic interactions.
2. The simultaneous application of F. oxysporum and R. etli tested through a triple split-root experiment showed that the simultaneous but spatially-separated inoculation of both endophytes did not lead to a significant additive effect with respect to reducing M. incognita penetration.
3. Furthermore, this co-inoculation showed a significant reduction in root colonization by F. oxysporum and thereby a of additive or synergism in biocontrol which may be due to systemic forms of incompatibility.
4. It was demonstrated that systemic induced resistance that signals the plant to start the actual mechanisms of action that leads to the biocontrol of nematodes can move acropedal and then basalpedal from the root to the shoot and then back down to the root system of active control mechanisms in the plant.
5. Depending on the timing of application of an endophyte and the type of signals they induce, there can be a reduction in the effectiveness of cocktails of biological control agents due in cross interference thereby limiting their overall influence on the target pest.

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Therefore research is needed into the type of signals induced, the timing of signal induction and the actual mechanisms of action involved and how these actions affect different biological control agents present on the plant at the same time.

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In: Schneider C, Leifert C, Feldmann F (Eds), Endophytes for plant protection: the state of the art. © 2013 Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-11-2.

5-2 Interaction between the endophytic Methylobacterium, host plant and its microbiome with respect to the plant fitness

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ABSTRACT

The structure of the lant endophytic micorbiome, is primarily determined by the host organism: it genotype and physiological state. At the same time it can be shaped by various abiotic and biotic factors, including exogenously applied beneficial bacteria. Nowadays increasing number of evidences confirms that the physiological outcome of environmental factors influencing the plant superoorganism is mediated to the large extend via dynamically reacting microbial communities. Viable but nonculturable forms - the dormant state found for many bacterial endophytes - is the population reserve to overcome the harsh environmental conditions for plant resident microbial communities, and genetic reserve for the plant host to alleviate environmental stress, including the pathogen infection. This concept explains *Methylobacterium* physiology and it priming capacities. Thus *Methylobacterium* sp. IMBG290 was in the dormant state in potato tissues until being activated by the plant treatment with the growth promoting *Pseudomonas* strain. The presence of M. *extorquens* DSM13060 in pine buds was season-dependent. Both endophytes had direct plant growth-promoting capacities *in vitro*, while their biocontrol capacities (primarily characterized for IMBG290 on potato) depended on the plant genotype, pathogen / herbivore type and environmental conditions (*in vitro*, greenhouse or field cultivation and different hydrothermal conditions regarding the last). We have found that the biocontrol capacities of *Methylobacterium* may be associated with the activation (appearance and increase in relative abundance) of potentially protective bacterial and fungal endophytes. Even though *Methylobacterium* strains tested did not produce any known phytohormon-like compounds and did not demonstrate microbial antagonism, they could increase the efficacy of another microbial fertilizers and biocontrol agents possibly due to their capacity for biofilm formation and production of the quorum-sensing signals. The growth-promoting effect of these *Methylobacterium* strains may be primarily attributed to methanol removal from the phyllosphere. Thereon they could be a valuable source for design of the complex biofertilizers, where they could synchronize exogenously applied microbial inoculants with the plant resident microbial communities.

Persistent Identifier: urn:nbn:de:0294-sp-2013-ppphe-2

Romeralo C et al, Fungal endophytes reduce necrosis length produced by *Gremmeniella abietina* in *Pinus halepensis* seedlings. In: Schneider C, Leifert C, Feldmann F (Eds), Endophytes for plant protection: the state of the art, pp. 150-155.
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5-3 Fungal endophytes reduce necrosis length produced by *Gremmeniella abietina* in *Pinus halepensis* seedlings

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ABSTRACT

Gremmeniella abietina (Lagerberg) Morelet is a pathogenic fungus which causes severe damage in coniferous forests, producing the death of the trees, in Central and Northern Europe, North America and Japan. In Spain, where *G. abietina* has only been isolated from symptomatic *Pinus halepensis* trees, the fungal infections have not led to epidemic outbreaks so far. Biological control (*i.e.* the use of biological antagonisms) is being considered an alternative and eco-friendly method to deal with plant diseases. Among such organisms several fungal endophytes have been successfully used to reduce or inhibit the growth of the pathogens. Thus, the aim of this study was to test the capacity of fungal endophytes to reduce the *G. abietina* advance on seedlings. The experiment was carried out on two-year old *P. halepensis* seedlings under greenhouse conditions. Five fungal endophytes obtained from healthy *P. halepensis* trees were used to evaluate their effect on six *G. abietina* isolates. The pathogen and the endophyte were both inoculated in every seedling leaving a space of 2 cm between them. Two months after the inoculation the visual severity was assessed by following a severity scale (from 0 healthy to 5 dead). At the end of the experiment seedlings were cut and brought to the laboratory where the necrosis length was measured. We evaluated the results in terms of visual severity and necrosis length produced by the pathogen. There were no significant differences in visual severity among endophyte-inoculated plants and the control ones. Nevertheless, control plants

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had a lower visual severity than the plants inoculated with pathogens. In addition, the presence of endophytes significantly reduced the necrosis caused by *G. abietina*. These results suggest that fungal endophytes could be an effective way of protection against *G. abietina* infections.

INTRODUCTION

The ascomycete fungus *Gremmeniella abietina* (Lagerberg) Morelet (anamorph *Brunchorstia pinea* (P. Karsten) Höhnel) is a pathogen whose infection produces cankers in stems and trunck, dieback and the death of the trees (Donaubauer 1972). In some of the countries severe damage has been registered in natural forests and plantations, where it has even caused devastation of huge areas (Kaitera et al. 1998; Wulff *et al.* 2006). Trees from genera *Picea* and *Pinus* are their principal host although some damages have been also found in *Abies*, *Larix* and *Pseudotsuga* trees. The fungus has been divided into three races: European, Northamerican and Asian. In Spain, *G. abietina* was detected for first time in 1929 from *Pinus pinaster* (Martinez 1933), and from *Pinus halepensis* in 1999 in forest plantations (Santamaria *et al.* 2003). Although no epidemic outbreaks have been registered yet it causes serious damages in trees. The main symptoms of the infection are crown defoliation, dieback and distorsion of terminal twigs, which occasionally leads to the death of the tree (Santamaria *et al.* 2003).

Biological control is considered an alternative method to control plant diseases. Since the use of chemical products has been reduced due to the harmful effect on the environment, the biological antagonisms are expected to become an important part of the control methods against plant pathogens (Cook 1993). The foremost mechanisms of biological control are based on different types of organisms-antagonist interaction: micoparasitism, antibiosis, metabolites production, nutrients competition and resistance induction (Heydari & Pessarakli, 2010). Fungal endophytes live inside the tissue and maintain a neutral, beneficial or detrimental relationship with the plant (Backman & Sikora, 2008). They have been previously used as biological control agents because they can inhibit or reduce the pathogen growth.

The reduction of the pathogen growth because of the endophyte presence has been previously studied in *in vitro* experiments (Santamaria *et al.* 2007) nevertheless no inoculation tests have been carried out yet. Further investigation about the effects of the endophytes in *Pinus halepensis* seedlings is recommended. Thus, the main objectives of this study were (i) to evaluate the influence of the endophytes on the visual severity produced by *G. abietina* isolates inoculated on *P. halepensis* seedlings, (ii) to observe the effect of the presence of endophytes on the necrosis produced by *G. abietina* on the seedlings.

MATERIALS AND METHODS

The experiment was carried out in the greenhouse located at the University of Valladolid. Two-year old seedlings of *Pinus halepensis*, six Spanish isolates of *G. abietina* and five fungal endophyes were used. Fungal endophytes were isolated from symptomless parts of the trees (Botella & Diez, 2011) and corresponded to *Trichoderma* sp., endophye Fungal sp. BL-11, *Aureobasidium* sp., *Aureobasidium pullulans* (de Bary) G. Arnaud and *Dothideomycete* sp. species. These isolates had previously showed reduction of mycelial growth of *G. abietina* in the in vitro experiments (Santamaria *et al.* 2007). Isolates were subcultivated in culture media some weeks before the experiment started in order to have enough mycelium.

It was a completely ramdom experiment with eight repetitions for treatment. Every treatment consisted of inoculation of every 30 combination of "isolate*pathogen" and an extra control treatment. Firstly the pathogen was inoculated at 10 cm from the top and secondly the endophyte at 8 cm from the top. A wound was made with a scalpel, then a little piece of culture medium with mycelium was placed there and finally the wound was covered with Parafilm®. Control treatments were performed with sterile culture media. Inoculations were made in December in order to mimic the natural behaviour of the fungus.

Two and a half months after inoculations some of the plants started to show symptoms of decline. A visual evaluation was made over 5 weeks to measure the disease severity according to the following scale: 0, symptomless; 1, chlorosis; 2, advanced chlorosis; 3, dieback; 4, necrotic; 5, dead. Afterwards, plants were cut and carried to the laboratory in order to measure the necrosis produced by the pathogens and the plant length because it is known that is a good indicator of disease severity (Virtanen et al. 1997). Relationship among necrosis and total length was defined as relative necrosis length and was used, with the severity index, as response variable in the statistical analyses.

All statistical analyses were done with SAS program. Results obtained from severity were analysed with non-parametric statistic (Kruskal-Wallis test) because data were categorical and they do not adjust to a normal distribution. Besides, the effect of isolates and endophytes on necrosis length was evaluated by a two-way analysis of variance (ANOVA). Normality, lineality and homocedasticity of the residuals were checked by the Kolmogorov-Smirnov test and graphical procedures. A significance of 95% was taken in all of the analyses. A Tukey HSD test was used on means of factors when significant differences were found in the ANOVA model.

RESULTS

The first symptoms of *G. abietina* infection were observed few weeks after the inoculation. Severity was used as response variable and endophyte and isolate as explanatory variables in the Kruskal-Wallis test. The test showed that there were no significant (p value = 0.9504)

differences between the severity in the plants inoculated with endophytes and isolates of *G. abietina* together than the visual severity that presented the plants inoculated only with *G. abietina* (the endophyte-control ones). Nevertheless it was revealed that there were significant differences (*p* value <0.001) among the severity of the plants inoculated with *G. abietina* isolates and the ones without pathogen (the pathogen-control plants). In addition, the effect of the endophytes and the isolates in the relative necrosis length were all significant in the ANOVA model.

Table 1: Two way ANOVA of relative necrosis length at the end of he experiment.

Source	DF	Mean square	F-value	Pr > F
Endophyte	5	0,0677	6,32	<0,0001
Isolate	6	0,4791	44,69	<0,0001
Endophye*Isolate	30	0,0125	1,17	0,2539

The Tukey-Kramer test revealed that seedlings inoculated with endophytes and *G. abietina* isolates together presented lower relative necrosis length (Figure 1) than the endophyte-control plants (inoculated only with *G. abietina*). Furthermore the pathogen-control plants (without pathogen) had lower relative necrosis length than the ones inoculated with most of the *G. abietina* isolates.

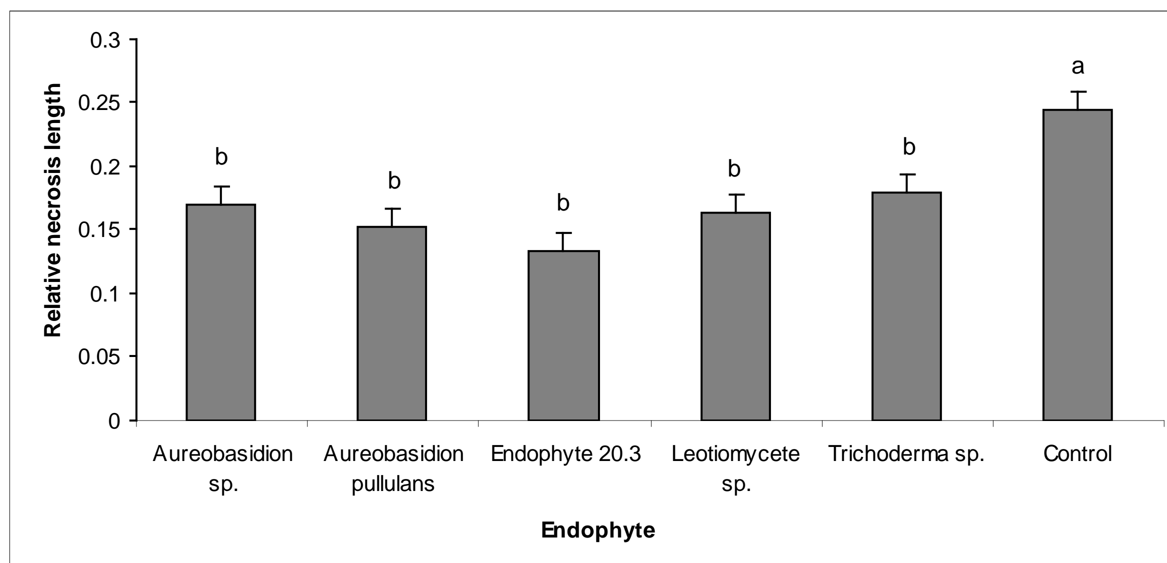

Figure 1: Mean relative necrosis length for every endophyte. Bars show standar error. Different letter means significant diferences with p<0.05 (Tukey HSD test)

DISCUSSION

This study explores the effectiveness of biological control against *G. abietina* in Aleppo pine seedlings. Preliminary results showed that the inoculation of *G. abietina* produced symptoms

Persistent Identifier: urn:nbn:de:0294-sp-2013-ppphe-2

of chlorosis, dieback, necrosis and the death of some plants. The presence of the endophytes did not reduce the severity of the symptoms caused by the fungus in the seedlings. Coversely, it could be due to the fact that the seedlings were placed in an outdoor greenhouse and thus exposed to the environmental conditions. Seedlings' wilt could have been produced by a combination of other factors and not only by *G. abietina*.

Results from our experiment showed that the presence of all the endophytes reduced the necrosis length produced by *G. abietina*. It can be explained by the fact that biological control agents may produce substances that attack directly to the pathogens or that induce their own systemic resistance (Gao *et al.* 2010; Akila *et al.* 2011). In our study isolates of *Aureobasidium* genus produced a reduction in the necrosis length caused by *G. abietina*. Previous studies have also pointed out the antagonistic activity of the species *Aureobasidium pullulans* against *Penicillium expansum* Link (Mounir *et al.* 2007) and *Aspergillus carbonarius* (Bainier) Thom (Dimakopoulou *et al.* 2008). Isolates from genus *Trichoderma* have ben shown before as effective tools against pathogens before. For instance, it has success against *Fusarium circinatum* Nirenberg & O'Donnell (Martínez-Álvarez *et al.* 2012), and *Botrytis cinerea* Pers. in Scots pine seedlings (Capieau *et al.* 2004).

This study provides additional knowledge about the effects of the inoculation of *G. abietina* and the fungal endophytes in Aleppo pine seedlings. Fungal endophytes reduced or inhibited the advance of the pathogen into the plant. Thus they may be used to control the disease. Nevertheless, a further study about these biological antagonisms is recommended, particularly about the mechanisms employed to reduce the disease.

ACKNOWLEDGEMENTS

The study was financed by the project of the Ministry "Biological control of *Gremmeniella abietina* in Spain (AGL2008-03622)". We are indebted to El Serranillo Nursery from the Ministry of Agriculture and Environment and the Central Nursery from the regional government of Castilla y León for their contribution of the Aleppo pine seedlings. We want to deeply thank Alan Benitez for his inestimable help during the inoculation process.

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In: Schneider C, Leifert C, Feldmann F (Eds), Endophytes for plant protection: the state of the art. © 2013 Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-11-2.

5-4 Development of spray formulations for an endophytic *Beauveria bassiana* strain

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ABSTRACT

The entomopathogenic fungus Beauveria bassiana causes diseases on a range of pest insects for example pests of oilseed rape plants. Furthermore, B. bassiana strain ATP 04 is able to colonize oilseed rape plants endophytically and protect them from insect pests by causing mycosis. The aim of our research is to develop novel formulations that deliver the fungus onto and then into the plant. First, three formulation strategies, namely encapsulation, seed treatment and spraying, were evaluated in the project. Best colonization was obtained with a treatment containing 106 submerged spores/ml and adjuvants that was placed on the 7th secondary leaf of plantlets (n=10). After 5 weeks, B. bassiana could be identified by PCR in the 12th secondary leaf of 70 % of the plantlets. Based on the data the systematic construction of aqueous spray formulations seemed most promising. Hence, different wetters, humectants, nutrients, sunscreens and other adjuvants were combined with 106 submerged spores/ml and investigated with regard to surface tension, contact angle, viability, germination, colonization and insect mortality. Wetters mixed into aqueous spore suspensions were sprayed on sunflower wax ("artificial leaf") with a smooth surface and defined surface tension, and on 32 days old 3th secondary leaf of natural oilseed rape plants. It could be shown that wetters based on non-ionic surfactants could decrease the contact angle from 110° to <25° resulting in an increase of the wetted leaf area compared to the control based just on water. However, some wetters decreased viability of spores by >90 %. It is known that B. bassiana submerged spores are sensitive against solar radiation in field. Compared to the water treatment the use of sunscreens such as reflectors and adsorbers could increase the viability of fungal submerged spores after UV radiation (365 nm) for 60 min by 60%. Finally more data on combinations of adjuvants, colonization and insect mortality tests will be presented. As a conclusion, much more systematic research on novel formulation materials, methods and technology in close collaboration with other fields such as entomology, molecular biology and biochemical engineering is needed to realize the biopesticide potential of entomopathogenic endophytic fungi.

Persistent Identifier: urn:nbn:de:0294-sp-2013-ppphe-2

In: Schneider C, Leifert C, Feldmann F (Eds), Endophytes for plant protection: the state of the art. © 2013 Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-11-2.

5-5 Differences in endophytes diversity among grapefruit (*Citrus paradisi*) trees irrigated with fresh water compared to treated sewage water.

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ABSTRACT

Approximately 70% of the water in Israel is used for agriculture. Due to the constant deficiency in fresh water in this arid area, exploitation of treated sewage water for irrigation of orchards and other crops have become one of the main water sources for agricultural. Currently more than 70% of the sewage water, in Israel, are treated and used for agricultural and other purposes. Citrus orchards are irrigated with treated water in many areas of the country. Trees in some of these orchards are going through slow decline and eventually die. One possible explanation is that the composition of the water is causing the soil to become hydrophobic so that although the orchard is watered, the water are running through the upper layer of the ground, away from the trees root system, creating a situation in which the tree cannot exploit the water. Another possibility is that the microbial community on and by the roots changes due to the presence of heavy metals and other contaminants in the water. This change, influences the health of the trees. Many aspects of physical, chemical and biological approaches have and are being studied, concerning the soil and root system. No attention was given to endophytic and saprophytic microorganisms and their involvement in the phenomenon. The decline of the trees, from a phytopathological view, resembles the development of a very week disease. Our study is amid at finding the differences in endophytes diversity, present or absent from trees of the same species, grafted on the same stock species, planted in the same type of soil with the only difference of being irrigated by different sources of water: fresh and treated sewage water. We are studying the culturable and non-culturable communities in the roots and canopy parts of the trees. Different diversity of endophytes in the trees or different ratio of the endophytes identified may be a possible explanation to the trees decline in the treated sewage water irrigated orchards. Absence or reduction in endophytes, in the trees, may cause interference in the tree-endophytes symbiotic relations. One possibility is that the dissolved in the treated water, exterminates the endophytes in the trees. The absence of the endophytes weakens the tree, causing slow decline. Another explanation is that in the absence of the endophytes, or due to the direct influence of the treated water on the tree, weak opportunistic pathogens invade the tree causing disease like decline. Our latest results will be presented.

Persistent Identifier: urn:nbn:de:0294-sp-2013-ppphe-2

In: Schneider C, Leifert C, Feldmann F (Eds), Endophytes for plant protection: the state of the art. © 2013 Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-11-2.

5-6 The entomopathogenic fungus *Beauveria bassiana* as an endophyte in grapevine *Vitis vinifera* (L.) plants

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ABSTRACT

Entomopathogenic fungi are naturally-occurring pathogens of arthropods which have gained recognition as biological control agents for a variety of insect pests. Besides colonizing insects, some entomopathogenic fungi such as *Beauveria bassiana* (Balsamo) Vuillemin may also live endophytically inside plants. Currently, it is unknown whether *B. bassiana* can exist as an endophyte in grapevine plants (*Vitis vinifera*) and still maintains its antagonistic potential against insect pests. In the present study, greenhouse experiments were conducted to optimize endophytic establishment of the entomopathogenic fungus *B. bassiana* in grapevine plants. Furthermore, the antagonistic potential of endophytic *B. bassiana* against pest insects was evaluated. *Beauveria bassiana* was either applied as a conidial suspension of two strains (GHA and ATCC 74040) or as the formulated product Naturalis® (3% and 5%). Different spore concentrations were tested for endophytic establishment of *B. bassiana* in grapevine plants: Conidial suspensions or Naturalis® were applied via handspraying on the upper and lower leaf surfaces of grapevine plants. Water served as a control. A subset of plants was wrapped with plastic bags for the first 24 h after inoculation to increase relative humidity. To determine if endophytic colonization of grapevine leaves by *B. bassiana* was successful, leaf disks of surface sterilized control and inoculated plants were obtained and placed on a selective medium. Any fungal tissue visible after two weeks was analyzed by molecular methods using *B. bassiana* strain specific microsatellite markers. The antagonistic activity of endophytic *B. bassiana* against putative target pest insects like the vine mealybug *Planococcus ficus* was assessed using surface sterilized leaves for a bioassay. In addition, possible effects on the feeding preference of black vine weevil *Otiorhynchus sulcatus* choosing between control and inoculated plants were examined through bioassays. Endophytic presence of *B. bassiana* was confirmed up to 28 days after inoculation, but did not influence the growth of the potted grapevine plants negatively. The different inoculation methods had no significant effect on the endophytic establishment of *B. bassiana* in grapevine plants. A significant effect of endophytic *B. bassiana* on growth but not on mortality of vine mealybugs was evident. Adult *O. sulcatus* chose significantly more often the control plants as a host plant compared to grapevine plants inoculated with *B. bassiana.*

Persistent Identifier: urn:nbn:de:0294-sp-2013-ppphe-2

In: Schneider C, Leifert C, Feldmann F (Eds), Endophytes for plant protection: the state of the art. © 2013 Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-11-2.

5-7 Genomic and molecular background of plant growth promotion in the endophyte *Enterobacter radicincitans* sp. nov. DSM 16656

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ABSTRACT

Endophytic plant growth promoting bacteria (PGPB) may have significant impact on both the plant physiology and the composition of the plant microbiome. Many different bacterial species proved to induce these plant growth-promoting effects on a variety of plant species. However, the regulatory pathways of the beneficial effects are still questionable. We described the strain *Enterobacter radicincitans* sp. nov. (DSM 16656) as a new species of the genus Enterobacter, which was isolated from the phyllosphere of winter wheat under temperate conditions. Growth promotion of root and shoot, along with increased yield, was conferred by inoculation of different crop and model plant species. Using a species specific molecular probe, quantitative real-time PCR and online emission fingerprinting at CLSM after *in situ* hybridization, we demonstrate the plant colonization behaviour and its high competitiveness against the native plant microbiome. Up to 20% of the total bacterial population was earned by the inoculated strain within Brassica oleracea root and shoot tissues. Recently, we have sequenced the genome of *E. radicincitans* to unravel the underlying molecular mechanisms of observed beneficial effects in host plants. Besides the presence of the complete *nif* operon, comprising the *nifUBALMVSNETKDHJ* genes, the genome sequence indicates phytohormone production with genes for indole-3-acetaldehyde synthesis and an auxin efflux carrier. Nitrogen fixation and auxin production of the strain was confirmed in pure culture. Current efforts aim at unravelling expression levels of genes and proteins related to the microbial association with plants.

Persistent Identifier: urn:nbn:de:0294-sp-2013-ppphe-2

In: Schneider C, Leifert C, Feldmann F (Eds), Endophytes for plant protection: the state of the art. © 2013 Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-11-2.

5-8 Endophytes - a source for biological control agents

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ABSTRACT

Plants are strongly associated and influenced by microbes. The composition of bacterial communities from different plant microenvironments (phyllosphere, endosphere, rhizosphere and endorhiza) of field-grown potato was studied using a multiphasic approach including cultivation dependent and independent analysis. *In vitro* dual culture assay was used for determination of the antagonistic potential of potato associated-bacteria against the important soil-borne pathogens *Verticillium dahliae* and *Rhizoctonia solani*. Interestingly, results revealed that composition and diversity of bacterial antagonists were mainly specific for each microenvironment. The main reservoir for antagonistic bacteria was the rhizosphere and the endorhiza. They were characterized by a highly similar composition of bacterial populations. Nearly identical fingerprints for the most prominent species *Pseudomonas putida* isolated from these micro-habitats confirmed the sharing of populations. Endophytes with antagonistic activity were mainly characterized as species belonging to *Pseudomonas, Bacillus*, *Agrobacterium* and *Serratia*. Endophytic bacterial isolates for biocontrol potential were evaluated on target-specific fungal antagonism and physiological characteristics. The endophyte *Serratia plymuthica* 3Re4-18 showed high inhibition effects against various pathogenic *R. solani* isolates. This strain is a competent root colonizer, which was analyzed using *gfp*-labeled mutants by confocal laser scanning microscopy. The high rhizosphere competence as a key factor for a successful disease suppression in the field was independent of the soil type and the crop species. The treatment of plants with the strain 3Re4-18 resulted in significant suppressing of Rhizoctonia diseases on field-grown lettuce, potato and sugar beet. This example showed that endophytes are an interesting group of plant-associated bacteria with high potential for pathogen management.

Persistent Identifier: urn:nbn:de:0294-sp-2013-ppphe-2

In: Schneider C, Leifert C, Feldmann F (Eds), Endophytes for plant protection: the state of the art. © 2013 Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-11-2.

5-9 The two most common endophytic bacteria hampering date palm large scalepropagation: identification and eventual relationship with the Brittle Leaf Disease

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ABSTRACT

Two strains of endophytic bacteria hampering date palm micropropagation were isolated from two cultivars ('Deglet Nour' and 'Barhee'). The isolates, which were orange-yellow and white of colors, were identified by API galleries as *Photobacterium damsela* and *Yersinia ruckeri*, respectively. Significant differences in bacterial population densities were observed in the cultures of the two genotypes and in cultures established from healthy and Brittle Leaf Disease-affected plants. Indeed, the percentage of contaminated tissue in 3-year-old in vitro tissue culture established from 'Deglet Nour' and 'Barhee' were 15 % and 30 %, respectively.

Moreover, we found that percentage of contaminated tissue in 3-year-old in vitro tissue cultures established from healthy and BLD-affected palms *cv.* Deglet Nour were 15% and 2%, respectively. Embryogenic and organogenic cultures were seriously damaged by these fastidious bacteria. They slowly turned yellow and brown and then died within three months. On the other hand, the two bacteria were not toxic for *in vitro* plants as growth rates were similar in *in vitro* plant growing with and without bacteria.

Persistent Identifier: urn:nbn:de:0294-sp-2013-ppphe-2

Bensaci O A, Lombarkia N, Laib D E, Initial evaluation of endophytic fungi, isolated from *Nerium oleander* L. for their biocontrol action against the bruchid *Acanthoscelides obtectus* (Say) (Coleoptera: Bruchidae) in Algeria. In: Schneider C, Leifert C, Feldmann F (Eds), Endophytes for plant protection: the state of the art, pp.162-167. © 2013 Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-11-2.

5-10 Initial evaluation of endophytic fungi, isolated from *Nerium oleander* L. for their biocontrol action against the bruchid *Acanthoscelides obtectus* (Say) (Coleoptera: Bruchidae) in Algeria.

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ABSTRACT

Insecticid activity of two endophytic fungal filtrates, isolated from leaves of oleander *Nerium oleander* (Apocynaceae: Gentianales) was experimentally demonstrated against the bean weevil *Acanthoscelides obtectus*, a serious harmful pest of beans and broad beans in Algeria. The filtrates (stock solutions) from Wickerham medium-cultivated colonies of *Paecilomyces* sp. and *Cladosporium* sp. showed variable insecticidal activities against the target insect, with an average mortality of 68% and 84% obtained with filtrates of *Paecilomyces* sp. and *Cladosporium* sp., respectively. The mortality of insects is proportionally positive with filtrate concentrations for *Cladosporium* sp. but it is not the case for *Paecilomyces* sp. The proteolytic activity is positively related to radial growth for both mycotaxons, however, for the chitinolytic activity, *Cladosporium* sp. exhibits an incompatibility with biomass, which implies high energy expenditure and disqualifies him to metabolize chitinous polymers, unlike *Paecilomyces* sp. proven his chitinolytic ability.These results, and even those relating to the relationship between the proteolytic and chitinolytic activities, show that *Paecilomyces* sp. can be used as contact mycoinsecticide, either in the form of filtrate or propagules, while *Cladosporium* sp. may reflect a different biological activity rather than enzymatic.

Persistent Identifier: urn:nbn:de:0294-sp-2013-ppphe-2

INTRODUCTION

Legume crops are on of the main protein sources in many developing countries. In Algeria, they occupy an important place with cereals in the daily citizen's diet. However, is suffers considerable losses by many pests and pathogenic microorganisms, especially in the post-harvest phase, without noting damages recorded usually in the fields, reducing in consequence land surfaces devoted to their culture. Indeed, many legume grains deteriorate rapidly if stored under adverse conditions. The bean weevil *Acanthoscelides obtectus* is considered in Algeria as a serious pest of beans and faba beans either in the field or in storage. While the chemical solution is widely considered to control this pest, its effectiveness remains insignificant due to the poor knowledge regarding the phytosanitary techniques of treatment by farmers, but also because of the harmful effects on the environment as well as human health, this is why their use is criticized the use of the conventional pesticides in this direction was largely criticized these last years.

In order to develop more reassuring alternatives to chemical methods, fungal endophytes are currently regarded as one most promising organisms in term of biological control and plant health conservation. In this context, the present study is focused in experimental bioprospecting of two endophytic fungi isolated from healthy leaves of *Nerium oleander* for their insecticidal activiy against the bean weevil *A. obtectus*.

MATERIALS AND METHODS

Individuals of *A. obtectus* was reared experimentally using glass jars (15,5 cm x 8 cm) containing for each 500 g of bean seeds without any chemical treatment. Jars are kept in the dark, at 27° C and 75 % RH. Insect individuals rising from a storage legume site in Jijel (Eastern of Algeria). Two endophytic mycotaxa: *Paecilomyces* sp. and *Cladosporium* sp. were selected from a group of pre-isolated mycoflora from healthy leaves of *N. oleander* (sampled from natural vegetation). Isolation procedures are adopted from Fisher & Petrini (1987); Suryanarayanan & Kumaresan (2000) and Huang *et al*. (2008).

An important biomass was obtained from fragments of colonies resulting from PDA (Potato Dextrose Agar) cultures in sterilized Erlenmeyer flasks, each containing 200 ml of Wickerham medium, closed and placed aseptically in the dark (25° C). Daily, the liquid cultures were subjected to intermittent agitations for one hour in order to homogenize the medium and the fungal biomass. Once reaching an important biomass (after 7 to 15 days), the fungal cultures were recovered, treated with ethyl acetate and filtered in series. Culture filtrates were obtained according to Saxena *et al*. (2008) then stored at 4° C.

Treatment was carried out by direct pulverization, applied to the insects put in limp of Petri dish of which each one contains five individuals. The filtrates are applied according to a gradient of concentration (100%, 75%, 50%, and 25%) with 5 repetitions for each concentration, and with a control unit, treated with sterile distilled water. The mortality rate

(%) was determined for each treatment after 2, 20, 24 and 48 hours after spraying. The calculation of mortality is based on the formula of corrected mortality (Abbott 1925).

In a second part, we tried to check the possible relation between proteolytic and chitinolytic enzyme activities and the insecticidal action of culture filtrates used, by the experimental induction of proteases and chitinases synthesis (Lopez-Llorca *et al*. 2002; Tikhonov *et al*. 2002) by using culture media having as specific substrate the gelatin and colloidal chitin, respectively. The proteolytic activity is evaluated by calculating the proteolytic index of Moscoso & Rosato (1987) determined ace following: PI = 1 - colony diameter/halo diameter (Lopez-Llorca *et al*. 2002) While the chitinolytic activity was evaluated by colorimetric method on fungal filtrates (Tikhonov *et al*. 2002). All statistical analysis was carried out using XLSTAT module 2009 of Microsoft Office.

DISCUSSION

Endophytic fungal filtrates showed a variable insecticid activity against *A. obtectus* (figure 1). The maximum of mortality (84%) was recorded from *Cladosporium* sp. for a concentration of 100% after 48h, and 68% for individuals treated by *Paecilomyces* sp. filtrates, for the same period (table 1). For *Cladosporium* sp. mortality rate is positively proportional with filtrate concentration. This is not the case for *Paecilomyces* sp. In addition, it seems that the influence of filtrate concentration on the mortality of the weevils is more outstanding for the treatments by *Cladosporium* sp. filtrates.

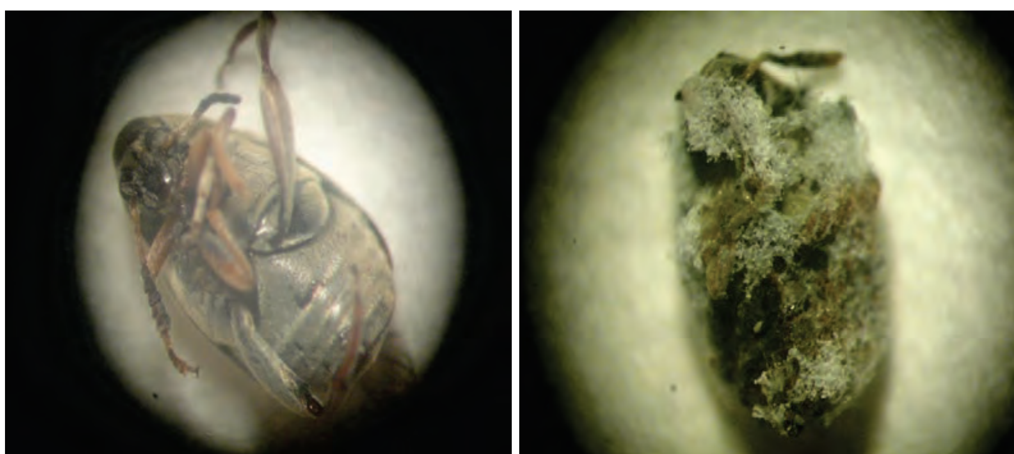

Figure 1 Dead bean weevil insects treated by culture filtrates of *Cladosporium* sp. (left) and *Paecilomyces* sp. (right)

For *Paecilomyces* sp., the proteolytic activity was noted as from the 3rd day (PI = 1,36) and was subject with the rise, to reach a maximum the 7th day (PI = 1,77) to slow down until the 12th day (figure 2). Whereas for *Cladosporium* sp. this activity was announced as from the 4th day (PI = 2,26), to reach a maximum the 12th day (PI = 2,96). Chitinolytic activity marked a clear difference between fungal filtrates, starts for *Paecilomyces* sp. at the 4th day to reach the maximum at the 9th day to decrease and stabilize gradually, whereas for

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Cladosporium sp. starting as of the first day of test but it decreases during the days which follow to be stabilized relatively between the 9th and the 12th day (figure 3).

Table 1. Insecticidal activity of different concentrations of endophytic *Paecilomyces* sp. (P) And *Cladosporium* sp. (C) culture filtrates against *A. obtectus*

Time (h)	Filtrate concentration (%) 25		50		75		100		Con-trol
	P	C	P	C	P	C	P	C	
2	0	0	4 ± 1.00	0	4 ± 1.00	8 ± 0.44	12 ± 1.13	22 ± 0.13	0
20	16 ± 0.89	4 ± 1.00	12 ± 0.56	5 ± 0.84	20 ± 1.03	23 ± 0.25	36 ± 0.98	45 ± 1.62	0
24	16 ± 0.21	22 ± 0.87	32 ± 1.25	26 ± 1.14	28 ± 0.56	45 ± 0.82	60 ± 0,45	62 ± 0.55	0
48	24 ± 0.94	39 ± 0.42	48 ± 1.56	41 ± 1.12	44 ± 0.58	61 ± 0.15	68 ± 0.3	84 ± 1.00	0

Values are represented in means ± standard errors at 0.05%

Proteolytic activity is positively related to the radial growth for both mycotaxons, while with the chitinolytic activity, we showed that *Cladosporium* sp. expose an incompatibility with its biomass, which implies a strong energy expenditure returning to him inapt to metabolize chitinous polymers, in opposite to *Paecilomyces* sp. having proven chitinolytic potentialities since many taxa are entomopathogenic, which suggests a facility of penetration of insect cuticle at early steps of its biological activity.

The proteolytic activity is extremely important for the endophytes, reflected by the production or induction of proteolytic enzymes such as proteases and polypeptidases. Thus, it was shown that the enzymatic activities and the virulence of fungi are two closely linked traits (Monod *et al*. 2002).

The obtained results proved that culture filtrates of endophytic *Paecilomyces* sp. and *Cladosporium* sp. isolated from oleander, present an effective biocontrol potentialities against *A. obtectus*. *N. oleander*, the “source” host-plant of endophytes, is already considered for its toxicity but also for its ethnobotanic and ecological roles among Algerian farmers. Taking in account the data drawn from the study of the enzymatic activities, it is possible to suggest an analysis thorough in order to select the most promising mycotaxon, especially when we make in consideration that each of both tested mycotaxa in this work, dissociates a specific enzymatic property.

In this framework, we propose the development of a screening system relating to mycoendophytes having a biological control potential to fight insect pests of local but strategic crops, by prospecting a so-called “endophyte plants sources” which must be of ecological and especially considerable ethnobotanic importance. In addition, a technical research on bioformulation must be realized for a better biological yield of mycoendophytic taxa for a long term and sustainable biological control program.

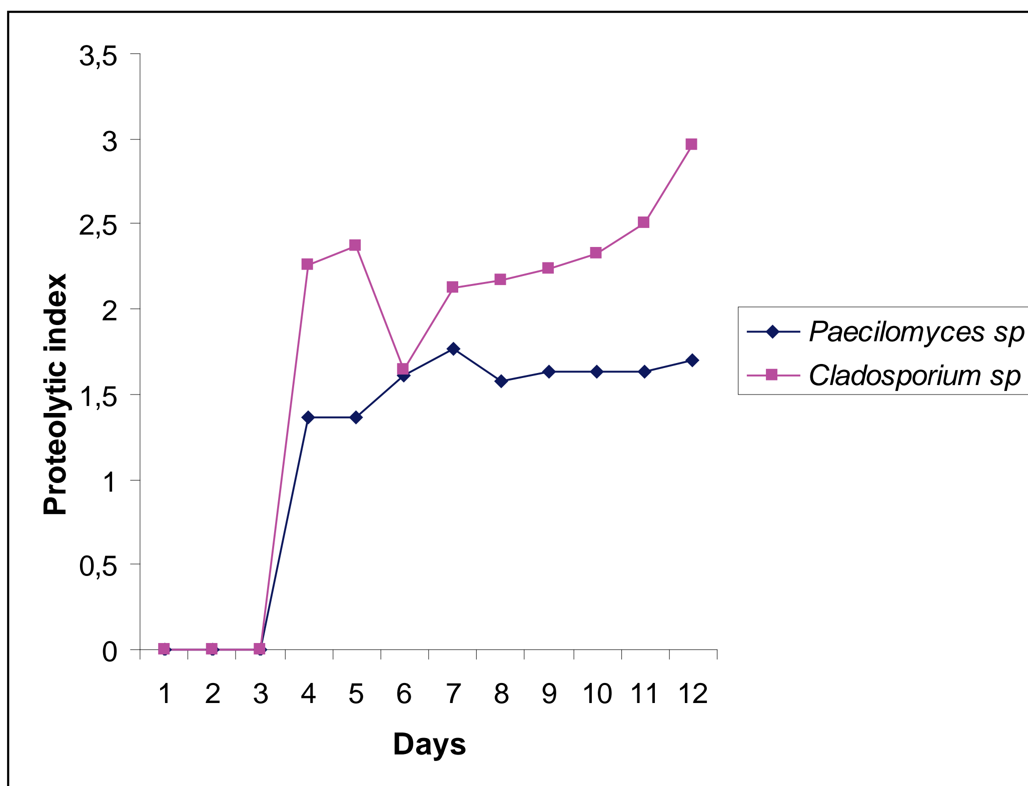

Figure 2 Proteolytic activity; expressed by the proteolytic index of studied fungal endophytes

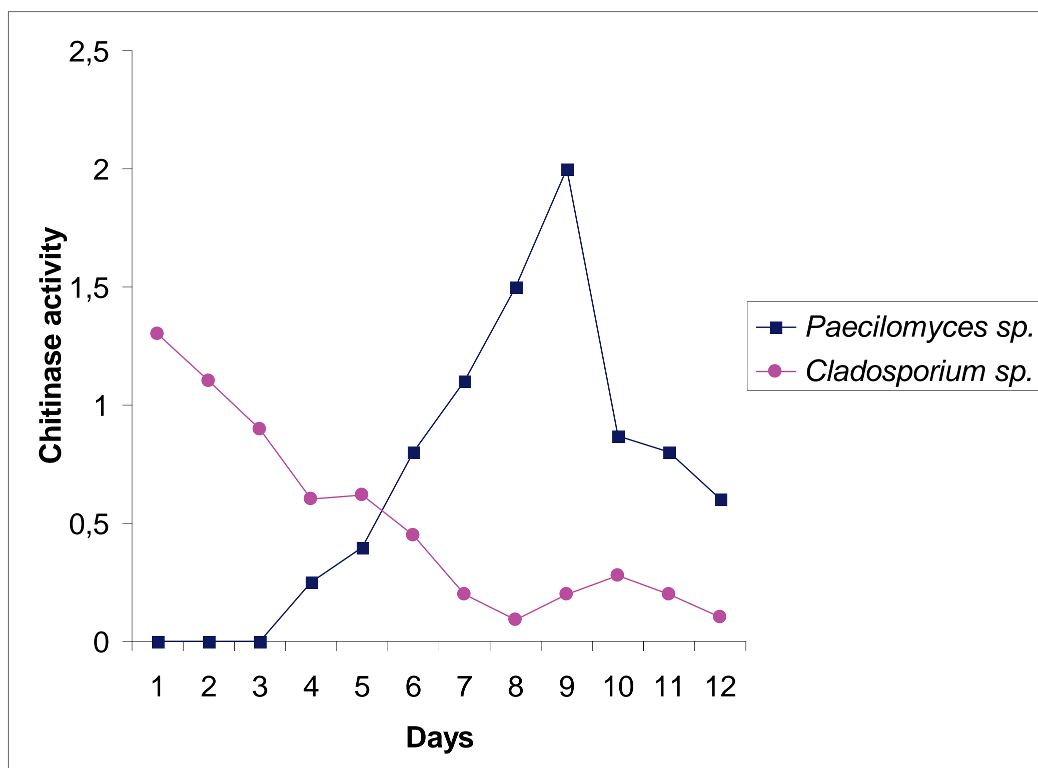

Figure 3 Chitinase activity of studied fungal endophytes (μmol N-acetylglucosamine per minute. ml^{-1} of extract)

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In: Schneider C, Leifert C, Feldmann F (Eds), Endophytes for plant protection: the state of the art. © 2013 Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-11-2.

5-11 Fungal endophytes of bean roots and their interactions with *Fusarium oxysporum* vascular wilts.

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ABSTRACT

Vascular wilt caused by *Fusarium oxysporum* f. sp. *phaeoli* is the single most important and persistent disease of bean crops in El Barco de Ávila, an important center of bean production in Spain. The purpose of our work is to find out if there are root endophytes capable of protecting bean plants against this disease. A survey of root endophytes from asymptomatic plants sampled at four locations was made in the summer of 2012. Fungal taxa belonging to approximately 20 genera have been identified. The most abundant are several *Penicillium* and *Fusarium* species, followed by *Corynespora casiicola*. Different ecological functions, like plant and animal pathogens, or producers of antimicrobial compounds, have been assigned to the set of endophytes identified. *In vitro* and *in planta* assays to detect antagonism between root endophytes and *F. oxysporum* f.sp. *phaseoli* will be performed with this set of fungi.

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5-12 Can endophytic *Beauveria bassiana* protect grapevine against *Plasmopara viticola*?

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ABSTRACT

Evidence has accumulated that the fungal entomopathogen *Beauveria bassiana* (Balsamo) Vuillemin (Ascomycota: Hypocreales) can endophytically colonize a wide array of plant species and offer protection against several insect pests. *B. bassiana* has also been recently reported to engage in plant-fungus interactions and effectively suppress plant diseases. Thus, the current study was carried out to investigate the endophytic establishment of *B. bassiana* in grapevine and its interaction with the grapevine downy mildew pathogen *Plasmopara viticola*. Three strains of *B. bassiana* were able to colonize grapevine plants following the artificial inoculation of leaves via foliar spray. However, percent colonization was significantly higher when the plants were inoculated with strain ATCC74040-based bioinsecticide Naturalis® (68%) and strain ATP01 (58%), as compared to strain EABb04/01-Tip (46%). Similarly, even though all three *B. bassiana* strains were able to reduced disease symptoms on inoculated plants, percent infection was significantly lower only when plants were inoculated with strain ATCC74040-based bioinsecticide Naturalis® (56%) and strain ATP01 (62%), in contrast to strain EABb04/01-Tip (75%) and the control (90%). On the other hand, none of the *B. bassiana* inoculated strains were as effective as the cupper treatment in reducing the disease severity. This study reports for the first time the endophytic establishment of *B. bassiana* in grapevine and its interaction with one of the most important grapevine pathogens, *P. viticola*. It is also one of a very few studies exploring the potential of endophytic *B. bassiana* to offer protection against plant diseases, a field that merits further investigation.

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Nguyen T T N et al, The rhizobacterium *Pseudomonas aeruginosa* $23_{1\text{-}1}$ protects watermelon against gummy stem blight caused by *Didymella bryoniae*. In: Schneider C, Leifert C, Feldmann F (Eds), Endophytes for plant protection: the state of the art, pp. 170-171. © 2013 Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-11-2.

5-13 The rhizobacterium *Pseudomonas aeruginosa* $23_{1\text{-}1}$ protects watermelon against gummy stem blight caused by *Didymella bryoniae*

Nguyen T T N[1,2]; Nguyen T G[2]; Nguyen T L[2]; Lübeck M[1,3]; Shetty N P[1,4]; de Neergaard E[1,5]; Tran T T T[2]; Pham V K[2]; Jørgensen H J L[1]

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ABSTRACT

Rhizobacterial strains were isolated from the roots of watermelon plants grown in the Mekong Delta of Vietnam in order to identify strains which could protect watermelon plants from infection by *Didymella bryoniae*, the cause of gummy stem blight.

A total of 190 strains were isolated and 68 were able to inhibit growth of *D. bryoniae* by production of antibiotics when tested *in vitro*. Four strains were able to reduce foliar infection of *D. bryoniae* by up to 70% when applied to watermelon seeds before sowing. Strain *Ps. aeruginosa* 231-1 was chosen as a model for studies of the ability to control disease under field conditions and for investigations of the mechanisms involved in protection. Field experiments showed that when the bacterium was applied as combinations of seed soaking, soil drenching and foliar spraying, disease levels were significantly reduced in two consecutive seasons and yield increased by up to 250%. Furthermore, it was found that the bacterium colonised watermelon plants endophytically, with higher numbers in plants infected by *D. bryoniae* than in non-inoculated plants. To elucidate the mechanisms involved in protection, the infection biology of the pathogen was compared between control

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plants and plants treated with bacteria. It was demonstrated that treatment with *Ps. aeruginosa* 231-1 inhibited pathogen penetration and this was associated with accumulation of H2O2, increased peroxidase activity and occurrence of new peroxidase isoforms, thus indicating that resistance was induced.

Due to the potential risk that *Ps. aeruginosa* 231-1 may cause disease in humans, its pathogenicity must be elucidated before practical use. However, the current study clearly demonstrates that endophytic, bacteria isolated from plant roots can control important plant diseases under greenhouse and field conditions as well as increase yield in the field. Furthermore, the mechanisms of control of *D. bryoniae* in watermelon involve antibiosis and induced resistance.

Persistent Identifier: urn:nbn:de:0294-sp-2013-ppphe-2

Kidd P et al., Bacterial endophyte isolated from corn kernels inhibits the growth of a fumonisin producing *Fusarium verticillioides*. In: Schneider C, Leifert C, Feldmann F (Eds), Endophytes for plant protection: the state of the art, pp. 172-177. © 2013 Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-11-2.

5-14 Bacterial endophyte isolated from corn kernels inhibits the growth of a fumonisin producing *Fusarium verticillioides*

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INTRODUCTION

Strains of *Fusarium verticillioides* produce mycotoxins such as fumonisins, which are common contaminants of maize and maize-based products worldwide and considered to be hazardous for human and animal health, due to their nephrotoxic, hepatotoxic, immunosuppressive and carcinogenic activity (references cited in Butron *et al.* 2006, Dalie *et al.* 2012).

Biological control of *Fusarium* infection is therefore of great interest for food safety and could contribute towards reducing the use of toxic chemicals with fungicide activity. Many plant-associated bacteria, both epiphytes and endophytes, are widely recognized as agents of biological control against plant diseases and infections (Compant et al. 2010). Numerous phylogenetically diverse bacterial strains showing inhibitory activity against different fungi have been isolated (Bacon & Hinton 2002, Compant *et al.* 2005, Dalie *et al.* 2010, McSpadden Gardener 2007, Yoshida *et al.* 2012).

MATERIAL AND METHODS

Corn kernels from lines which are resistant (A509) and sensitive (EP42) to infection by fumonisin- producing *Fusarium* spp. (Butron *et al*. 2006) were soaked in sterile water for 48 hours. After the soaking period, kernels were air-dried in a laminar flow bench and replicates of about 3 g were surface-sterilized for 3 minutes with NaClO solution supplemented with one droplet of Tween 40 per 100 ml solution. Two concentrations of NaClO (1% and 3%) were tested. The sterilized kernels were subsequently rinsed three times with sterile deionised water and macerated with 15 ml of 10 mM $MgSO_4$ using a Polytron PT1200E homogeniser (Kinematica AG) at medium speed for 2 minutes. Ten fold serial dilutions of

the macerated kernels were plated in EF869D media (Table 1) supplemented with cycloheximide (200 mg L^{-1}) to prevent fungal growth. The plates were incubated for 7 days at 28 ºC and 1-10 representatives of the different colony morphologies were purified by subculturing several times. The purified isolates were genotyped by BOX-PCR as described by Becerra-Castro *et al*. (2011) and one representative of each BOX-PCR profile was selected.

Table 1: Culture media used in this study

EF869D		SNA		284	
	g L^{-1}		g L^{-1}		g L^{-1}
Potato peptone	0.5	KH_2 PO_4	1	Tris HCl	6.06
Soy peptone	0.5	KNO_3	1	NaCl	4.68
Yeast extract	0.25	$MgSO_4 \cdot 7H_2O$	0.5	KCl	1.49
NaCl	0.5	KCl	0.5	NH4Cl	1.07
Glucose	0.1	Glucose	0.2	Na2SO4	0.43
$CaCl_2 \cdot 2H_2O$	0.034	Sacarose	0.2	$MgCl_2$ $6H_2O$	0.20
pH	6.7-7.0	pH	4.5-4.6	$CaCl_2$ $2H_2O$	0.03
				Na2HPO4	0,04
				$Fe^{III}NH_4$citrate, 17% Fe	0,005
				TE solution	1 ml
				C sources	See below
				pH	6.5-6.8

C sources (per liter): Sodium salts of lactate 0.35 g; gluconate, 0.62 g; succinate 0.42 g; glucose, 0.52 g; fructose, 0.54 g,. TE solution contained per liter: 37% HCl, 5.2 ml, $ZnSO_4$, 0.08 g, $MnCl_2 \cdot 4H_2O$, 0.1 g, H_3BO_3, 0.3 g; $CoCl_2 \cdot 6H_2O$, 0.19 g; $CuSO_4$, 0.017 g; $NiCl_2 \cdot 6H_2O$, 0.024 g; $Na_2MoO_4 \cdot 2H_2O$,0.036 g; $FeSO_4 \cdot 7H_2O$, 1.5 g.

Media 284AV consisted of media 284 supplemented with 10 ml per L of BME aminoacids solution 50X (Sigma-Aldrich) and 5 ml per L of BME vitamins solution 100X (Sigma-Aldrich). Solid media contained 1.8% agar (2.3% agar in the case of media supplemented with cycloheximide)

The ability of each representative bacterial strain to inhibit the growth of a fumonisin-producing *Fusarium verticillioides* was analysed. Bacterial strains were streaked in EF869D plates and incubated for 24h at 28ºC, thereafter, *Fusarium* was point inoculated at 1 cm from bacterial colonies and the plates were incubated at 28ºC. The inhibition of fungal growth, in comparison with control plates (not inoculated with bacteria), was checked every two days until the mycelium growth covered the plate surface.

The inhibition of fungal growth by 4 isolates of one selected strain (P3R) was further analysed using four media (EF869D, SNA, 284 and 284AV, Table 1). *Fusarium* was point inoculated in the center of the plates and allowed to grow at 28ºC for 48h. Afterwards, P3R isolates were point inoculated at four points, 1.5 cm distance around the border of the fungal

colony. The inhibition of fungal growth in comparison with control plates (not inoculated with bacteria) was periodically checked at 24, 48 and 72h of incubation. In a second experiment, the inhibition of the fungal growth was analysed by inoculating P3R isolates in the same way but at 0.5 and 1 cm distance from the border of the fungal colony. The inhibiton of fungal growth was checked at 3, 6, and 18 h of incubation at 28ºC. Finally a third experiment was carried out by streaking the bacterial isolate along the diameter of the plate and incubating the plates for 48 h at 28ºC. Thereafter, *Fusarium* was point inoculated at 0.5, 1 and 2 cm from the line formed by grown bacterial cells and the fungal growth was analysed at 2 and 4 days of incubation.

The influence of potential volatile compounds produced by isolate P3R on the growth of *Fusarium* was checked by incubating EF869D plates streaked with P3R isolates and plates point inoculated with *Fusarium* in a sealed container. The growth of the fungal colony was compared with that on plates incubated in similar containers in the absence of PR3 as well as with that on plates incubated in the presence of other bacterial strains of the colection (which in previous experiments did not show any effect on *Fusarium* growth)

The P3R isolates were also cultivated in liquid EF869D, SNA, 284 and 284AV until stationary phase (60 h). The incubation was carried out in a rotary shaker at 150 rpm and 28ºC. In addition the isolates were co-inoculated with fumonisin-producing *Fusarium* in these liquid media and incubated in the same way. The cell-free supernatants of these bacterial and bacterial+fungal cultures (centrifugation at 6000 g) were filtered through 0,2 µm sterile nitrocellulose filters. The inhibition of *Fusarium* growth by these supernatants was checked by adding up to 300 µl of supernatant at four points at 0,5 cm distance around the border of *Fusarium* colonies (previously grown for 48h).

The strain P3R was identified by PCR amplification and sequencing of the gene coding 16S rRNA (16S rDNA) as described by Becerra-Castro *et al.* (2011).

RESULTS AND DISCUSSION

The treatment of corn kernels with either 1% or 3 % NaClO for 3 minutes successfully eliminated cultivable bacteria from the seeds surface. Nevertheless, in corn kernels treated with 1% NaClO some fungi survived the sterilisation process and grew in EF869D plates (even in the presence of cycloheximide)

The density of bacterial endophytes in soaked corn kernels of the *Fusarium* resistant line A509 was $3{,}5 \times 10^{6} \pm 5{,}0 \times 10^{5}$ colony forming units per g (cfu/g) and that of the sensitive line EP42 $1{,}2 \times 10^{7} \pm 1{,}3 \times 10^{6}$ cfu/g. The 32 isolates obtained from line A502 were grouped in 7 BOX-PCR profiles and the 16 isolates from line EP42 showed 6 different BOX-PCR profiles. Most of the isolates, both from line A509 and from line EP42 presented the same BOX-PCR profile. In line 509 other profiles were also represented by various isolates, while in line EP42 most of the other profiles had only one representative in the collection.

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Among the representatives of all the BOX-PCR profiles only one isolate showed the ability to inhibit the growth of a fumonisin-producing *Fusarium*. This isolate was obtained from the *Fusarium* resistant line A509. The partial 16S rDNA sequence (about 800 bp) was aligned using the tool provided by Ribosomal Database Project (RDP) and compared with aligned sequences of type strains in the RDP database (Cole *et al*. 2009). The closest sequences to that of P3R (99.6 % similarity) were members of the genus *Pseudomonas* such as *P. tolaasii* (AF255336), *P. palleroniana* (AY091527), *P. moraviensis* (AY970952) and *P. lutea* (AY364537) *P. extremorientalis* (AF405328). Interestingly one of the closest relatives found (*P. tolaasii*) was isolated from mushrooms affected by brown blotch disease Yoshida *et al*. (2012) isolated *Pseudomonas* strains from *Fusarium* head blight-infected wheat spiklets, which were able to inhibit the growth of the fungal pathogen. Suppressive effects of several fluorescent *Pseudomonas* against soil-borne diseases have also been previously reported (McSpadden Gardener 2007).

The isolates P3R induced a strong inhibition of the growth of fumonisin-producing *Fusarium* when inoculated simustaneously in the same plate (Figs. 1 and 2). After two days of incubation, the size of fungal colonies where up to 35±3% smaller (in EF869D medium) in the presence of P3R than in those plates where the bacterial isolate was absent. One day later, the % of inhibition in the presence of P3R was between 45±4% (in 284 medium) and 55±2% (in EF869D medium).

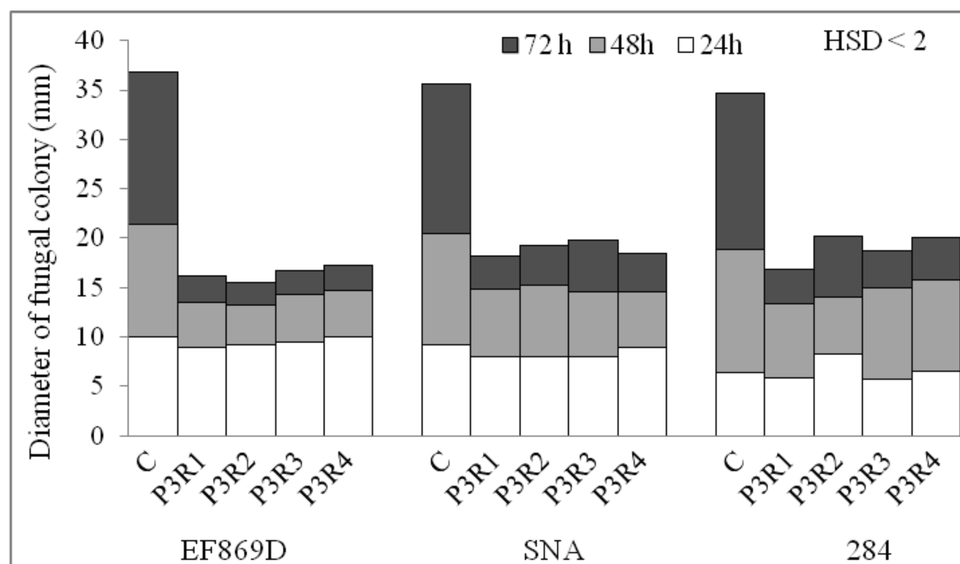

Figure 1: Fungal growth in the absence (C) and presence of 4 diferent isolates of the strain P3R (P3R1, P3R2, P3R3 and P3R4). HSD: Honestly significant difference

The negative effect of P3R on *Fusarium* growth was evident 24 h after bacterial inoculation at 0,5 cm from the border of a previously grown fungal colony, in all 4 culture media used (reduction of fungal growth was between 20% and 24%). Nevertheless, in the 1.8% agar plates used and with bacteria inoculated at 1 cm from the fungal colony, the inhibition was detectable only when the fungal colony grew close to the bacterial inocula (distance < 0.5

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cm). After sixty hours of incubation, the reduction of *Fusarium* growth inoculated at 0,5 cm from previously grown bacterial colonies was up to 80% in EF869D and 284AV media, while in SNA and 284 media, the reduction was up to 70% and 55%, respectively. An inhibition of fungal development when bacteria were inoculated at larger distances from *Fusarium* was also detectable after 60 h of incubation, although in this case it was less intense. In plates where *Fusarium* was inoculated at 2 cm from the bacteria the inhibition reached up to 40% in EF869D and 284AV plates, and up to 20% in SNA plates.

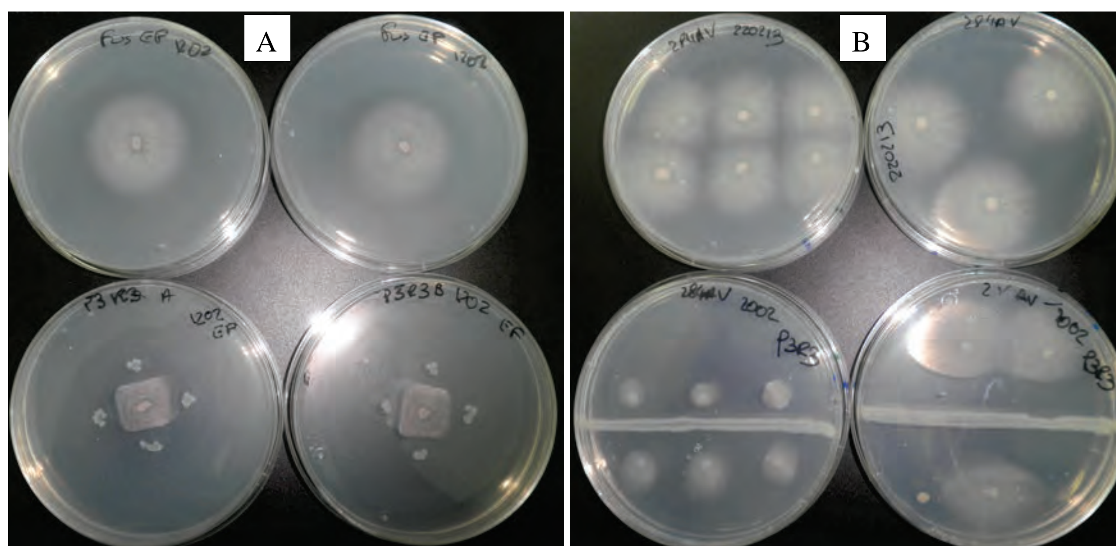

Figure 2 Inhibition of *Fusarium* growth by isolated strain P3R when fungus and bacteria were inoculated simustaneously in EF869D plates and incubated for 72 h (A) and when *Fusarium* was inoculated at 0,5, 1 and 2 cm from bacteria previously grown in 284AV plates and incubated for 60 hours (B). The plates in the first row show the growth of *Fusarium* in the absence of bacteria.

In the experiments carried out, we did not detect any inhibition of *Fusarium* growth in the presence of volatile compounds produced by P3R. Moreover, the fungal growth was not inhibited by metabolites present in non-concentrated cell-free supernatant of liquid bacterial cultures or liquid bacterial+fungal cultures.

Strong reduction of fungal growth by *Pseudomonas* strains (percentages of growth inhibition 50-75%) was also observed by Yoshida *et al*. (2012). These authors tested the fungal growth using a method by which inhibition of fungal mycelia could occur through bacterial attachment to fungal hyphae. In addition, in other experiments they observed smashed necrosis of fungal mycelia by attachment of bacterial isolates. In our study, the *Pseudomonas*-induced inhibition of fungal growth seems to be due to metabolites secreted by the bacteria that difuse into the surrounding agar. The colonization and attachment by bacterial cells to mycelia does not seem necessary to inhibit fungal growth. Nevertheless, the negative results obtained with cell-free supernantants of liquid bacterial and bacterial+fungal cultures indicates that further experiments are needed to confirm this hypothesis and to identify potential toxic compounds excreted by bacteria. Other studies have identified

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bacteria able to produce antibiotics and other allelochemicals which are active against plant pathogens (Compant *et al*, 2005).

The endophyte strain isolated from corn kernels of a *Fusarium* resistant line may be useful as biocontrol agent against plant diseases and grain contamination caused by this fungus, although additional and extensive research is need to confirm its potential application.

ACKNOWLEDGEMENTS

This research was funded by the Spanish Ministerio de Ciencia e Innovación (CTM2009-14576-CO2-01) and Ministerio de Economía y Competividad (CTM2012-39904-C02-01)

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Saar K et al., Characterisation of *Acremonium*-like fungi from wheat and tall fescue. In: Schneider C, Leifert C, Feldmann F (Eds), Endophytes for plant protection: the state of the art, pp. 178-180. © 2013 Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-11-2.

5-15 Characterisation of *Acremonium*-like fungi from wheat and tall fescue

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INTRODUCTION

Species of *Acremonium, Verticillium* and other *Acremonium*-like fungi are ubiquitous soil inhabitants (Domsch et al. 1980) and have also been reported as endophytes (Wicklow et al. 2005; You et al. 2009). We here report results obtained with *Acremonium*-like fungi isolated from monocots. The aim of the study was to characterize supposed *Acremonium*-like fungal isolates by morphology and molecular taxonomy and to evaluate their antagonistic capabilities that may potentially play a role in the interaction with other fungi.

MATERIALS AND METHODS

The fungi studied were isolated from surface sterilised wheat (*Triticum aestivum;* seeds, nodes, ears) and tall fescue (*Festuca arundinacea;* leaves) and identified based on morphological characters. Three isolates each identified as *Simplicillium lamellicola* and *Sarocladium bactrocephalum,* respectively, were selected for further studies. The complete rRNA operon internal transcribed spacer (ITS) region and a partial sequence of the large subunit (LSU) rRNA gene were amplified by PCR, sequenced, and the phylogenies of the respective sub-groups of *Acremonium*-like fungi were reconstructed.

In order to study the effect of metabolites on mycelial growth, the isolates were cultured on cellophane sheets placed over PDA. After five days of pre-culture the cellophane sheets with the adhering mycelia were removed, and the media were inoculated with the different isolates. In a second set of experiments the isolates were grown in shake culture in potato dextrose broth, and the culture filtrates were incorporated into potato dextrose agar plates (final concentration 30%). The plates were then inoculated with different phytopathogenic fungi. In a third experiment, suspensions in culture filtrate containing mycelia and conidia of

F. graminearum were used to inoculate wheat leaf segments placed on filter paper in Petri dishes.

RESULTS AND DISCUSSION

Based on morphological criteria, the isolates were tentatively identified as Simplicillium lamellicola (syn. Verticillium lamellicola), Sarocladium bactrocephalum (syn. Acremonium bactrocephalum), Sarocladium strictum (A. strictum) and A. curvulum. In the molecular studies, the six selected isolates clustered in two groups according to their respective morphological identification as S. lamellicola (Group I) or Sarocladium bactrocephalum (Group II). However, whereas molecular taxonomy confirmed the tentative assignment for the S. lamellicola strains to the genus Simplicillium, the supposed Sarocladium bactrocephalum strains were identified as members of the species Acremonium sclerotigenum by LSU gene based phylogenetic reconstruction.

On PDA pre-cultured with the Group II isolates, mycelial growth of the isolates belonging to Group I was reduced by at least 50% compared to growth on non pre-cultured plates (controls), whereas the isolates of Group II where not or only slightly affected. On PDA pre-cultured with the isolates belonging to Group I, mycelial growth of the isolates from both groups was only reduced by about 20%. In the experiment with PDA amended with culture filtrate, *Phoma lingam* and *Fusarium graminearum* were generally inhibited most. Inhibition of *Pythium ultimum* and *Botrytis cinerea* was only temporary or nil, respectively. The filtrates from the isolates of Group II tended to promote mycelial growth of *R. solani* and *B. cinerea* whereas those of Group I inhibited the mycelial growth. Following transfer of the inhibited mycelia to non-amended PDA, all isolates resumed growth, indicating that the inhibition was fungistatic rather than fungicidal. Inoculation of wheat leaf segments placed on filter paper with *F. graminearum* suspended in water resulted in 100% disease incidence. Application in 30% culture filtrate reduced the disease incidence by about 65% and 35% in the case of the filtrates of Group I and Group II, respectively. Culture filtrate alone (100%) did not cause any effect.

It can be concluded that the two groups of isolates that are clearly assigned to two different taxa by both morphological and molecular taxonomic characterization differ in various aspects. Firstly, the metabolites from Group II affected growth of the Group I isolates more than the Group II isolates were affected by the metabolites from Group I. Moreover, the culture filtrates from the Group I isolates were in most cases more inhibitory to *in vitro* growth of the tested phytopathogenic fungi than the filtrates from the Group II isolates. This was confirmed in a test on leaf segments where the disease incidence was lower when the *Fusarium* inoculum was applied suspended in culture filtrates of Group I than in filtrates of Group II isolates. Further experiments are needed to evaluate if the inhibitions observed *in vitro* also play a role in interactions between *Acremonium*-like fungi and phytopathogens within the plant.

Persistent Identifier: urn:nbn:de:0294-sp-2013-ppphe-2

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Döring M, Overview about the research of endophytes as biocontrol agents against phytopathogens. In: Schneider C, Leifert C, Feldmann F (Eds), Endophytes for plant protection: the state of the art, pp.181-182. © 2013 Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-11-2.

5-16 Overview about the research of endophytes as biocontrol agents against phytopathogens

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ABSTRACT

Biocontrol of plant diseases can be defined as the reduction of inoculum or disease-causing activity of a plant pathogens through the use of organisms and typically involves an active human role (Cook & Baker 1983). Reduction of chemical inputs in agriculture and forestry is the major impetus for developing biological controls for the suppression of phytopathogens. Another task is that biocontrol agents can be an alternative to pesticides which may have a negative impact on human health and environment.

The following steps are important for the development of biocontrol agents with endophytic microorganisms, endophytes can have biocontrol effects on the 10 most important bacterial and fungal phytopathogens in molecular plant pathology (Dean *et al.* 2012; Mansfield *et al.* 2012):

Isolation of putative competent endophytes:

There is an isolation strategy where competent endophytes can be isolated from crop plants which look healthy in comparison to plants of the same species which show disease symptoms. Biodiversity can be also a resource for selection of microbes for biocontrol aspects.

Screening test on different strategies of endophytes to control phytopathogens:

In vitro tests via dual culture plate assays, plate diffusion tests and inoculation tests on pot plants are commonly used. Experiments on plants in the field are rare. Here is a need for more effective and standardized bioassays for antibiosis, antagonism, competition, induced systemic resistance and colonization competence of endophytes.

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Mass propagation, formulation and application of endophytes to plants:

Different abiotic (type of medium, temperature, soil condition) and biotic (autochthonous endophytic microbes and epiphytes of inoculated plants, population dynamics of plant microbiome) factors can change the growth characteristics and biocontrol effects of *in vitro* produced endophytes. Those factors have to taken into account for successful product development.

Commercialization of products:

Biocontrol products (including *Trichoderma*, *Pseudomonas*, *Bacillus*, *Streptomyces*, *Actinomyces,* grass endophytes) already found a niche in biocontrol market, others will follow in the near future.

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5-17 The influence of bacteria from an insect vector on plants infected with phytoplasma

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ABSTRACT

The control of phloem restricted pathogens needs new strategies given that conventional application of chemical spray is inefficient. The role of endophytes in the induction of plant resistance against such pathogens including phytoplasma has been suggested. However, in order to proceed towards practical application, such a candidate should be cultivable and able to penetrate and survive within the plant for a reasonable time. We hypothesized that since phytoplasma is transferred by phloem feeding insects, the vectors, as well as the host plants, may serve as reservoirs of potential micro-organisms. Such a potential and beneficial bacterium was isolated from an insect vector (*Hyalesthes obsoletus* (Hemiptera: Cixiidae)), and grown on artificial medium. The isolate, belonging the bacterial family Xanthomonadaceae, was introduced to healthy and phytoplasma-infected plants (periwinkle and grapevine) and its presence in plant tissues was confirmed by PCR analysis three weeks post inoculation. A change in the plant morphology was observed four and eight weeks post inoculation. The presence of the bacteria seems to affect the morphology of the infected plants but not the healthy ones. In some parameters, the effect of phytoplasma on plant morphology was markedly reduced. Further study is needed to examine the potential use of this isolate as a bio control agent against phytoplasma.

Persistent Identifier: urn:nbn:de:0294-sp-2013-ppphe-2

In: Schneider C, Leifert C, Feldmann F (Eds), Endophytes for plant protection: the state of the art. © 2013 Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-11-2.

5-18 Impact of *Piriformospora indica* on the growth of different tropical vegetables and on *Fusarium oxysporum* f. sp. *niveum*

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ABSTRACT

Piriformospora indica is a root endophytic fungus, which was originally isolated from the spore of an arbuscular mycorrhizal fungus (Fakhro *et al*. 2010). It has been demonstrated that this isolate has plant-promoting properties in numerous plants species and induces resistance against root and shoot pathogens in barley, wheat, Arabidopsis and other crops. In order to test its application potential to tropical vegetables, the impact of *P. inidca* on the growth of five different vegetable crops (sweet melon, water melon, luffa, bitter gourd, and cowpea) and on interaction with *Fusarium oxysporum* f. sp. *niveum* (Fon), an important soil-borne pathogen in cucurbits. The results by microscopic observation showed that *P. indica* did not colonised on the roots of all the cucurbit crops tested, but well in the roots of cowpea in 14 days post inoculation (dpi). Chlamidospores were clearly observed inside the epidemic cells of cowpea roots. The growth of roots and stems between inoculated and un-inoculated plants in all tested the vegetable crops had no significant differences in 14 dpi. One reason may be that the time between inoculation and investigation was too short. Therefore, a further test of *P. indica* on cowpea plants has been performing. The test results on PDA medium showed that *P. indica* had no any direct inhibition to Fon. No clear effect was observed, when the watermelon plants were inoculated with Fon after 14 dpi with *P. indica*. Further tests about the effect on Fon are needed in green house and in field conditions.

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5-19 Effect of selected fungal endophytes and pathogens on the growth of *Solanum tuberosum* L. infected with Potato virus Y (PVY)

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ABSTRACT

Potato virus Y (PVY) is one from the most important plant viruses, resulting in reduced yields of *Solanum tuberosum* L. up to 80%. Main goal of our study was to investigate the effect of fungal inoculation with selected strains of plant endophytes (*Glomus intraradices, Trichoderma viride, Trichoderma harzianum*) and pathogens (*Colletotrichum sp*.) on the growth and development of infected with PVY and control plants of *Solanum tuberosum* L. We have analysed the level of H2O2, (oxidative stress) in tissues of *S. tuberosum* L. to control the answer of PVY-infected and control host-plants for fungal inoculation. Moreover, analysis of roots with transmission electron microscope (TEM) was done to reveal potential participation of G. intraradices in transmission of PVY in plant tissues.

Persistent Identifier: urn:nbn:de:0294-sp-2013-ppphe-2

In: Schneider C, Leifert C, Feldmann F (Eds), Endophytes for plant protection: the state of the art. © 2013 Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-11-2.

5-20 Root endophytic bacterial community in *Phragmites australis* plants exposed to carbamazepine

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ABSTRACT

Carbamazepine is an antiepileptic and mood-stabilizing drug used widely in Europe and North America. In the environment, it is found as a persistent and recalcitrant contaminant, being one of the most prominent compounds in the list of hazardous PPCPs found in Waste Water Treatment Plants (WWTPs). Usual concentrations in WWTPs range between 0,968 µg/L in influents and 0,674 µg/L in effluents, revealing a low removal efficiency of activated sludge processes. In some cases, the ultimate fate of this compound is the groundwater. A study in 2010 revealed concentrations up to 16 ng/L in confined and semi-confined groundwaters in France.

Until now, the bioremediation approach has focused in the role of the plant in the carbamazepine uptake. Studies have been performed in edible plants (cucumber, soybean and *Brassica* sp.) and also in some grass and macrophyte species. Nevertheless, investigation about the degradation of carbamazepine by endophytes is scarce. Since endophytes have been proposed to play an important role in phytoremediation, this study focuses on the identification and characterization of endophytic candidates for biodegradation of carbamazepine in wetland systems.

Phragmites australis was exposed to carbamazepine (5 mg/L) for 9 days in semi-hydroponic conditions. After surface sterilization, different bacterial strains were isolated from rhizomes and roots. These strains were able to grow either in PDA or in R2A media supplemented with 10 µM carbamazepine. The species were identified by sequencing of the 16S rDNA. Growth in minimal medium with carbamazepine as sole carbon source was studied. Phylogenetic studies and identification of non-cultivable strains will shed more light on the behavior of the bacterial community in response to this important pharmaceutical.

Persistent Identifier: urn:nbn:de:0294-sp-2013-ppphe-2

In: Schneider C, Leifert C, Feldmann F (Eds), Endophytes for plant protection: the state of the art. © 2013 Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-11-2.

5-21 Microorganisms as biological agents against pathogenic fungi of agricultural interest

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ABSTRACT

Use of biological control agents, such as plant growth promoting rhizobacteria (PGPR), and Lactic Acid Bacteria (LAB) can be a suitable approach in control of disease in vegetable crops to reduce and limit the widespread use of chemicals. PGPR, such as *Pseudomonas* and *Bacillus* strains, are the major root colonizers that can elicit plant defense and compete with pathogens. Lactic Acid Bacteria [LAB] are worldwide known as probiotic microorganisms and one of the most important probiotic feature is their activity against a wide range of harmful bacteria/fungi. Their use as “effective microorganisms” is increasing as active components of soil amendments for sustainable agriculture. This work focuses on the *in vitro* screening of 32 PGPR and LAB strains isolated from environmental sources against *Fusarium* spp, *Sclerotinia* spp. and *Rhizoctonia* spp which are widely distributed fungi affecting plant, animal and human health. In the agricultural field, they are considered important plant pathogenic fungi, with a record of devastating infections in various economically important crop plants. Fungal inhibition was performed using spot agar test in a triplicate assay with different conditions for LAB and PGPR. All LAB strains (except one) showed an antifungal activity against all *Fusarium* species tested (inhibition halo between 23.7- 65.3mm); the highest halos were obtained against *F. graminearum* on which 50% of the strains showed a total growth inhibition of the colony. Among PGPR, preliminary results showed a strong inhibition by some *B. subtilis* strains against all fungi tested. These results are promising for the formulation of a commercial product that could be used for a wide spectrum of plant crops. *In vivo* application with the most successful strains is foreseen towards wheat and lettuce to confirm *in vitro* results.

Persistent Identifier: urn:nbn:de:0294-sp-2013-ppphe-2

In: Schneider C, Leifert C, Feldmann F (Eds), Endophytes for plant protection: the state of the art. © 2013 Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-11-2.

5-22 The effects of endophytic *Fusarium* treatment on wheat

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ABSTRACT

Endophytes are microorganisms that form symptomless infection with in healthy plants tissues. The Fusarium is one of the most important genus which includes some species to showing saprophytic and/or endophytic characters. In this study, a pathonegicity test was carried out on Momtchill cv. using totally 35 isolates of different *Fusarium* species which were obtained from wheat production areas of Turkey. The pathogenic *F. culmorum* isolate which had obtained from previous study was used as a disease control and sterile distilled water was used as checking. Fusarium crown rot index, plant length and plant fresh weight were assessed to find out endophytic or pathogenic effects of *Fusarium* isolates tested. After pathogenicity test, none of *Fusarium* has pathogenic characters, some isolates also improved plant lengths and plant weight. While *F. sporotrichoides* had the biggest effect on plant length, the plants inoculated with *F. acuminatum* had the highest plant fresh weight value. These results support that some of our endophytic *Fusarium* isolates can have a potential to use as a biological agent encouraging plant growth. We will focus on using possibilities of some *Fusarium* species against pathogenic *Fusarium* and other fungi in the future.

Persistent Identifier: urn:nbn:de:0294-sp-2013-ppphe-2

In: Schneider C, Leifert C, Feldmann F (Eds), Endophytes for plant protection: the state of the art. © 2013 Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-11-2.

5-23 Effects of Fungicides on Endophytic Fungi in Timothi-Grass (*Phleum pratense*)

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ABSTRACT

All plants in natural ecosystems appear to be in symbiotic relationships with endophytic fungi [1] that live inside plant tissues without causing any apparent symptoms. This highly diverse group of fungi has been remained to date poorly characterized, as estimably less than 1% of all the possible endophytic species are known to science so far [2]. Endophytes are seen as promising alternatives to agrochemicals increasing fitness of plants by conferring abiotic and biotic stress tolerance, increasing biomass, and decreasing water consumption. It may be possible that overuse of agrochemicals, e.g. fungicides, may decrease overall plant fitness in long-term usage by unbalancing the ecosystem of microorganisms associated to plants. In our experiment, five different fungicides were selected to treat timothy-grass plants and experiment was carried out in three different locations in Estonia. Plants were sprayed with fungicides in agronomically recommendable concentrations, samples were collected after 1.5-2 weeks from spraying, surface sterilized and cultured on PDA agar. Noticeable decrease in colonization of plants by endophytes treated with fungicides compared to control-plants was detected. The most effective was systemic fungicide tebuconazole ('Folicur 250 EW') decreasing endophytic colonization more than half compared to control-plants, effective were also azoxystrobine ('Amistar') and sulphur as an active ingredient consisting contact fungicide ('Thiovit Jet'), decreasing endophytic colonization to half compared with control-plants. Less effective were systemic fungicides propiconazole ('Bumper 25 EC') and epoxiconazole ('Opus EC'). Endophytic species that grew out from plant samples were determined by morphological and ITS rDNA analysis. Four endophytes were identified to species level, twelve to genus level so far and one possible new species discovered. Noticeable was also that older plants tend to have more endophytes.

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5-24 Potentialities of endophytic fungi of olive tree as biological control agents against *Colletotrichum acutatum* and *Verticillium dahliae*

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ABSTRACT

Fungal endophytes grow asymptomatically within the tissues of all vascular plants and some are known to provide their host plants with tolerance to different types of environmental stress, including fungal pathogens. Anthracnose and Verticillium wilt are the most important olive diseases in worldwide caused by the fungus *Colletotrichum acutatum* and *Verticillium dahliae.* The present study aimed to determine the antagonistic ability of endophytic fungi against both casual agents, to be further used as biological control agents. The endophytic fungi studied were isolated from leaves, branches and roots of olive tree Cv. Cobrançosa which is moderately tolerant to both diseases. Dual cultures of five endophytes (namely *Hypocrea lixii*, *Paecilomyces lilacinus, Fusarium oxysporum, Penicillium commune, Penicillium roseopurpureum*) with phytopathogens were carried out on potato dextrose agar medium, and the internal radial fungal growth, hyphae morphology, sporulation and spore viability were evaluated during 15 days. Dual cultures of the same species were used as controls. Among the endophytes tested, *H. lixii, P. lilacinus*, have showed the highest inhibitory effect on mycelia growth of *C. acutatum* (50% and 38%) and *V. dahliae* (55% and 32%), respectively. *Penicillium commune*, have reduced significantly the *C. acutatum* sporulation between 27-78%, and germination between 37-82%, when compared to control. The number and the viability of the spores produced by *C. acutatum* challenged by endophytes were significantly reduced compared to control, with values above 80% and 68%, respectively. In *V. dahliae* the production and the viability of spores were only slightly reduced when compared to controls. Observations made by microscope in the interacting zone showed several morphological alterations including growth of thin hyphae compared to control cultures, vacuolation and lysis of hyphal of the pathogens. The results obtained support the potential of fungal endophytes as biological control agents againts both *C. acutatum* and *V. dahliae*.

ACKNOWLEDGMENTS

This work has been supported by FCT (reference grant PTDC/AGR-PRO/4354/2012).

Persistent Identifier: urn:nbn:de:0294-sp-2013-ppphe-2

In: Schneider C, Leifert C, Feldmann F (Eds), Endophytes for plant protection: the state of the art. © 2013 Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-11-2.

5-25 Grapevine bacterial endophytes control multiple grapevine pathogens

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ABSTRACT

A collection of bacterial endophytes isolated from wild and domesticated grapevine was characterised for 30 features, arranged in five major groups: plant growth promotion (PGP), antibiotic resistance (AB), secretion of enzymes (ENZ), quorum sensing (QS) and biocontrol (BICO). Many bacterial strains showed a high biocontrol potential against known grapevine pathogens *in vitro* (*Botrytis cinerea, Botryosphaeria dothidea, Botryosphaeria obtusa, Phaeomoniella chlamydospora, Phaeoacremonium aleophilum*) and *in vivo* (*B. cinerea* and *Plasmopara viticola*). Interestingly, effective biocontrol agents were classified into several, often distantly related genera. By comparing bacterial strains from wild and cultivated grapevines, we show that the strains with the best biocontrol activity against multiple pathogens were mostly isolated from wild grapevine. Conversely, strains from domestic grapevines were generally more efficient in the *in vitro* biocontrol of *P. chlamydospora*. We also discuss here the other groups of analysed features, their distribution and possible ecological significance.

Persistent Identifier: urn:nbn:de:0294-sp-2013-ppphe-2

Ngwene B et al., Phosphate solubilization and plant growth promotion of the fungal root endophyte Piriformospora indica. In: Schneider C, Leifert C, Feldmann F (Eds), Endophytes for plant protection: the state of the art, pp.192-193. © 2013 Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-11-2.

5-26 Phosphate solubilization and plant growth promotion of the fungal root endophyte *Piriformospora indica*

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ABSTRACT

Plant symbiotic associations range from pathogenic to mutually beneficial and may depend on the nutritional status of the host. Root colonizers such as arbuscular mycorrhizal fungi (AMF) support plant mineral nutrition in exchange for photosynthetic carbon. The fungus Piriformospora indica belonging to the order Sebacinales (Basidiomycota) is a root endophyte with a broad host spectrum. In contrast to AMF, P. indica is able to colonize roots and promote plant growth independent of phosphate concentrations in the soil. Its impact on mineral nutrition is currently under debate. Improved phosphate or nitrogen uptake could not be shown in barley plants, but reduced phosphate amounts in maize plants were observed when they were colonized by a phosphate transporter knocked out P. indica mutant. In order to get more insights into the relationship between P. indica and plants concerning mineral nutrition, and to assess their role in phosphate nutrition and uptake in tomato plants, the solubilisation activity, the production of extra-radical mycelia and the plant growth promoting effect of the endophyte Piriformospora indica were investigated in in-vitro and in-vivo experiments with compartment systems.

When cultured in agar medium, P. indica growth was enhanced in the when supplied with Tri-calcium phosphate or phytin as the P source. Fungal growth was significantly increased at higher concentrations but the fungus did not produce hydrolysis halo as parameter for P solubilization. Interestingly, in liquid medium, Pi was released from Tri-calcium phosphate and phosphate rock into the medium although no extracellular acid phosphatase activity of P. indica was evident. The fresh fungal biomass was similar in liquid medium supplemented with soluble phosphate, tri-calcium phosphate and rock phosphate after 30 days of incubation; however the dry biomass and the P content in mycelium were significantly higher when the fungus grew in Tri-calcium and rock phosphates. In pot experiments, shoot biomass and P content in tomato plants supplied with 180mg/L rock phosphate was

Persistent Identifier: urn:nbn:de:0294-sp-2013-ppphe-2

significantly higher when plants were colonized by P. indica than mock plants. In a separate experiment, P. indica inoculatuion also had a positive effect on these parameters when plants where supplied with soluble P. A synergistic effect with G. intraradices was also partly observed. However AMF colonization was negatively affected in a co-inoculation treatment at high concentration of localized (compartment) Pi. Further analysis is needed to understand the interaction between P. indica and AMF at low and high concentrations of Pi in substrate. We also demonstrated by PCR and trap plants that P. indica is able to produce extra radical mycelium (ERM) in a soil-substrate, and the growth seems to be higher in the presence of a host plant. Quantification of fungal mycelium at different distances from the host plant under conditions that promotes saprophytic or symbiotic behavior will give more information of P. indica ERM growth in soil.

Persistent Identifier: urn:nbn:de:0294-sp-2013-ppphe-2

In: Schneider C, Leifert C, Feldmann F (Eds), Endophytes for plant protection: the state of the art. © 2013 Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-11-2.

5-27 Competitive properties of *Neotyphodium lolii* – a perennial ryegrass (*Lolium perenne* L.) endophyte towards *Fusarium poae*

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ABSTRACT

Perennial ryegrass is one of the most important grasses in Poland, used for pasture and for turf. It is often attacked by numerous pathogens, which can decrease yield and its quality. Use of fungicides for plants protection against diseases is often limited. The other way of protection of perennial ryegrass can be use of an endophyte - *Neotyphodium lolii*. Antifungal activity of the fungus is often reported. It is mainly associated with antibiotic chemicals produced by the symbiont. However, other mechanism are also responsible for inhibition of pathogens' development. Detailed experiments in controlled conditions were conducted to determine whether there is an effect of endophyte mycelium density in leaf sheaths and blades on development of disease symptoms caused by *Fusarium poae*. Three endophyte infected (E+) and non-infected (E–) perennial ryegrass genotypes were artificially infected by *F. poae*. Ten days after inoculation the degree of tillers infestation was evaluated based on a five-degree scale. Density of endophyte mycelium was estimated in the following cross-sections: leaf sheath base, 0.5 cm below the ligule section of leaf sheaths, 0.5 cm above the ligule section of leaf blade, 3.0 cm above the ligule section of leaf blade. The cross-sections were stained with aniline blue. The stained hyphae were counted under the microscope (200x). Mycelium density was calculated as an average number of hyphae per 1 mm of the width of leaf blade or leaf sheath cross-sections. The relationship between endophyte hyphal density and disease index was determined using Pearson's correlation coefficient. There was a significant effect of the endophyte presence on the intensity of disease symptoms. Density of mycelium of the endophyte strains differed between cross-sections studied. There was not a significant correlation between *F. poae* disease indexes and *N. lolii* mycelium density. The inhibitory mechanism does not appear to be based on competition between endophyte and pathogen hyphae inside the plant, but rather through other defence mechanisms.

Persistent Identifier: urn:nbn:de:0294-sp-2013-ppphe-2

Schroers H-J et al., Assessing the biological control potential of *Clonostachys solani*. In: Schneider C, Leifert C, Feldmann F (Eds), Endophytes for plant protection: the state of the art, pp.195-196. © 2013 Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-11-2.

5-28 Assessing the biological control potential of *Clonostachys solani*

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ABSTRACT

Species of *Clonostachys* (*Bionectriaceae*, *Hypocreales*, *Ascomycota*) are known to occur as soil saprotrophs but also as destructive mycoparasites. The ability of some *Clonostachys* species to parasitize insects, ticks, and nematodes has less often been reported. Inventories of the teleomorphs of *Clonostachys* mainly in tropical regions have suggested that it also accommodates endophytes because the sexual fruiting bodies of several species are routinely encountered on recently dead trees and formed on stromata erumping through bark. For biological control purposes only one of the more than 30 described species in *Clonostachys*, *C. rosea* (cited also as *Gliocladium catenulatum*), has until now been used. *Clonostachys solani* is the second most commonly isolated soil inhabitant of the genus in temperate regions and forms sexual fruiting bodies on woody substrata similar to those of *C. rosea*. Recently we isolated *C. solani* from an unidentified insect larva. Within the context of screenings for novel biological control agents, we studied the entomopathogenic ability of *C. solani* against the cabbage root fly *Delia radicum*, an economically important pest of brassica crops. We also tested its ability to survive on the rhizoplane or as an endophyte in the tissue of cauliflower (*Brassica oleracea* Botrytis group) roots.

In-vitro tests lasting 14 days were designed to screen aggressiveness of *C. solani* against *D. radicum.* Insect eggs were drenched with ca. 5 x 10^6 conidia. Laboratory soil bioassays lasting 35 d mimicked natural exposure pathways of *C. solani* to different insect life stages starting with eggs and evaluated as numbers of pupae or surviving larvae. Spore concentrations used in soil tests were comparable to economic rates for in-furrow applications (3.85 x 10^6 spores/g dry soil). In a field experiment, compatibility or pathogenicity to cauliflower was inferred after introducing ca. 23 x 10^6 *C. solani* conidia to the soil near stems. In a lab experiment, seed germination and seedling survival was measured after coating seeds with *C. solani* inoculum (0.5 x 10^8 conidia/ml). Preliminary

results from root washing tests with water or with water and 70 % ethanol were also gathered to infer the survival of *C. solani* on the rhizosphere or as root endophytes.

The tested strain of *C. solani* caused significant mortality of either eggs or larvae in soil tests (47.9±12.8 Abbott's corrected soil mortality, ACM). Although eggs of *Delia radicum* were colonized by *C. solani*, only insignificant ACM of 15.56±11.76 was measured in *in-vitro* tests. The tested strain was not plant pathogenic but reduced, however insignificantly, cauliflower plant mortality and increased plant weight. The preliminary results indicated that *C. solani* can colonize the rhizosphere and occasionally also the root tissue.

Persistent Identifier: urn:nbn:de:0294-sp-2013-ppphe-2

Burlak O et al., Photosynthetic Effects in *Kalanchoë daigremontiana* Hamet & Perr. Protected with Bacterial Strains under Mars-Like Conditions. In: Schneider C, Leifert C, Feldmann F (Eds), Endophytes for plant protection: the state of the art, pp.197-202. © 2013 Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-11-2.

5-29 Photosynthetic effects in *Kalanchoë daigremontiana* Hamet & Perr. protected with bacterial strains under mars-like conditions

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ABSTRACT

The effects of pretreatment of plants of *Kalanhoë daigremontiana* Hamet & Perr. with endophytic bacterium *Methylobacterium* sp. on photosynthetic activity (PA) under Mars simulated condition (low atmospheric pressure, high CO_2 concetration, ultra violet (UV) of near-Martian suface spectrum and low nutrients availability) were studied in short-duration experiments using a Mars simulation facility (MSF) (DLR, Berlin). Parameters of the photosystem II (PSII) photochemistry efficiency such as maximum quantum yield, F_v/F_m; operating efficiency, Φ_{PSII}; photochemical quenching coefficient, *qP;* maximum quantum efficiency in the light, F_v'/F_m'; nonphotochemical quenching, *NPQ*, were monitored *in situ* during the diurnal CAM (Crassulacean Acid Metabolism) cycle with MINI-PAM fiberoptical mounted in the MSF. The value of F_v/F_m in dark-adapted leaves was measured also before and after exposure to simulated martian conditions. The multifactor abiotic stressors inside the MSF decreased the PSII photochemistry efficiency and enhanced NPQ as compared to control plants grown without stressors in the MSF. In kalanchoe plants treated with *Methylobacterium* sp., parameters of the PSII photochemistry efficiency were higher and NPQ was lower than in non-inoculated plants with stressors at the end of light period. The superior F_v/F_m and Φ_{PSII} values in these plants correlated with enhanced expression of the genes, encoding phosphoenolpyruvate carboxylase, and increased nocturnal malate accumulation under stressful conditions. Plants

Persistent Identifier: urn:nbn:de:0294-sp-2013-ppphe-2

inoculated with *Methylobacterium* sp, exhibited better accommodation (higher F_v/F_m) after exposure to Martian-like conditions than non-inoculated plants. The results suggest that Mars-simulated stress in kalanchoe plants was alleviated partially by the inoculation with selected bacterial strains.

INTRODUCTION

The atmospheric CO_2 concentrations and global surface temperatures increase permanently (IPCC Climate Change 2007) that leads to reducing soil water content. Under these circumstances, it seems practical to use CAM plants that have more adapted photosynthetic systhem and possess a higher water-use efficiency than C_3 and C_4 plants during water stress (Borland *et al.* 2009). Thus CAM-plants could be used on soil fallow for enrichment of bioavailable organic compounds or terraforming of barren land on Earth or Martian and Lunar greenhouses. Numerous studies on plant growth under Mars-like conditions indicate that plants tolerate low atmospheric pressure but sensitive to low O_2 concentration (Tang *et al.* 2010). CAM species show an average increase in biomass productivity in response to increased atmospheric CO_2 concentration (Drennan & Nobel 2000). The performance of the CAM photosynthetic apparatus can be estimated by measuring chlorophyll fluorescence.It was shown that the photosynthetic activity (PA) served as the indicator of functionality of eukaryotic photosystem under simulated Mars-like parameters (de Vera *et al.* 2010).

Environmental stresses (non-optimal temperature, salinity, drought, irradiation) enhance the extent of photoinhibition, a process that is determined by the balance between the rate of photodamage to photosystem II (PSII) and the rate of its repair. The exposure to environmental stresses does not affect photodamage but inhibit the repair of PSII through suppression of the synthesis of PSII proteins (Takahashi & Murata 2008). To deal with stresses, plants have evolved several defence mechanisms. Endophytic bacteria, both exogenously applied for plant inoculation and resident ones, possess a potency to alleviate effect of stressors on PSII (Burlak *et al.* 2010; 2013).

The concept of using microbial technology, including resident endophytes, for the alleviation of environmental stressors effects may provide a novel low cost strategy for plant growing/protosoil formation for Lunar/Martian greenhouses (Kozyrovska *et al.* 2006; Zaets *et al.* 2011). Earlier, we showed that plant-growth promoting bacteria (PGPB) (*Klebsiella oxytoca* and *Paenibacillus* sp.) assisted Kalanchoë plant to accommodate after acute irradiation with γ-quanta (^{60}Co) (Burlak *et al.* 2010). The main objective of this study was to assess a photoprotective effect of bacterial species on CAM plants grown in the Martian Regolith Simulant (MRS) under near-Mars simulated conditions. For Kalanchoë plants inoculation by endophytic bacterium *Methylobacterium* sp. IMBG290 showed earlier positive protection effects (Ardanov *et al.* 2011; 2012) have been chosen.

Persistent Identifier: urn:nbn:de:0294-sp-2013-ppphe-2

MATERIALS AND METHODS

The Mars simulation facility and chlorophyll fluorescence analyses

In HUMILAB experimental the Mars simulation facility (MSF) (DLR Berlin), the increasing CO_2 content with partial pressure from 39 Pa to 114 Pa (at low atmosphere pressure 12 mbar) and a LED of the visible light spectra and UV-irradiation were used as described earlier (de Vera *et al.* 2010). Chlorophyll fluorescence in MSF were measured fluorometer MINI-PAM (Heinz WALZ GmbH, Effeltrich, Germany) as described previously (Burlak *et al.* 2010).

Plant material and microbial species

Kalanchoë daigremontiana plants were grown in the commercial soil, and 3 days before exposure to stressful conditions the plants were replaced into MRS. PGPB strains were taken from the collection of Institute of Molecular Biology and Genetics (Kyiv, Ukraine), *Methylobacterium* sp. IMBG290 *Paenibacillus* sp. IMBG156, *Pseudomonas fluorescens* IMBG163 were cultured and used for the inoculation of plants as described in (Burlak *et al.* 2010).

RNA isolation, cDNA synthesis, semi-quantitative RT-PCR

were done as recommended by Fermentas (EU). Data were standardized for the housekeeping glyceraldehydes-3-phosphate dehydrogenase (GAPDH) gene using specific primers.

The total malate content in Kalanchoë leaves

was measured by Malate Assay Kit (BioVision, USA). Statistical tests of the significance of differences between means from two to three experiments were based on Student's t-test ($p < 0.05$).

RESULTS AND DISSCUSION

The Mars environment stressors have influenced the photosynthetic machinery, and PA of Kalanchoë plants was changed within the MSF, decreasing operating quantum efficiency, Φ_{PSII}, and maximum quantum efficiency, F_v/F_m, at the end of light period (Fig. 1b) compared to normal conditions (Fig. 1a). *In situ* chlorophyll fluorescence measurement in the MSF showed positive impact of endophytic bacterium *Methylobacterium* sp. (Fig. 1c), as well as *Paenibacillus* sp. (data not shown) inoculation on plants which expressed in higher F_v/F_m and Φ_{PSII} values compared to non-inoculated plants (Fig. 1b). However, plants inoculated with *P. fluorescens* (Fig. 1d) exhibited lower values of F_v/F_m and Φ_{PSII} at the end of light period than untreated plants. The *qP*value of non-inoculated control plants under Martian conditions was lower than in non-inoculated control plants inside the facility under ambient

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conditions. This supposes the decrease in electron flow rate through the electron transport chain of chloroplasts, probably resulting in ATP and NADPH synthesis impairment. Although the loss of PSII efficiency under dark might be attributed to the photosynthetic membrane disturbance, major impairment to PSII function caused in the light was related to the photo-oxidative damage of PSII reaction centres. The non-photochemical quenching *NPQ,* was higher in stressed untreated Kalanchoë plants (Fig. 1b) than in unstressed control plants within the MSF (Fig. 1a), exhibiting enlarged de-exciting by thermal dissipation. At the beginning of light period (Fig. 1b, the values from 5:30 h to 8:00 h), high *NPQ* represented the most likely protective mechanism/s of the photosythetic electron transport down-regulation induced by decreased CO_2 assimilation so that production of ATP and NADPH would match the actual demand (Muller *et al.* 2001). However, the increase in *NPQ* in a second half of light period (Fig. 1b, the values from 8:00 h to 12:00 h) may indicate the accumulation of photo-damage in PSII reaction centres. After exposure to multi-factor stress, both inoculated and control Kalanchoë plants were dark-adapted (0.5 h). In the period of plant accommodation to post-stress conditions, F_v/F_m in Kalanchoë leaves in control was lower than in inoculated plants (Burlak *et al.* 2013). The maximum quantum yield of PSII in Kalanchoë plants inoculated with *Methylobacterium* sp. exhibited the best accommodation capacity of PSII after exposure plants to abiotic stressors.

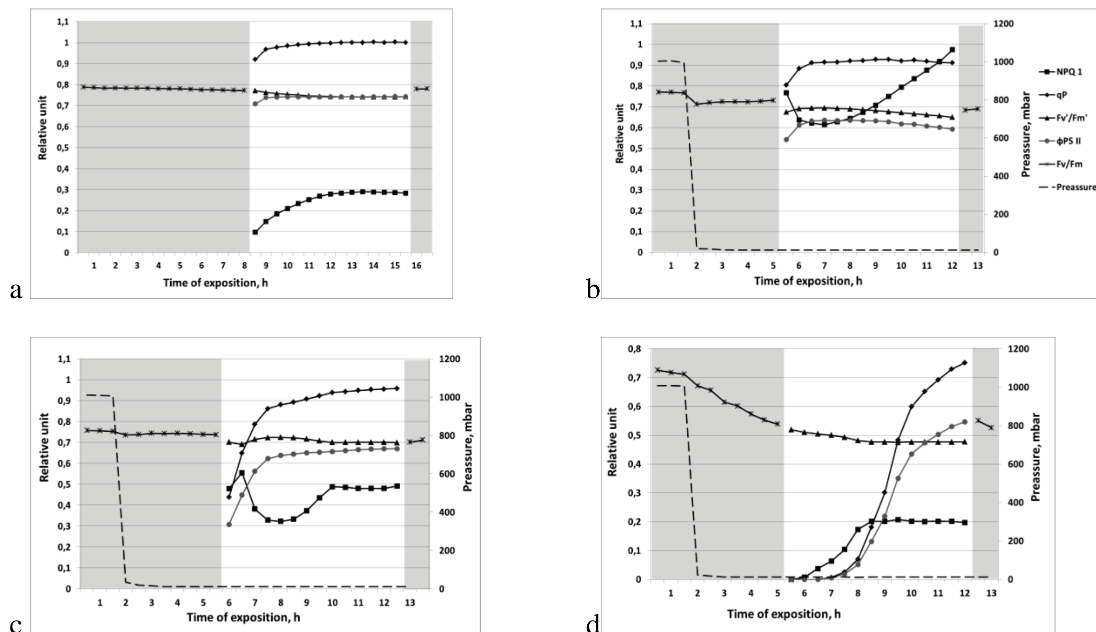

Figure 1. Maximum quantum efficiency of PSII in the Kalanchoe leaves under the dark (Fv/Fm; visible in the greyish areas) and in exposed to the light (Fv′/Fm′; visible in the white areas), ΦPSII, qP and NPQ in MSF. Non-inoculated control plants inside MSF under normal conditions (a) and under simulated Martian conditions (b); plants treated with Methylobacterium sp. (c), Pseudomonas fluorescence (d).

The CAM cycle is tightly regulated with a number of control points, and the delayed induction of electron transport traced by Φ_{PSII} kinetics may be related to protracted Rubisco

activation and/or malate decarboxylation. Inoculated with *Methylobacterium* sp. and *Paenibacillus* sp. plants had increased nocturnal malate accumulation under LED illumination, as well as inoculated variants under UV within the MSF compared to non-inoculated control plants (Fig. 2). It is known that CAM plants exhibit increased nocturnal malate accumulation under non-optimal conditions (e.g. elevated atmospheric CO_2 concentrations) (Drennan & Nobel 2000).

In CAM plants, a dark CO_2-uptake is mediated by phosphoenolpyruvate carboxylase (PEPC), an enzyme that can be regulated by environmental factors at transcriptional and posttranslational levels. Under the effect of factors, limiting normal growth, plants inoculated with *Methylobacterium* sp. and *Paenibacillus* sp. had higher expression of the Ppc gene, comparing with plants grown outside the facility under normal conditions (Fig. 3). In this study, the level of the Ppc transcripts correlated positively with malate accumulation under stressful conditions in exposed Kalanchoë plants.

Figure 2

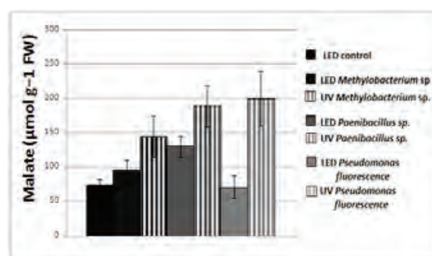

Figure 3

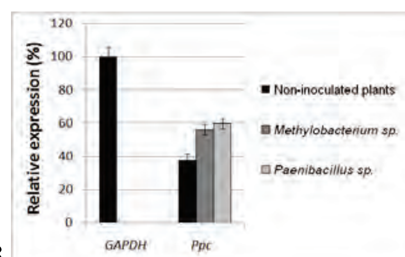

Figure 2. Malate content in K. daigremontiana plants grown on the MRS after MSF expose lighted with LED (PAR) or UV during a day period. Error bars indicate the SD of the means (n = 3). Plants were treated with various bacterial strains.

Figure 3. The relative expression of the phosphoenolpyruvate carboxylase gene (Ppc) in treated with bacteria Kalanchoë plants in MSF (n = 2).

Short-duration exposure to Mars-like conditions led to a slight decrease in the photosystem II activity in Kalanchoë plants. However, after inoculation with *Methylobacterium* sp. PSII acquired a protection under stressful conditions. It is possible to assume that microorganisms stimulated the electron flow rate through the electron transport chain of chloroplasts and could enhance the resistibility to stress by the activating of plant defence system. The processes reflected by *NPQ* and *qP* in plants help to minimize the production of reactive oxygen species (ROS) in chloroplasts. Chlorophyll triplets readily react with oxygen to produce ROS that cause damage to PSII-mediated electron transport and the induction of protein degradation (Hoyo *et al.* 2011). We may assume that some bacterial species could complement the deficient antioxidative systems of the plant with their own ROS-eliminating systems (Zaets & Kozyrovska 2008). It was well documented, that *Methylobacterium* sp. IMBG290 activated multiple plant antioxidant enzymes that combated oxidative stress during plant defence (Ardanov *et al.* 2011). The ability of microorganisms to confer optimized stress tolerance of plants may provide a strategy for mitigating the impacts of adverse environmental conditions on growing plants in Lunar or Martian habitats/greenhouses.

Persistent Identifier: urn:nbn:de:0294-sp-2013-ppphe-2

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In: Schneider C, Leifert C, Feldmann F (Eds), Endophytes for plant protection: the state of the art. © 2013 Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-11-2.

5-30 Endophytic bacteria for improving biomass production of poplars

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ABSTRACT

Endopytic bacteria may promote plant growth by useful metabolic capabilities such as the contribution of assimilabe nitrogen, the production of phytohormones and siderophones or the suppression of pathogens. In our study we analyzed the growth promoting effect of selected endophytic bacteria strains on in vitro meristem plants. Parallel their influence was measured using cuttings of different poplar clones in the field. Inoculation of bacteria free meristem plants with Stenotrophomonas sp. 169 induced an enhanced root growth resulting in significantly increased root and shoot weights. However, the growth promoting effect was reduced by using meristem plants already colonized with endophytic Paenibacilli. An increased plant growth and yield could also be revealed on field grown poplar cuttings inoculated with Stenotroph¬omonas sp. 169, Paenibacillus sp. 22 and Pseudomonas 3RE 2-7, respectively. Here, the result of inoculation was found to be influenced by the poplar clone used and the growth conditions during the experiment. To throw light on potential modifying factors further research is needed.

Persistent Identifier: urn:nbn:de:0294-sp-2013-ppphe-2

In: Schneider C, Leifert C, Feldmann F (Eds), Endophytes for plant protection: the state of the art. © 2013 Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-11-2.

5-31 Preliminary study of endophyte infection rate in timothy-grass at different nitrogen treatments

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ABSTRACT

Endophytic fungi are highly diverse microorganisms that live within plant tissues, usually asymptomatically, and are considered to be mostly mutualists. Timothy-grass (*Phleum pretense* L.) is a perennial grass common to most of Europe, US and Canada, but has been studied little in regards to endophytes.

Nitrogen is a common currency in endophyte/host interactions, limiting the growth of the host plant and fungi both. It is suggested that endophytes act as a kind of "nitrogen sinks" and might mean an extra metabolic cost for the host grass, since the endophyte might compete with the host for nutrients under nutrient limited conditions.

In this study, the effect of different levels of nitrogen fertilization on the abundance and diversity of endophytic fungi were studied. A field study of sowed timothy grass was established in 2010 in Tartu County, Estonia. Three levels of nitrogen fertilization, 60 kg/ha, 120 kg/ha and 180 kg/ha, and a control of no fertilization were used. During the autumns of 2011 and 2012, timothy-grass was sampled from all plots and processed for fungal endophyte study. Leaves were surface sterilized with ethanol and bleaching agent and leaf segments of 5 x 5 mm plated on malt extract agar (MEA). Species determination of isolated fungi, using morphotaxonomical criteria and rDNA sequencing, is currently ongoing.

Endophytic fungi were isolated in all fertilization treatments. Overall colonization frequency (CF- number of segments colonized by endophytes compared to the total number of segments observed) of timothy-grass by fungal endophytes was under 50% in both years (32% in 2011 and 48% in 2012). Colonization frequency in the plants collected from the control plots (no N treatment) was 30% in both years. In 2011 CF was 33%, 50% and 43% (N treatments 60 kg/ha, 120 kg/ha and 180 kg/ha, respectively) and in 2012 the CF was 50%, 47% and 65%.

Persistent Identifier: urn:nbn:de:0294-sp-2013-ppphe-2

In: Schneider C, Leifert C, Feldmann F (Eds), Endophytes for plant protection: the state of the art. © 2013 Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-11-2.

5-32 Influence of drought on the root exudation of *Plantago lanceolata* and *Lotus corniculatus*

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ABSTRACT

Plant roots exudate a large number of compounds into the rhizosphere, which is defined as that part of soil that is directly influenced by plant roots. Many of these exudates, mostly low molecular weight compounds, have an impact on various processes within the rhizosphere. This can be interactions between different plant species as well as plant-microbe or plant-insect interactions.

Abiotic stress can cause a change the quality and quantity of plant root exudates which might on the other hand influence the composition of the microbial community in the soil. Pathogenic or beneficial bacteria can might be attracted or avoided by changes of the chemical milieu in the rhizosphere.

The first aim of this study was to characterize the composition of root exudates by the two grassland species *Plantago lanceolata* and *Lotus corniculatus.* The second goal was to investigate changes in root exudation that are caused by exposing these plants to drought conditions. Both species were grown in rhizotrons under green house conditions. Roots exudates were collected using micro suction cups and a controlled vacuum pump which allows a continuously and non-invasive collection of exudates while growing the plants in a natural soil system.

After solid phase extraction (SPE) samples were directly subjected to LC-MS analysis which represents a powerful tool for the rapid and sensitive detection of a wide range of analytes.

First measurements revealed the occurrence of different aliphatic dicarboxylic acids as well as some phenolic compounds. The composition of exudates as well as methodological issues will be critically discussed.

Persistent Identifier: urn:nbn:de:0294-sp-2013-ppphe-2

Sas Paszt L et al., The collection of beneficial soil microorganisms held in the SYMBIO BANK. In: Schneider C, Leifert C, Feldmann F (Eds), Endophytes for plant protection: the state of the art, pp.206-207. © 2013 Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-11-2.

5-33 The collection of beneficial soil microorganisms held in the SYMBIO BANK

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ABSTRACT

An important part the project called EcoTechProduct, which is carried out at the Research Institute of Horticulture in Skierniewice (Poland), is to establish and maintain a Bank of Symbiotic Microorganisms, called the SYMBIO BANK. At present, the collected material of isolated spores of mycorrhizal fungi and PGPR bacteria comes from organic orchards and plantations located around Skierniewice, (Poland), and organic orchards and plantations in the Bieszczady and Białowieza areas (Poland). Results of studies to date have shown that there are large differences in the occurrence of mycorrhizal fungi depending on the species and plant cultivation method. At present, the material collected in the bank of isolated spores of mycorrhizal fungi and the bank of PGPR bacteria comes from less polluted regions in Poland. Trap cultures have been used to isolate and identify spores of the following species of arbuscular mycorrhizal fungi: *Ambispora fennica, A. gerdemannii, Gigaspora margarita, Glomus aggregatum, G. caledonium, G. claroideum, G. constrictum, G. drummondii, G. fasciculatum G. macrocarpum, G. microaggregatum, G. mosseae, G. pallidum, G. rubiforme, Scutellospora dipurpurescens*. The collection in the SYMBIO BANK contains (approximately): Spores isolated from the soil of the following plant species: strawberry 16.0 thousand, apple 6.5 thousand, sour cherry 1.1 thousand pear 8.1 thousand. Isolates of bacteria: *Pseudomonas fluorescens* -170, dissolving phosphorus compounds - 40, digesting cellulose - 40, producing spores - 110, fixing atmospheric nitrogen - 10, *Actinomycetes* - 40. Isolates of microscopic fungi - 50, including Trichoderma sp. - 30. The work of isolating and identifying species and strains of AM fungi and PGPR bacteria is continued. They are collected, catalogued and stored in a Bank of Symbiotic Microorganisms, called SYMBIO BANK, specially established for this purpose. The collected strains and species are identified, characterized and stored in a cryoprotectant (glycerol) at the temp. of -80°C. In the near future, a website of the SYMBIO BANK will be launched, which will contain a list of the isolates held in the collection and their descriptions, which will serve as a source of key information for the identification of the species of AM fungi and PGPR bacteria in

Poland. This will contribute to the knowledge of the biodiversity of these symbionts and help in the formulation of microbiologically-enriched bioproducts for use in fruit-growing practice. The most effective strains and species of microorganisms will be patented and registered in Poland as bacterial and mycorrhizal inocula to be used in fruit production and in phytoremediation of heavy metal pollution. The establishment of the bank of PGPR bacteria and spores of mycorrhizal fungi will contribute to the understanding and maintenance of the biodiversity of these symbionts, the knowledge of their biology and ecology, as well as to the development of ecological technologies of fertilization of horticultural plants in Poland and the protection of the natural environment and human health.

The work has been supported by a grant from the EU Regional Development Fund through the Polish Innovation Economy Operational Program, contract No. UDA-POIG.01.03.01-10-109/08-00.

Persistent Identifier: urn:nbn:de:0294-sp-2013-ppphe-2

In: Schneider C, Leifert C, Feldmann F (Eds), Endophytes for plant protection: the state of the art. © 2013 Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-11-2.

5-34 Identification of endophytic mycorrhizal fungi colonizing the roots of wild fruit plants with nested PCR

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ABSTRACT

Development of new mycorrhizal inocula for fruit crops makes it necessary to acquire and identify species of AM fungi colonizing the roots of fruit trees and shrubs. As part of the research carried out in the Rhizosphere Laboratory of the Institute of Horticulture in Skierniewice, strains of native AM fungi have been acquired. Evaluation of the usefulness of these strains for use in the cultivation of plants requires that the species be first identified. A study was carried out to identify the species of arbuscular mycorrhizal fungi colonizing the root zone of several species of wild fruit plants in non-polluted areas of Podlasie (eastern Poland) and the Bieszczady region (southern Poland), using molecular techniques. The plant material consisted of the roots of plantain from trap cultures established with samples of rhizosphere soil of strawberry plants and apple trees growing near Białowieża, and apple, sweet cherry, plum and pear trees growing in the Bieszczady region. Fungal sequences were amplified by nested PCR using primers which target the region of the large subunit rRNA gene (LSU rRNA). The PCR products were cloned with pGEM-T vector system (Promega) and 151 clones were digested with *Hinf*I, *Hsp*92 and *Taq*I restriction enzymes. The restriction patterns allowed to classify the fungi into 4 different groups. Examples of each group were sequenced and compared to the sequences available in an NCBI database (www.ncbi.nlm.nih.gov). Analysis of the obtained RFLP patterns and the sequencing of DNA fragments revealed the presence of *Funneliformis caledonium* and *Rhizophagus intraradices* fungi in the rhizosphere soil of apple trees and strawberry plants from Podlasie. The fungi identified in the rhizosphere zone of apple trees growing in Bieszczady were *Rhizophagus intraradices*, *Rhizophagus irregularis* and *Funneliformis mosseae*. Identification of the fungi in the roots of sweet cherry, plum and pear trees revealed the presence of *Rhizophagus irregularis*. The tests made it possible to identify the species of mycorrhizal fungi colonizing the roots of selected fruit plants. The results will be used to conduct further research on the application of AMF strains in mycorrhizal inocula for fruit crops. 'Development of innovative products and technologies for organic fruit production'. The work has been supported by a grant from the EU Regional Development Fund through the Polish Innovation Economy Operational Programme.

Persistent Identifier: urn:nbn:de:0294-sp-2013-ppphe-2

In: Schneider C, Leifert C, Feldmann F (Eds), Endophytes for plant protection: the state of the art. © 2013 Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-11-2.

5-35 The influence of bioproducts on root growth and mycorrhizal occurrence in the rhizosphere of strawberry plants cv. 'Elsanta'

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ABSTRACT

An experiment was carried out in a greenhouse of the Research Institute of Horticulture (RIH) to evaluate the effect of new organic fertilizers and amendments on root growth and mycorrhizal abundance and biodiversity in the rhizosphere of strawberry plants cv. 'Elsanta'. The plants were grown in rhizoboxes (sized 37 cm x 1.8 cm x 20 cm), filled with 1.85 kg of a podsolic soil collected from an uncultivated field of an experimental organic orchard of the RIH. The soil characteristics were: pH 5.5, organic matter content 1.5%, P content 51 mg P kg^{-1}, K content 158 mg K · kg^{-1}. The plants were treated with different organic fertilizers and amendments: dry granulated bovine manure (Doctor O'grodnik), extract of vermicompost (Humus UP), extract of humates (Humus Active + Aktywit PM), plant extract (BioFeed Amin), extract from several seaweed species reinforced with humic and fulvic acids (BioFeed Quality), a consortium of beneficial soil organisms (Micosat), a stillage from yeast production (Vinassa) and a solution of titanium (Tytanit). Plants treated with BioFeed Amin, BioFeed Quality, Micosat, Vinassa and Tytanit received also half dose of dry manure. A standard NPK fertilization (NPK control) and a not fertilized control were also included. The following parameters were measured: root growth and morphological parameters, number of Arbuscular Mycorrhizal Fungi (AMF) spores, mycorrhizal frequency of AMF in the roots. The chemical composition of the applied products and of soil were also determined. The treatment inducing the highest development of mycorrhizas in the roots of strawberry plants cv. 'Elsanta' were Micosat and BF Amin. The treatments BF Quality and BF Amin had the most beneficial effect on the formation of AMF spores in the rhizosphere. Application of the bioproducts had a positive effect on root growth parameters in comparison with the plants fertilized with NPK. The use of BF Quality and Humus UP induced a reduction of the amount of mineral nutrients in the soil.

The work has been supported by a grant from the EU Regional Development Fund through the Polish Innovation Economy Operational Program, contract No. UDA-POIG.01.03.01-10-109/08-00.

Persistent Identifier: urn:nbn:de:0294-sp-2013-ppphe-2

In: Schneider C, Leifert C, Feldmann F (Eds), Endophytes for plant protection: the state of the art. © 2013 Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-11-2.

5-36 Rhizosphere bacteria affecting the colonization of strawberry roots by arbuscular mycorrhizal fungi

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ABSTRACT

With the development of organic farming, new commercial bio-stimulators containing beneficial microorganisms (bacteria, mycorrhizal fungi) have appeared on the market. Such formulations often contain only one type of microorganisms, and thus their effectiveness is largely dependent on the environmental conditions. Bio-stimulators containing many types of microorganisms with different environmental requirements and several lines of action will be more effective and less dependent on external factors.

The aim of this study was to assess the impact of ten bacterial strains on the colonization of strawberry roots (cultivar 'Elsanta') by arbuscular mycorrhizal fungi. The strains used in this study have useful properties such as the ability to dissolve phosphorus compounds, synthesize siderophores and auxins, and have antagonistic action against the pathogenic fungus Verticillium dhaliae. The strawberry plants were grown under greenhouse conditions for four months. The impact of the bacterial strains was assessed by measuring the degree of mycorrhizal frequency [% F], relative mycorrhizal intensity [% M], and the abundance of arbuscules [% A]. The results will help to develop new bio-fertilizers containing bacterial or bacterial-mycorrhizal consortia enhancing plant growth and yielding.

Persistent Identifier: urn:nbn:de:0294-sp-2013-ppphe-2

In: Schneider C, Leifert C, Feldmann F (Eds), Endophytes for plant protection: the state of the art. © 2013 Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-11-2.

5-37 The disease-resistant and growth-promoting effect of a PGPR strain coordinated with AMF

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ABSTRACT

The antagonistic bacterium strain S-13, which was isolated from field was planted for years, could resistant pathogens of Fusarium Wilt Tomato and was identified as Bacillus amyloliquefaciens (GU323369.1) approximate species by phylogenetic analysis of 16S rDNA was identified strains S-13 of the species. A mutant strain of S-13 was obtained which could withstand 300μg / mL rifampicin. Pot results show that irrigating group and spraying group of S-13 have better control effects for tomato wilt within 45 days compared with control which up to 100% and 93% separately; the strain S-13 could colonize in tomato rhizosphere soil, roots, stems, leaves after inoculation with the higher colonization level in soil and root; the strain S-13 was tested by DGGE analysis to be the dominant bacteria in the soil which reduce the diversity of soil bacteria in 25 days; the compost inoculum of S-13 and AMF is tested to have obvious effect on inhibiting the occurrence and spread of Fusarium Wilt and promoting growth for tomato.

Persistent Identifier: urn:nbn:de:0294-sp-2013-ppphe-2

In: Schneider C, Leifert C, Feldmann F (Eds), Endophytes for plant protection: the state of the art. © 2013 Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-11-2.

5-38 Increased tolerance to the white root rot fungus *Armillaria mellea* in mycorrhizal *Lavandula angustifolia*

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ABSTRACT

Lavandula angustifolia, native of the western Mediterranean, is the economically most important among herb crops due to its versatile use in essential oil industries. As most drought tolerant woody shrubs, lavender is highly susceptible to white root rot, a fungal disease that seriously damages agricultural production in replant soils and disrupts the establishment of plants in revegetation sites for landscape purposes.

The response of mycorrhizal *L. angustifolia* to a strongly virulent *Armillaria mellea* isolate from an orchard in southern Catalonia has been evaluated under controlled conditions. Plants inoculated with *Rhizophagus irregularis* (former Glomus intraradices BEG 72) achieved higher biomass and higher number of flowers despite the pathogen's infection in the root system, and mycorrhizal colonization caused a decrease in *A. mellea* disease symptoms and plant mortality.

Persistent Identifier: urn:nbn:de:0294-sp-2013-ppphe-2

In: Schneider C, Leifert C, Feldmann F (Eds), Endophytes for plant protection: the state of the art. © 2013 Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-11-2.

5-39 Field response of three Mediterranean fruit tree species to mycorrhizal inoculation

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ABSTRACT

Replant syndrome is a major challenge for agricultural systems in the Mediterranean area. The survival and growth performance in the field of three fruit crops of increasing economic importance, namely fig tree (*Ficus carica*), carob tree (*Ceratonia siliqua*) and pomegranate (*Punica granatum*) were monitored after inoculation with two arbuscular mycorrhizal fungi isolates: *Rhizophagus irregularis* and *Glomus mosseae*. The experiments were conducted in two different sites: one is located in a botanical garden on the coast line while the second one is a former replant vineyard infested with the soilborne pathogenic fungus *Armillaria mellea*. Both mycorrhizal isolates were equally effective at increasing growth in the noninfested area, where survival rates were high in all treatments. In the replant soil, fruit tree species had different responses to mycorrhizal inoculation. There were no significant differences in pomegranate growth and survival while a thirty percent growth increase was obtained in mycorrhizal fig tree plants. Mycorrhizal colonization in carob trees promoted a fourfold increase both in plant height and in survival.

Persistent Identifier: urn:nbn:de:0294-sp-2013-ppphe-2

Feldmann F, Hommes M, Endophytes for Plant Protection: the Registration Process at a Glance. In: Schneider C, Leifert C, Feldmann F (Eds), Endophytes for plant protection: the state of the art, pp. 214-222. © 2013 Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-11-2.

5-40 Endophytes for plant protection: the registration process at a glance

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INTRODUCTION

Micro-organisms are widely used as plant protection agents in plant production. Recently, plant protection products are registered in several European countries, in USA or Canada containing strains of e. g. *Coniothyrium minitans* against *Sclerotinia* spp., *Pseudomonas chlororaphis* against *Fusarium* spp., *Ampelomyces quisqualis* against *Erysiphe cichoracearum*, *Bacillus thuringiensis* subspecies *kurstaki* against e. g. *Ostrinia nubilalis, Bacillus thuringiensis* subspecies *aizawai* against Noctuidae, *Bacillus thuringiensis* subspecies *tenebrionis* against *Leptinotarsa decemlineata* or *Cydia pomonella Granulo-Virus* (CpGV) against *Cydia pomonella*.

'Micro-organisms' means any microbiological entity, including lower fungi and viruses, cellular or non-cellular, capable of replication or of transferring genetic material (Reg. EU 1107/2009).

Following a common definition, endophytes are „Microbes that colonize living, internal tissues of plants without causing any immediate, apparent negative effects". Endophytes on one hand induce secondary metabolites in plants or growing media which can be extracted for several purposes. On the other hand these substances may interfere with potentially pathogenic organisms of the produced plant and may, therefore, cause enhanced tolerance of plant, increased resistance or changed quality of products. Endophytic fungi are, therefore, an important group of potential plant protection agents and have to undergo the same registration process if their use is intended and if they fell under the same EU legislation like the already registered organisms cited above.

There are several theoretical modes of endophyte action related to plant pathogens:

a) direct hyperparasitism of target organisms
b) induced resistance in host plants against target organisms
c) production of own secondary substances toxic to attacking pathogens
d) competition with target organisms on resources

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e) influence on secondary substances of plants later used as extracts against target organisms
f) influence to fertilization status of target organisms

It is the mode of action and the intention of use of endophytes which decides about the classification of the micro-organisms and the related registration requirements and processes.

Cases a), b) and c) are clearly covered by the definition of plant protection products (PPP). Case d) may be a matter of specific discussions: If the protective character due to the mode of action is clearly demonstrated by experimental trials it could be called PPP. But if the micro-organisms, e.g. yeasts, are normally used as foodstuff but would have some protective effect they might be classified as "basic substance". In case e) the endophyte would enhance some secondary substances later extracted as so called "botanicals". The endophyte in that moment would not have to be registered but the extract. Finally, in case f) fertilization effects do not require registration of those micro-organisms as plant protection agents. For instance, mycorrhizal fungi remain soil improvers or biofertilizers if they are not intended to be used as plant protection agents.

The intention of this paper is to summarize roughly the most important documents which are relevant for the registration process of endophytes if they have been classified or are intended to be used as plant protection agents.

RELEVANT EU-REGULATIONS AND HARMONIZED DOCUMENTS

It is the Regulation (EC) 1107/2009 which sets in article 2 the definition of PPP: Products, in the form in which they are supplied to the user, consisting of or containing active substances, safeners or synergists, and intended for one of the following uses:

a) protecting plants or plant products against all harmful organisms or preventing the action of such organisms, unless the main purpose of these products is considered to be for reasons of hygiene rather than for the protection of plants or plant products;
b) influencing the life processes of plants, such as substances influencing their growth, other than as a nutrient;
c) preserving plant products, in so far as such substances or products are not subject to special Community provisions on preservatives;
d) destroying undesired plants or parts of plants, except algae unless the products are applied on soil or water to protect plants;
e) checking or preventing undesired growth of plants, except algae unless the products are applied on soil or water to protect plants.

These products are referred to as 'plant protection products'.

Regulation (EC) 1107/2009 determines that "Substances [here: micro-organisms including virus] should only be included in plant protection products where it has been demonstrated that they present a clear benefit for plant production and they are not expected to have any harmful effect on human or animal health or any unacceptable effects on the environment" (Recital 10).

Because "incentives should be given for the placing on the market of low-risk plant protection products" (Recital 17) alternatives for chemical-synthetic PPP are under development and applications for registration are carried out. Specifically, micro-organisms often are allowed to be used in organic agriculture as natural pesticides.

Pesticides, whether chemical-synthetic or natural, can be harmful not only to the target organism but as well to the operator, the by-stander, the consumer, and the environment. To minimize the risk the active substances of plant protection products are assessed in the EU by a community procedure in a first step; Plant protection products containing such active substances are then assessed on a community level and are authorized on a national level. They may only be distributed and used if they have been authorized. The authorization of commercial products is a matter of the individual Member States. Important aims of authorization are a sufficient protection of crops, avoiding harmful effects on human health and avoiding unacceptable effects on the environment[1].

During the authorization procedure the competent authority of each member state works together with assessment authorities or units. As an example, in Germany the Julius Kühn-Institut (JKI) evaluates efficacy, crop tolerance, resistance and practical applications. The Federal Institute for Risk Assessment (BfR) assesses the impact on human and animal health. The Federal Environment Agency (UBA) assesses the product with regard to possible effects on the environment. The Federal Office of Consumer Protection and Food Safety (BVL), the designated authority, assesses the composition and the physico-chemical characteristics of the product in question[2] and is responsible for the risk management.

European regulations (e.g. Regulation (EC) 1107/2009; Regulation (EC) 544/2011 and 545/2011), directives (e.g. Directive 2009/128/EC) and technical guidelines[3] stipulate how these assessments are performed.

A comprehensive dossier including data and tests is part of the authorization application (active substances and plant protection products). Documentation on physical and chemical characteristics, analytics and efficacy, toxicology, residue behavior and environmental behavior is required. The studies must be performed according to specified standards and by certified testing facilities. The requirements for micro-organisms are to some extent different from those for chemical substances due to different production processes and properties.

After approval the list of active substances is contained in Commission Implementing Regulation (EU) No 540/2011. The first inclusion is for a maximum period of 10 years but is renewable, and can be subject to conditions and reviewed at any time.

Member States can only authorise the marketing and use of plant protection products after an active substance (here the micro-organism) has been added to the list of approved active substances.

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[1] http://www.bvl.bund.de

[2] http://www.bvl.bund.de/EN/04_PlantProtectionProducts/01_AuthorizationReviewActSub/PlantProtectionProducts_authorizationReviewActSub_node.html

[3] http://ec.europa.eu/food/plant/protection/resources/publications_en.htm

Persistent Identifier: urn:nbn:de:0294-sp-2013-ppphe-2

The Regulation (EU) No 1107/2009 and its Implementing Regulations set out common rules and guidance on data requirements; data evaluation; risk assessment; the protection of commercial information (data protection); and public access to information on pesticides.

The submitted dossier is assessed by a rapporteur member State on behalf of the EU to determine the intrinsic hazard of the active substance, safeners and synergists, and the risks of their use in the plant protection product. The rapporteur's draft assessment report is peer reviewed by the European Food Safety Authority, which provides its conclusion to the Commission and may also suggest options for risk management. In light of this conclusion, a proposal for approval or non-approval is made by the European Commission which is subject to a vote by all Member States in the Standing Committee on the Food Chain and Animal Health before adoption. Existing active substances subject to a non-approval decision must be withdrawn from the EU market.

Commission Regulation (EU) No 544/2011 provides a comprehensive list of the tests and studies required to support an active substance for Approval. Part A deals with requirements for chemical substances and Part B for micro-organisms including viruses.

Commission Regulation (EU) No 545/2011 provides a comprehensive list of the tests and studies required for the formulated plant protection product. As before, Part A of the Annex deals with requirements for chemical preparations and Part B for preparations of micro-organisms including viruses.

Uniform Principles for evaluation and authorisation of plant protection products (Commission Regulation (EU) No 546/2011) are the harmonised criteria for evaluating plant protection products at a national level. Application of the Uniform Principles ensures that authorisations issued in all Member States are assessed to the same standards. Part I deals with the evaluation and authorisation of chemical plant protection products, and Part II deals with the evaluation of plant protection products containing micro-organisms.

Furthermore, labelling requirements for plant protection products (Commission Regulation (EU) No 547/2011) are covered by the 'Dangerous Substances Directive' and the 'Dangerous Preparations Directive', which are gradually being replaced by the EU Regulation on Classification, Labelling and Packaging of Substances and Mixtures (known as the CLP Regulation). However, Commission Regulation (EU) No 547/2011 provides additional specific phrases for special risks and safety precautions for plant protection products.

Furtherly, Part B of the Commission Regulations (EU) No 544/2011 and 545/2011 allow the Commission, to adopt or amend technical and other guidance documents such as explanatory notes or guidance documents on the content of the application concerning micro-organisms, pheromones and biological products, for the implementation of the Regulation. The Commission may ask the Authority to prepare or to contribute to such guidance documents.

Such a Guidance Document is e.g. SANCO/11470/2012 "GD on botanicals used in PPP". Plant and animal oils and extracts differ from synthesized chemicals in their origin. Synthesized chemicals are based on chemical reactions whereas oils and extracts are

manufactured by physically processing material of biological origin. The significant difference between oils/extracts and synthetic chemicals is the composition or specification. The composition of an extract / oil, however depends on the material of biological origin, the manufacturing process(es) and may depend on further processing. Therefore extracts /oils have a larger variation in the qualitative and quantitative composition than synthetic chemicals. The production of ingredients in the (living) biological origin depends on the climatic conditions, e.g. time of sunshine, rain, soil etc. and differs each year. Therefore the concentrations and kinds of the ingredients are varying naturally and affecting the quantitative and qualitative composition of the extract/oil produced from the biological material. With respect to the mode of action of endophytes cited above it may be interesting to check whether the induction of “botanicals” may be an interesting use of those micro-organisms.

Another guidance document potentially relevant here is SANCO/10363/2012 “GD Guidance on the procedure for application of basic substances to be approved in compliance with Article 23 of Regulation 1107/2009”. Regulation 1107/2009 introduces the new category of "basic substances" which are described in recital as "active substances, not predominantly used as plant protection products but which may be of value for plant protection and for which the economic interest of applying for approval may be limited". It is not predominantly used for plant protection purposes but nevertheless is useful in plant protection either directly or in a product consisting of the substance and a simple diluent; and is not placed on the market as a plant protection product.

RISK ASSESSMENT DURING THE REGISTRATION PROCESS OF PLANT PRODUCTION PRODUCTS BASED ON MICRO-ORGANISMS

Part B MICRO-ORGANISMS INCLUDING VIRUSES of the Commission Regulations (EU) No 544/2011 and 545/2011 provide detailed information on data required for the registration of micro-organisms as active substances and as plant production products. A wide range of matters is covered including e. g.

- the identity of the micro-organism and the plant protection product based on that micro-organism;
- biological properties of the micro-organims and the physical, chemical and technical properties of the plant protection product;
- function, efficacy on target pests and data on application;
- analytical methods
- effects on human health, residues and fate and behaviour in the environment and
- effects on non-target organisms

All EU Member States apply the same evaluation procedures and authorization criteria. A joint positive list of active substances permitted in plant protection products is also part of

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the harmonization process. Active substances are evaluated in a joint procedure and a decision on their inclusion in the positive list is made.

Besides plant protection products, recent EU legislation regulates the authorization of safeners, synergists and adjuvants. Adjuvants are added to plant protection products, for instance, in order to improve wetting of leaf surfaces. Plant resistance improvers which are not acting directly but via the plant´s resistance reaction are authorized under Regulation 544/2011 EC as well.

Data of application and further information about the uses

Already during the assessment of active substances but as well of plant protection products several data on application have to be presented to provide information about the intended uses. The data should describe how the product should be used to minimize the risk of the operator, by-stander and consumer to come in contact with harmful substances.

The field(s) of use, existing and proposed, for preparations containing the active substance must be specified from among the following: field use, such as agriculture, horticulture, forestry and viticulture, protected crops, amenity, weed control on non-cultivated areas, home gardening, house plants, plant products storage practice or others[4].

The nature of the effects on harmful organisms must be stated: contact action, stomach action, inhalation action, fungitoxic action, fungistatic action, desiccant, reproduction inhibitor, or others must be specified. It must be stated whether or not the product is translocated in plants.

Details of the intended use must be provided. Where relevant, effects achieved, e.g. sprout suppression, retardation of ripening, reduction in stem length, enhanced fertilisation, etc. must be reported.

For each method of application and each use, the rate of application per unit (ha, m^2, m^3) treated, in terms of g or kg of both preparation and active substance, must be provided[5].

The method of application proposed must be described fully, indicating the type of equipment to be used as well as the type and volume of diluent to be used per unit of area or volume.

The maximum number of applications to be used and their timing must be reported. Where relevant the growth stages of the crop or plants to be protected and the development stages of the harmful organisms must be indicated. Where possible the interval between applications, in days, must be stated.

The duration of protection afforded both by each application and by the maximum number of applications to be used, must be indicated.

[4] The following chapter is widely cited from the relevant EU-regulations
[5] EN 11.6.2011 Official Journal of the European Union L 155/75

Where relevant, minimum waiting periods between last application and sowing or planting of succeeding crops, which are necessary to avoid phytotoxic effects on succeeding crops, must be stated. Limitations on choice of succeeding crops, if any, must be stated.

The proposed instructions for use of the preparation, to be printed on labels and leaflets, must be provided.

Packaging must be designed in accordance with the criteria and guidelines specified in the FAO 'Guidelines for the Packaging of Pesticides'.

The suitability of the packaging, including closures, in terms of its strength, leakproofness and resistance to normal transport and handling, must be determined and reported and the resistance of the packaging material to its contents must be reported in accordance with GIFAP Monograph No 17[6].

Cleaning procedures for both application equipment and protective clothing must be described in detail. The effectiveness of the cleaning procedure must be fully investigated and reported.

Where relevant pre-harvest intervals, re-entry periods or withholding periods necessary to minimise the presence of residues in or on crops, plants and plant products, or in treated areas or spaces, with a view to protecting man or livestock, must be specified, e.g.: pre-harvest interval (in days) for each relevant crop, re-entry period (in days) for livestock, to areas to be grazed, re-entry period (in hours or days) for man to crops, buildings or spaces treated, withholding period (in days) for animal feedingstuffs, waiting period (in days), between application and handling treated products, or waiting period (in days), between last application and sowing or planting succeeding crops.

Where necessary, in the light of the test results, information on any specific agricultural, plant health or environmental conditions under which the preparation may or may not be used must be provided.

The recommended methods and precautions concerning handling procedures (detailed) for the storage, at both warehouse and user level of plant protection products, for their transport and in the event of fire must be provided. Where available, information on combustion products must be provided. The risks likely to arise and the methods and procedures to minimise the hazards arising, must be specified. Procedures to preclude or minimise the generation of waste or leftovers must be provided.

This list is not complete. Other details and methods to dispose of plant protection products, packaging and contaminated materials, where proposed, must be fully described. Data must be provided for such methods, to establish their effectiveness and safety.

Efficacy

Efficacy studies play an important role during the authorization process of micro-organisms as plant protection products. The data supplied must be sufficient to permit an evaluation of

[6] http://ec.europa.eu/food/plant/protection/resources/10393_rev4_2004.pdf

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the plant protection product to be made. In particular it must be possible to evaluate the nature and extent of benefits that follow use of the preparation, where they exist in comparison to suitable reference products and damage thresholds, and to define its conditions of use [7].

Sufficient data must be generated and submitted to confirm that patterns determined hold for the regions and the range of conditions, likely to be encountered in the regions concerned, for which its use is to be recommended. Where an applicant claims that tests in one or more of the proposed regions of use are unnecessary because conditions are comparable with those in other regions where tests have been carried out, the applicant must substantiate the claim for comparability with documentary evidence.

In order to assess seasonal differences, sufficient data must be generated and submitted to confirm the performance of the plant protection product in each agronomically and climatically different region for each particular crop (or commodity)/harmful organism combination. Normally trials on effectiveness or phytotoxicity, in at least two growing seasons must be reported. These data are the basis for the formulation of the so called Good Agricultural Practice of the use (the same expression is used for the description of entire production standards; see below). They are at the same time the basis for the necessary amount of a plant protection product for a certain use at a given site.

Furthermore, the information provided for the assessment “efficacy” report should allow deciding how to use a plant protection product without creating negative effects on the processing of the target plant, and without leading to side effects on non-target organisms, including soil organisms as well as bees or beneficial organisms.

Finally, laboratory data and field information relating to the occurrence and development of resistance or cross-resistance in populations of harmful organisms to the active substance(s), or to related active substances, must be provided. Where such information is not directly relevant to the uses for which authorisation is sought or to be renewed (different species of harmful organism or different crops), it must, nevertheless be provided, as it may provide an indication of the likelihood of resistance developing in the target population. Where there is evidence or information to suggest that, in commercial use, the development of resistance is likely, evidence must be generated and submitted as to the sensitivity of the population of the harmful organism concerned to the plant protection product. In such cases a management strategy designed to minimise the likelihood of resistance or cross-resistance developing in target species must be provided.

Generally, all planning, testing and reporting has to be carried out according to guidelines of the European and Mediterranean Plant Protection Organisation (EPPO)[8].

[7] The following chapter is widely cited from Regulation 545/2011 EC

[8] http://www.eppo.int/

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FINAL REMARK

Agricultural use of endophytes is intended especially on the field of plant protection. The control of plant-pathogenic organisms is receiving increasing attention as alternative to synthetic pesticides. Research groups around the world recently develop active substances from endophytes which can be used in plant protection.

The bottleneck for practical use is the proof that the products developed from endophytes are neither harmful to human health nor to the environment, and are characterized by stable efficacy. The cited regulations give guidance through the registration process which assesses the risk of pesticide use.

A rising option for endophytes might be the classification as “Low Risk Products”. Article 22, Regulation (EC) No 1107/2009 introduces the new category of "low-risk active substances" which are described in recital 17 as active substances that present “considerably less of a risk than other substances". Specific criteria are laid down in Annex II.5 to identify a substance as low risk. It is expected that low risk substances would be preferably used in Organic Agriculture, Integrated Pest Management (IPM) programmes, home and garden uses, and in areas with public access.

Probably, possible candidate substances will in many cases be simple organic molecules, simple plant extracts, semiochemicals, microorganisms or mineral molecules. However, neither must the scope of low-risk active substances be limited to this non-exhaustive list of substance groups, nor can all substances belonging to these groups be considered as low-risk substances without further assessment. In addition, the scope of the low risk concept is not restricted to naturally occurring substances, but also includes synthetized molecules.

The classification of active substances as “basic substance” or “low risk substance” is mainly not basing on the level of possible risk, but in the ways to market the products and their economic potential.

Plant extracts seems to be the most diversified group of substances, some being harmless, others being highly toxic. They are often complex mixtures and their composition is not fully characterised.

Micro-organisms are also a complex group, for which data requirement and uniform principles might need further adaptation. The criteria surely have to be adapted to those specificities to promote the large group of endophytes adequatley.

If the endophytes pass this assessment the wide field of practical use is open.

Persistent Identifier: urn:nbn:de:0294-sp-2013-ppphe-2

Feldmann F, Host spectrum of a commercial mycorrhizal inoculum. In: Schneider C, Leifert C, Feldmann F (Eds), Endophytes for plant protection: the state of the art, pp.223-239. © 2013 Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-11-2.

5-41 Host spectrum of a commercial mycorrhizal inoculum

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BIOLOGICAL PHYTOMEDICAL FACTORS IN THE CONCEPT OF INTEGRATED PLANT PROTECTION

Integrated plant protection as the most important component of integrated plant production approaches is the most promising attempt for the transformation of conventional patterns of pesticide use to more sustainable and environmentally friendly plant protection procedures in all areas of plant production (agriculture and horticulture). The European plant protection acts define the integrated plant protection approach as "a combination of methods in which the use of chemical plant protection products is limited to the essential minimum by paying particular attention to biological, biotechnical, plant breeding and cultivation-related measures". Here, we define the underlieing science of integrated plant production as “phytomedicine”.

Biological factors are integrated as direct protectives against disease and parasites or as plant strengtheners inducing and supporting plant defense reactions or plant disease pre-disposition. Mycorrhizal fungi are acting indirectly and are, therefore, commercialized as "soil improvers", "bio-fertilizers" or "plant strengtheners". Presently, an increasing amount of examples of “best plant production practice” in horticulture (Feldmann, 2007) realise all principles of integrated plant protection e.g. that in practice biological soil improvers are increasingly considered. Since many years symbiotic mycorrhizal fungi are well known to reduce negative effects of abiotic and biotic stresses strengthening the plant health by inducing complex physiological changes (Varma & Hock 1995) in the host.

AIM OF THIS STUDY

In Germany, the first company selling AMF inoculum was founded already in 1989 but did not succeed in commercializing inoculum targeted to professional producers of horticultural products. Only in the last 15 years producers in Germany developed interesting projects which show promising results especially for urban horticultural aspects: as some examples,

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(i) a producer of high value in vitro propagated medicinal plants significantly reduced the loss of plant material using AMF and optimized the eco-balance of the plant production system (Grotkass *et al.* 2000, Schneider et al, 2008); (ii) Producers of ornamentals are able to provide their customers with plant material for balcony, garden or indoor use (Weissenhorn & Feldmann 1999; Backhaus & Feldmann 1999; Feldmann 1998) which is characterized by higher stress tolerance. Additionally, the company of such producers have a better energy-balance, because of shorter service lives and earlier selling times. (iii) On roof tops, the loss of plants was reduced and the surviving plants were more tolerant to water stress (Busch & Lelley 1997).

Due to such examples, the German production and trade with AMF inoculum increased between 1999 and 2003 to 1700%. The same could be observed world wide (Feldmann, 2003). Since 2003 estimates of the consumption of mycorrhizal inoculum has become very difficult because new strategies of marketing were introduced by the inoculum producers. Nowadays producers offer their standard inoculum to distributors which mix it with substrates, fertilizers or other carrier materials and enhance the quality of those products without explicitly advertising the microbes.

Recently, the German horticultural market dominated by hobby gardeners is discovering the advantages of AMF applications by comparing labels of substrates. Labels citing “contains living microbes” are assumed to be “biologically worthful” and are therefore tested. Especially the considerations of AMF as plant strengtheners in homegardens and organic farming systems certainly have the potential to promote future developments in the mycorrhizal sector.

Together with the new interest crucial information is required by the inoculum consumer: he wants to know whether “his” plant of interest will be colonized by a commercial inoculum. The intention of this paper was to summarize our knowledge about the mycorrhizal status of useful plants with a focus on Germany to provide it to the consumer. Therefore, names of the plants are cited in German and in Latin language.

Colonization data were collected and analysed following standard clearing and staining procedures between 1989 and 2011 on three levels: (1) trees or bushes were mainly surveyed in urban parks of Braunschweig, Germany, or in botanical gardens without inoculation. In these cases roots were collected (approximately 10-100cm) in the field. (2) annual plants were inoculated with commercial inoculum containing mainly *Glomus etunicatum, Glomus mosseae* and *Glomus intraradices* directly in home gardens or planting systems following the instructions or (3) studied in pot experiments with not-inoculated reference plants. In this latter case the data were already published elsewhere by the author.

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RESULTS OF THE SCREENING

Tab. 1: Mycorrhizal status of important vegetables (except cabbage as non-mycorrhizal plants) after inoculation with arbuscular mycorrhizal fungi (*Glomus* spp.); propagation by sawing in standard substrate EE Type 0 or vegetatively in EE Type 1; DRC: Degree of Root Colonization [%]

	DRC [%]
Gurke (*Cucumis sativus* L., Cucurbitaceae)	45
Melone (*Cucumis melo* L., Cucurbitaceae)	34
Wassermelone (*Citrullus lanatus* (Thunb.) Matsum. & Nakai., Cucurbitaceae)	51
Gartenkürbis (*Cucurbita pepo* L., Cucurbitaceae)	76
Zucchini (*Cucurbita pepo* ssp. *pepo* convar. *giromontiina* L., Cucurbitaceae)	56
Riesen- und Hokkaidokürbis (*Cucurbita maxima* L., Cucurbitaceae)	43
Moschuskürbis (*Cucurbita moschata*, L.; Cucurbitaceae)	37
Avocado (*Persea americana MILL.*, Lauraceae)	38
Zuckermais (*Zea mays convar. saccharata* L., Poaceae)	57
Tomate (*Lycopersicon esculentum* L., Solanaceae)	89
Aubergine (*Solanum melongena* L., Solanceae)	78
Paprika (*Capsicum annuum* L., Solanaceae)	55
Knollenfenchel (*Foeniculum vulgare* Mill., Apiaceae)	55
Stangensellerie (*Apium graveolens* L. *var. dulce,* Apiaceae)	45
Spargel (*Asparagus officinalis* L., Asparagaceae)	64
Bindesalat (*Lactuca sativa* L. var. *longifolia,* Asteracea)	65
Kopfsalat (*Lactuca sativa* L. var. *capitata,* Asteracea)	48
Schnittsalat (*Lactuca sativa* L. var. *crispa,* Asteracea)	39
Spargelsalat (*Lactuca sativa* L.var. *angustana,* Asteracea)	27
Chicorée (*Cichorium intybus* L. *var. foliosum*, Asteracea)	22
Endivie (*Cichorium endivia* L., Asteracea)	18
Löwenzahn (*Taraxacum officinale* F.H.Wigg., Asteraceae)	12
Brunnenkresse (*Nasturtium officinale* R.BR., Brassicaceae)	+?
Rucola, Rauke (*Eruca sativa*, Brassicaceae)	0
Gartenmelde (*Atriplex hortensis* L., Chenopodiaceae)	15
Mangold (*Beta vulgaris subsp. vulgaris*, Chenopodiaceae)	5
Spinat (*Spinacia oleracea* L*.,* Chenopodiaceae)	0
Rhabarber (*Rheum rhabarbarum* L., Polygonaceae)	+?
Portulak (*Portulaca ssp. sativa* (HAW.) ČEL., Portulacceae)	17
Winterportulak (*Claytonia perfoliata* Donn. Ex Willd., Portulacaceae)	0
Feldsalat (*Valerianella locusta* Mill., Valerianaceae)	31
Zwiebel (*Allium cepa* L., Amaryllidaceae)	67
Frühlingszwiebel, (*Allium fistulosum* L., Amaryllidaceae)	87
Knoblauch (*Allium sativum* L., Amaryllidaceae)	56
Schalotte (*Allium ascalonicum* Strand., Amaryllidaceae)	96
Porree, Lauch (*Allium porrum* L., Amaryllidaceae)	77
Perlzwiebel (*Allium porrum* L. var. sectivum, Amaryllidaceae)	45
Bärlauch (*Allium ursinum*, Amaryllidaceae)	34
Schnittlauch (*Allium schoenoprasum*, Amaryllidaceae)	100

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Table 1 continued	52
Mohrrübe (*Daucus carota* L. (Hoffm.) Schübl. et G. Martens, Apiaceae)	32
Pastinake (*Pastinaka sativa*, Apiaceae)	29
Sellerie (*Apium graveolens* L., Apiaceae)	13
Knolliger Kälberkopf (*Chaerophyllum bulbosum* L., Apiaceae)	25
Schwarzwurzel (*Scorzonera hispanica* L., Asteraceae)	82
Topinambur (*Helianthus tuberosus* L., Asteraceae)	57
Wurzelpetersilie (*Petroselinum crispum* (Mill.) Nym. & A.W.Hill, Apiaceae)	0
Meerrettich (*Armoracia rusticana* Gaert. Mey. & Scherb., Brassicaceae)	0
Radieschen (*Raphanus sativus* L. subsp. sativus, Brassicaceae)	0
Schwarzer Rettich (*Raphanus sativus* L. subsp. niger var. niger, Brassicaceae)	+?
Wasabi (*Wasabia japonica* Matsum., Brassicaceae)	75
Kartoffel (*Solanum tuberosum* L., Solanaceae)	
Feuerbohne (*Phaseolus coccineus* L.)	34
Gartenbohne, Buschbohne, Stangenbohne, (*Phaseolus vulgaris* L.)	46
Sojabohne (*Glycine max* (L.) Merill.)	42
Schalerbse (*Pisum sativum* L. convar. sativum)	34
Markerbse (*Pisum sativum* L. convar. medullare Alef. emend. C.O. Lehm)	23
Zuckererbse (*Pisum sativum* L. convar. axiphium Alef emend. C.O. Lehm)	22
Riesenerbse (*Pisum granda sneida* L. convar. sneidulo p. shneiderium)	45
Gewöhnliche Luzerne, Luzerne (*Medicago sativa* L.)	55
Linsen (*Lens culinaris* Medik.)	18
Ackerbohne, Dicke Bohne, Saubohne (*Vicia faba* L.)	56
Saat-Platterbse (*Lathyrus sativus* L.)	43
Knollen-Platterbse (*Lathyrus tuberosus* L.)	46
Augenbohne (*Vigna unguiculata* (L.) Walp.)	61
Anis (*Pimpinella anisum* L., Apiaceae)	24
Artischocke (*Cynara scolymus* L., Asteracea)	21
Baldrian (*Valeriana officinalis* L., Valerianaceae)	19
Basilikum (*Ocinum basilicum* L., Lamiceae)	56
Beifuss (*Artemisia vulgaris* L., Asteracea)	27
Beinwell (*Symphytum officinale* L., Boraginaceae)	17
Pimpinelle (*Sanguisorba minor* L., Rosaceae)	32
Bohnenkraut (*Satureja hortensis* L., Lamiaceae)	45
Borretsch (*Borago officinalis* L., Boraginaceae)	34
Breitwegerich (*Plantago major* L., Plantaginaceae)	89
Brennnessel (*Urtica dioica* L., Urticaceae)	0
Brunnenkresse (*Nasturtium officinale* R. Br., Brassicaceae)	0
Dill (*Anethum graveolens var. hortorum*, L., Apiaceae)	39
Eberraute (*Artemisia abrotanum* L., Asteracea)	32
Efeu (*Hedera helix* L., Araliaceae)	17
Eibisch (*Althaea officinalis* L., Malvaceae)	27
Estragon (*Artemisia dracunculus* L., Asteracea)	30
Fenchel (*Foeniculum vulgare* Mill., Apiaceae)	30
Fingerkraut (*Potentilla reptans* L., Rosaceae)	54
Frauenmantel (*Alchemilla vulgaris* L., Rosaceae)	44
Gänseblümchen (*Bellis perennis* L., Asteraceae)	57
Gartenkresse (*Lepidium sativum* L:, Brassicaceae)	0
Goldrute (*Solidago virgaurea* L., Asteracea)	47

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Table 1 continued

Gundermann (*Glechoma hederaceum* L., Lamiaceae)	78
Günsel (*Ajuga reptans* L., Lamiaceae)	23
Huflattich (*Tussilago farfara* L., Asteracea)	12
Kamille (*Matricaria chamomilla* L., Asteraceae)	39
Kapuzinerkresse (*Tropaeolum minus* Juss. ex DC., Tropaeolaceae)	19
Katzenminze (*Nepeta cataria* L., Lamiaceae)	25
Kerbel (*Anthriscus cerefolium* ssp. cerefolium	28
Klatschmohn (*Papaver rhoeas* L., Papaveraceae)	24
Klette (*Arctium lappa* L., Asteracea)	+?
Knoblauchsrauke (*Alliaria petiolata* (MB.) Cavara et Grande, Brassicacea)	+?
Königskerze (*Verbascum thapsus* L., Scrophulariaceae)	22
Koriander (*Coriandrum sativum* L., Apiaceae)	67
Krauseminze (*Mentha spicata* L. emend. Harley)	60
Kreuzkraut (*Senecio vulgaris* L., Asteraceae)	40
Kümmel (*Carum carvi* L., Apiaceae)	65
Lavendel (*Lavandula officinalis* Chaix et Vill., Lamiaceae)	38
Lein (*Linum usitatissimum* L., Linaceae)	67
Liebstöckel (*Levisticum officinale* Koch, Apiaceae)	31
Majoran (*Origanum majorana* L., Lamiaceae)	78
Malve (*Malva silvestris* L., Malvaceae)	31
Märzveilchen (*Viola odorata* L., Violaceae)	80
Mittlerer Wegerich (*Plantago media* L., Plantaginaceae)	90
Oregano (*Origanum vulgare* L., Lamiaceae)	75
Petersilie (*Petroselinum crispum* (Mill.) Nyman & A.W.Hill, Apiaceae)	46
Pfefferminze (*Mentha piperita* L., Lamiaceae)	39
Quendel (*Thymus serpyllum* L., Lamiaceae)	33
Rosmarin (*Rosmarinus officinalis* L., Lamiaceae)	66
Salbei (*Salvia officinalis* L., Lamiaceae)	18
Sauerampfer (*Rumex acetosa var. hortensis* L., Polygonaceae)	0
Schafgarbe (*Achillea millefolium* L., Asteracea)	36
Schlüsselblume (*Primula veris* L., Primulaceae)	65
Schöllkraut (*Chelidonium majus* L., Papaveraceae)	45
Spitzwegerich (*Plantago lanceolata* L., Plantaginaceae)	100
Thymian (*Thymus vulgaris* L., Lamiaceae)	55
Tripmadam (*Sedum reflexum* L., Crassulaceae)	10
Walderdbeere (*Fragaria vesca* L., Rosacea)	34
Waldmeister (*Galium odoratum* (L.) Scop., Rubiacea)	12
Wegrauke (*Sisymbrium officinale* (L.) Scop., Brassicaceae)	0
Wegwarte (*Cichorium intybus* L., Asteracea)	11
Weinraute (*Ruta graveolens* L., Rutaceae)	16
Wermut (*Artemisia absinthum* L., Asteraceae)	20
Ysop (*Hyssopus officinalis* L., Lamiaceae)	45
Zitronenmelisse (*Melissa officinalis* L., Lamiaceae)	38

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Tab. 2: Mycorrhizal status of ornamentals after inoculation with mycorrhizal fungi; DRC: Degree of root colonization (/ 0; * 1-33%, ** 34-66%; >66%); if not especially mentioned: AM Arbuscular Mycorrhiza, else: EM Ectomycorrhiza, EC Ericacious mycorrhiza, OM Orchidacious mycorrhiza.

German Name	***Latin Name***	**Family**	**DRC**
Acker-Schachtelhalm	*Equisetum arvense*	Equisetaceae	*
Akelei	*Aquilegia vulgaris, A.cv.*	Ranunculaceae	*
Aloe	*Aloe vera*	Aloaceae	**
Alpenveilchen	*Cyclamen persicum*	Primulaceae	**
Amaryllis	*Hippeastrum cv.*	Amaryllidaceae	*
Armenische Traubenhyazinthe	*Muscari armeniacum*	Hyacinthaceae	*
Aufrechter Schwertfarn	*Nephrolepis exaltata*	Nephrolepidaceae	**
Aukube	*Aucuba japonica*	Cornaceae	**
Australisches Gänseblümchen	*Brachyscome multifida*	Asteraceae	***
Bartlilien, Schwertlilien	*Iris cv.*	Iridaceae	*
Bartnelke	*Dianthus barbatus*	Caryophyllaceae	/
Basilikum	*Oncimum basilicum*	Lamiaceae	**
Becherprimel	*Primula obconica*	Primulaceae	**
Benjamin Feige	*Ficus benjamina*	Moraceae	**
Bergenie	*Bergenia cv.*	Saxifragaceae	*
Blaue Fächerblume	*Scaevola saligna*	Goodeniaceae	*
Blaue Lobelie / Männertreu	*Lobelia erinus*	Campanulaceae	*
Blaue Passionsblume	*Passiflora caerulea*	Passifloraceae	**
Blaue Prachtwinde, Prunkwinde	*Ipomoea tricolor*	Convolvulaceae	/
Blaue Stechfichte	*Picea pungens 'Glauca'*	Pinaceae	* EM
Blaues Lieschen	*Exacum affine*	Gentianaceae	***
Blaugummibaum, Eukalyptus	*Eucalyptus gunnii, E. globulus*	Myrtaceae	**
Blaukissen	*Aubrieta cv.*	Brassicaceae	/
Blaumäulchen,Schnappmäulchen	*Torenia fournieri*	Scrophulariaceae	*
Bogenhanf, Zimmerheld	*Sansevieria trifasciata*	Dracaenaceae	**
Braut-, Fliederprimel	*Primula malacoides*	Primulaceae	*
Brautmyrte, Gemeine Myrte	*Myrtus communis*	Myrtaceae	**
Buchsbaum; Buchs; Bux	*Buxus sempervirens*	Buxaceae	*
Buntnessel	*Plectranthus scutellarioides*	Lamiaceae	**
Cattleya	*Cattleya cv.*	Orchidaceae	* OM
Chin.Roseneibisch; Hibiskus	*Hibiscus rosa -sinensis*	Malvaceae	*
Chinaschilf, Chinagras, Silbergras	*Miscanthus sinensis*	Poaceae	*
Christrose, Nieswurz	*Helleborus niger*	Ranunculaceae	*
Christusdorn, Mäusedorn	*Euphorbia milii (var. milii)*	Euphorbiaceae	*
Cinerarie/Kreuzkraut	*Pericallis x hybrida*	Asteraceae	*
Columnea/Rachenrebe	*Columnea microphylla*	Gesneriaceae	*
Cymbidie, Kahnorchidee	*Cymbidium cv.*	Orchidaceae	* OM
Dickblatt	*Crassula ovata*	Crassulaceae	/
Dipladenie/Mandevilla	*Mandevilla sanderi*	Apocynaceae	*
Drachenbaum	*Dracaena fragrans*	Dracaenaceae	**

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Table 2 continued

Drehfrucht	*Streptocarpus cv.*	Gesneriaceae	*
Dreimasterblurne	*Tradescantia fluminensis*	Commelinaceae	/
Drillingsblume	*Bougainvillea glabra*	Nyctaginaceae	***
Duftwicke	*Lathyrus odoratus*	Fabaceae	***
Echeverie	*Echeveria elegans*	Crassulaceae	/
Echte Kapuzinerkresse	*Tropaeolum majus*	Tropaeolaceae	***
Echte Zitrone	*Citrus limon*	Rutaceae	*
Echter Jasmin, Duftjasmin	*Jasminum officinale*	Oleaceae	***
Echter Salbei	*Salvia officinalis*	Lamiaceae	**
Edel-/Land- / Nelke	*Dianthus caryophyllus*	Caryophyllaceae	/
Edelpelargonie	*Pelargonium grandiflorum*	Geraniaceae	***
Edelrose, Teehybriden	*Rosa cv.*	Rosaceae	*
Efeu	*Hedera helix*	Araliaceae	**
Efeuaralie	*x Fatshedera lizei*	Araliaceae	*
Efeupelargonie/ Hängegeranie	*Pelargonium peltatum*	Geraniaceae	*
Einjahrsphlox	*Phlox drummondii*	Polemoniaceae	*
Eisenkraut	*Verbena cv.*	Verbenaceae	**
Elatiorbegonie	*Begonia cv.*	Begoniaceae	*
Elefantenfuß	*Beaucarnea recurvata*	Dracaenaceae	*
Elfenspiegel	*Nemesia cv.*	Scrophulariaceae	**
Elfensporn	*Diascia vigilis*	Scrophulariaceae	*
Engelstrompete	*Brugmansia suaveolens*	Solanaceae	**
Erika/Glockenheide/Kapheide	*Erica gracilis*	Ericaceae	* EC
Ewige Liebe/Eisbegonie	*Begonia cucullata var. hookeri*	Begoniaceae	*
Falsche Gloxinie	*Sinningia speciosa*	Gesneriaceae	*
Federbuschcelosie	*Celosia argentea var. plumosa*	Amaranthaceae	**
Feigenbaum, Echte Feige	*Ficus carica*	Moraceae	**
Fensterblatt	*Monstera deliciosa*	Araceae	*
Fingeraralie	*Schefflera elegantissima*	Araliaceae	*
Flammenblume, Stauden-Phlox	*Phlox paniculata*	Polemoniaceae	*
Flammendes Käthchen	*Kalanchoe blossfeldiana*	Crassulaceae	/
Flammendes Schwert	*Vriesea splendens*	Bromeliaceae	*
Fleißiges Lieschen	*Impatiens walleriana*	Balsaminaceae	*
Forsythie	*Forsythia x intermedia*	Oleaceae	***
Frauenhaarfarn	*Adiantum raddianum*	Adiantaceae	**
Freesie / Kapmaiblume	*Freesia cv.*	lridaceae	**
Frühlingskrokus	*Crocus vernus*	Iridaceae	*
Fuchsie	*Fuchsia culitvars*	Onagraceae	***
Funkie, Hosta	*Hosta cv.*	Hostaceae	**
Gardenie-; Kap-Gardenie	*Gardenia jasminoides*	Rubiaceae	*
Garten-/ Gewöhnlicher Flieder	*Syringa vulgaris*	Oleaceae	***
Gartenaster / Sommeraster/	*Callistephus chinensis*	Asteraceae	***
Gartendahlie, Georgine	*Dahlia cv.*	Asteraceae.	*
Garten-Edel-Pfingstrose	*Paeonia lactiflora*	Paeoniaceae	**
Gartenfuchsschwanz, Amarant	*Amaranthus caudatus*	Amaranthaceae	**

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Table 2 continued

Löwenmäulchen	*Antirrhinum majus*	Scrophulariaceae	*
Garten-Lupine	*Lupinus polyphyllus*	Fabaceae	***
Gartenmargerite	*Leucanthemum x superbum*	Asteraceae	*
Garten-Strohblume	*Helichrysum cv.*	Asteraceae	***
Gartentulpe, Tulpe	*Tulipa cv.*	Liliaceae	**
Gefleckte Taubnessel	*Lamium maculatum*	Lamiaceae	**
Gefüllter Schneeball	*Viburnum opulus 'Roseum'*	Caprifoliaceae	**
Gelbe Osterglocke, Narzisse	*Narcissus pseudonarcissus*	Amaryllidaceae	*
Gerbera	*Gerbera jamesonii, G. cv.*	Asteraceae	***
Geweihfarn, Hirschgeweihfarn	*Platycerium bifurcatum*	Polypodiaceae	/
Gladiole	*Gladiolus cv.*	Iridaceae	*
Glanzkölbchen	*Aphelandra squarrosa*	Acanthaceae	**
Glockenblume	*Campanula isophylla*	Campanulaceae	*
Glockenrebe	*Cobaea scandens*	Cobaeaceae	/
Glücksklee, Vierblättrig	*Oxalis tetraphylla*	Oxalidaceae	/
Goldene Efeutute, Goldtute	*Epipremnum aureum*	Araceae	*
Goldgarbe, Hohe Schafgarbe	*Achillea filipendulina*	Asteraceae	*
Goldkugelkaktus	*Echinocactus grusonii*	Cactaceae	*
Goldlack, Buntlack	*Erysimum cheiri*	Brassicaceae	/
Goldtaler, Sternauge	*Asteriscus maritimus*	Asteraceae	*
Goldzweizahn	*Bidens ferulifolia*	Asteraceae	***
Greisenbart, Luftnelke, Tillandsie	*Tillandsia cyanea*	Bromeliaceae	*
Greiskraut, Silberblatt	*Senecio cineraria*	Asteraceae	*
Großblumige Chrys.	*Chrysanthemum x grandiflorum*	Asteraceae	***
Große Flamingoblume	*Anthurium andraeanum*	Araceae	*
Grünlilie/Chlorophytum/Graslilie	*Chlorophytum comosum*	Anthericaceae	*
Gummibaum	*Ficus elastica*	Moraceae	**
Gundermann, Gundelrebe	*Glechoma hederaceae*	Lamiaceae	*
Guzmanie	*Guzmania lingulata*	Bromeliaceae	*
Hahnenkammcelosie	*Celosia argentea var. cristata*	Amaranthaceae	**
Hanfpalme	*Trachycarpus fortunei*	Arecaceae	*
Hebe/Strauchveronika, Strauchehrenpreis	*Hebe x andersonii*	Scrophulariaceae	*
Heidekraut	*Calluna vulgaris*	Ericaceae	* EC
Helexine / Bubikopf	*Soleirolia soleirolii*	Urticaceae	/
Hoher Rittersporn	*Delphinium elatum*	Ranunculaceae	*
Holländische Schwertlilie	*Iris x hollandica*	Iridaceae	*
Hortensie	*Hydrangea macrophylla*	Hydrangeaceae	*
Husarenknopf	*Sanvitalia procumbens*	Asteraceae	*
Hyazinthe	*Hyacinthus orientalis*	Hyacinthaceae	*
Indica-Azalee / Zimmerazalee	*Rhododendron simsii*	Ericaceae	* EC
Indisches Blumenrohr, Canna	*Canna cv.*	Cannaceae	*
Inkalilie; Peru-Lilie	*Alstroemeria cv.*	Alstroemeriaceae	**
Islandmohn	*Papaver nudicaule*	Papaveraceae	**
Jap.Kamelie	*Camellia japonica*	Theaceae	***

Table 2 continued

Japanische Myrte	*Cuphea hyssopifolia*	Lythraceae	**
Japanischer Sago	*Cycas revoluta*	Cycadaceae	**
Judenbart, Steinbrech	*Saxifraga stolonifera*	Saxifragaceae	*
Kaiserkrone	*Frittilaria imperialis*	Liliaceae	**
Kalifornische Washingtonie	*Washingtonia filifera*	Arecaceae	*
Kalla, Zimmerkalla, Calla	*Zantedeschia aethiopica*	Araceae	*
Kanar. Dattelpalme	*Phoenix canariensis*	Arecaceae	*
Känguruklimme /-wein	*Cissus antarctica*	Vitaceae	***
Kanonierblume	*Pilea cadierei*	Urticaceae	/
Kap-Bleiwurz	*Plumbago auriculata*	Plumbaginaceae	/
Kapkörbchen	*Osteospermum ecklonis*	Asteraceae	*
Kleine Flamingoblume	*Anthurium scherzerianum*	Araceae	*
Kleines Schneeglöckchen	*Galanthus nivalis*	Amaryllidaceae	*
Klematis, Waldrebe	*Clematis cv.*	Ranunculaceae	*
Kletterfeige	*Ficus pumila*	Moraceae	**
Kletternder Baumfreund	*Philodendron hederaceum*	Araceae	*
Knollenbegonie	*Begonia cv.*	Begoniaceae	*
Kolbenfaden/Aglaoneme	*Aglaonema commutatum*	Araceae	*
Kolbenlilie	*Cordyline fruticosa*	Dracaenaceae	**
Königs-, Blattbegonie; Buntblatt	*Begonia-Rex*	Begoniaceae	*
Königs-Strelitzie	*Strelitzia reginae*	Strelitziaceae	**
Korbmaranthe, Pfauenmaranthe	*Calathea makoyana*	Marantaceae	*
Kriechender Günsel	*Ajuga reptans*	Lamiaceae	*
Küchenschelle, Kuhschelle	*Pulsatilla vulgaris*	Ranunculaceae	*
Lakritz-Strohblume / Gnaphalium	*Helichrysum petiolare*	Asteraceae	***
Lampionblume, Blasenkirsche, Judenkirsche	*Physalis alkekengi var. franchetii*	Solanaceae	**
Lavendel	*Lavandula augustifolia*	Laminaceae	**
Leberbalsam/Ageratum	*Ageratum houstonianum*	Asteraceae	*
Leier- Gummibaum / Geigenfeige	*Ficus lyrata*	Moraceae	**
Levkoje	*Matthiola incana*	Brassicaceae	/
Lilie	*Lilium cv.*	Liliaceae	**
Lorbeerbaum, Gewürzlorbeer	*Laurus nobilis*	Lauraceae	**
Madagaskar-Immergrün	*Catharanthus roseus*	Apocynaceae	*
Madagaskar-Jasmin	*Stephanotis floribunda*	Asclepiadaceae	*
Mädchenauge	*Coreopsis grandiflora*	Asteraceae	***
Maiglöckchen	*Convallaria majalis*	Convallariaceae	/
Marante, Pfeilwurz	*Maranta leuconeura*	Marantaceae	*
Maßliebchen / Gänseblümchen	*Bellis perennis*	Asteraceae	*
Medinille	*Medinilla magnifica*	Melastomataceae	*
Mehlsalbei	*Salvia farinacae*	Lamiaceae	**
Mexikanische Bergpalme	*Chamaedorea elegans*	Arecaceae	*
Mittagsgold, Gazanie	*Gazania cv.*	Asteraceae	***
Weihrauch, Harfenstrauch	*Plectranthus forsteri*	Lamiaceae	**
Neu Guinea / Edel-Lieschen,NeuGuinea-Impatiens	*Impatiens cv.*	Balsaminaceae	*

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Neukaledonische Strahlenaralie	*Schefflera arboricola*	Araliaceae	**
Niedere-Studentenblume	*Tagetes patula*	Asteraceae	*
Nordmannstanne	*Abies nordmanniana*	Pinaceae	** EM
Oleander, Lorbeerrose	*Nerium oleander*	Apocynaceae	*
Olivenbaum	*Olea europaea*	Oleaceae	***
Osterkaktus	*Rhipsalidopsis gaertneri*	Cactaceae	*
Palmlilie	*Yucca elephantipes*	Agavaceae	**
Pantoffelblume, Löwenblume	*Calceolaria integrifolia*	Scrophulariaceae	*
Petunie, Hängepetunie	*Petunie x atkinsiana*	Solanaceae	**
Poinsettie / Weihnachtsstern	*Euphorbia pulcherrima*	Euphorbiaceae	*
Portulak-Röschen	*Portulaca umbraticola*	Portulaceae	*
Pracht-/ Feuer Salbei	*Salvia splendens*	Lamiaceae	**
Prachtscharte	*Liatris spicata*	Asteraceae	*
Prärie-Glockenblume	*Eustoma grandiflorum*	Gentianaceae	***
Purpurtüte, Fußblatt	*Syngonium podophyllum*	Araceae	*
Ranunkel,Schnitt-,Topfranunkeln	*Ranunculus asiaticus*	Ranunculaceae	*
Rauer Sonnenhut	*Rudbeckia hirta*	Asteraceae	*
Riesen-Lauch, Kugellauch	*Allium giganteum*	Alliaceae	**
Ringelblume	*Calendula officinalis*	Asteraceae	***
Rispiges Gipskraut / Schleierkraut	*Gypsophila paniculata*	Caryophyllaceae	/
Rosmarin	*Rosmarinus officinalis*	Lamiaceae	**
Rotblättriger Baumfreund	*Philodendron erubescens*	Araceae	*
Rutenhirse	*Panicum virgatum*	Poaceae	*
Sal-Weide, Palmkätzchen	*Salix caprea*	Salicaceae	*
Scheidenblatt	*Spathiphyllum- wallisii*	Araceae	*
Scheinbeere, Rebhuhnbeere	*Gaultheria procumbens*	Ericaceae	* EC
Schleifenblume	*Iberis sempervirens*	Brassicaceae	/
Schmalblättriger Drachenbaum	*Dracaena reflexa var. angustifolia*	Dracaenaceae	**
Schmetterlingsorchidee, Malaienblume	*Phalaenopsis cv.*	Orchidaceae	* OM
Schmuckkörbchen, Mexiko-Aster	*Cosmos bipinnatus*	Asteraceae	***
Schmucklilie, Liebesblume	*Agapanthus africanus*	Alliaceae	**
Schneeflocke, Bacoba	*Sutera grandiflora*	Scrophulariaceae	**
Schönmalve	*Abutilon cv.*	Malvaceae	*
Schwarzäugige Susanne	*Thunbergia alata*	Acanthaceae	**
Schwarzkümmel	*Nigella damascena*	Ranunculaceae	*
Schweigrohrwurzel	*Dieffenbachia seguine*	Araceae	*
Septemberkraut	*Aster ericoides*	Asteraceae	*
Silbervase	*Aechmea fasciata*	Bromeliaceae	*
Skabiose	*Scabiosa caucasica*	Dipsacaceae	**
Skimmie	*Skimmia japonica*	Rutaceae	*
Smaragd-Peperonie	*Peperomia caperata*	Piperaceae	**
Sonnenblume	*Helianthus annuus*	Asteraceae	***
Spinnenpflanze	*Cleome hassleriana*	Capparaceae	*
Statice, Widerstoß	*Limonium sinuatum*	Plumbaginaceae	/

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Table 2 continued

Stengellose Schlüsselblume	*Primula vulgaris*	Primulaceae	**
Stiefmütterchen	*Viola x wittrockiana*	Violaceae	***
Strahlige Sinnblume	*Aeschynanthus radicans*	Gesneriaceae	*
Strand-Silberkraut / Duftsteinrich	*Lobularia maritima*	Brassicaceae	/
Strauchige Sonnenwende	*Heliotropium arborescens*	Boraginaceae	*
Strauchmargarite	*Argyranthemum frutescens*	Asteraceae	*
Streifenfarn	*Asplenium nidus*	Aspleniaceae	*
Studentenblume	*Tagetes erecta*	Asteraceae	*
Sufinia-Petunie, Hängepetunie	*Petunia x atkinsiana 'Surfinia'*	Solanaceae	**
Trichterwinde	*Convolvulus sabatius*	Convolvulaceae	/
Usambaraveilchen, Afrika-Veilchen	*Saintpaulia ionantha*	Gesneriaceae	*
Venusschuh,Frauenschuh-Orchidee	*Paphiopedilum cv.*	Orchidaceae	* OM
Vergißmeinnicht	*Myosotis sylvatica*	Boraginaceae	*
Vogelmilch, Milchstern	*Ornithogalum thyrsoides*	Hyacinthaceae	*
Wachsblume/ Porzellanblume	*Hoya lanceolata subsp. bella*	Asclepiadaceae	*
Wandelröschen, Lantane	*Lantana camara*	Verbenaceae	*
Weihnachtskaktus	*Schlumbergera x buckleyi*	Cactaceae	*
Weißer Schwan, Jasmin Nachtschatten	*Solanum jasminoides*	Solanaceae	**
Wunderstrauch/Croton	*Codiaeum variegatum*	Euphorbiaceae	*
Zauberglöckchen, Minipetunien	*Calibrachoa cv.*	Solanaceae	**
Zierdost, Oregano, Wilder Majoran	*Origanum vulgare*	Lamiaceae	**
Zierspargel	*Asparagus densiflorus*	Asparagaceae	*
Zierspargel; "Plumosus"	*Asparagus setaceus*	Asparagaceae	*
Ziertabak	*Nicotiana x sanderae*	Solanaceae	**
Zimmeraralie, Fatsia	*Fatsia japonica*	Araliaceae	**
Zimmer-Lilie / Riemenblatt Klivie	*Clivia miniata*	Amaryllidaceae	*
Zimmerrebe Rauten -Klimme/ Russ.Wein, Efeu-Wein	*Cissus rhombifolia*	Vitaceae	***
Zimmertanne; Norfolk-Tanne	*Araucaria heterophylla*	Araucariaceae	*
Zinnie	*Zinnia elegans*	Asteraceae	*
Zonalpelargonie/Stehende Geranie	*Pelargonium zonale*	Geraniaceae	**
Zylinderputzer, Schönfaden	*Callistemon citrinus*	Myrtaceae	**
Zyperngras; Papyrusgras	*Cyperus alternifolius (subsp. flatelliformis)*	Cyperaceae	*

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Tab. 3: Mycorrhizal status of fruit plants in orchards; DRC: Degree of root colonization (/ 0; * 1-33%, ** 34-66%; >66%); if not especially mentioned: AM Arbuscular Mycorrhiza, else: EM Ectomycorrhiza, EC Ericacious mycorrhiza. The sampling did not reveal representative results.

German and Latin Name	Family	DRC
Apfel (Malus domestica)	Rosaceae	**
Birne (Pyrus communis)	Rosaceae	**
Quitte (Cydonia oblonga)	Rosaceae	**
Sauer-Kirsche (Prunus cerasus)	Rosaceae	**
Süß-Kirsche (Prunus avium)	Rosaceae	**
Pflaume, Zwetsche (Prunus domestica)	Rosaceae	**
Pfirsich (Prunus persica)	Rosaceae	**
Aprikose (Prunus armeniaca)	Rosaceae	**
Erdbeere (Fragaria x ananassa)	Rosaceae	**
Himbeere (Rubus idaeus)	Rosaceae	**
Brombeere (Rubus fructicosus)	Rosaceae	*
Stachelbeere (Ribes uva-crispa)	Grossulariaceae	*
Schwarze Johannisbeere (Ribes nigrum)	Grossulariaceae	*
Rote Johannisbeere (Ribes rubrum)	Grossulariaceae	*
Heidelbeere (Vaccinium spp.)	Ericaceae	* EC
Sanddorn (Hippophae rhamnoides)	Elaeagnaceae	**
Schwarzer Holunder (Sambucus nigra)	Adoxaceae	*
Haselnuss (Corylus avellana)	Betulaceae	* EM
Walnuss (Juglans regia)	Juglandaceae	* EM
Weinrebe (Vitis vinifera)	Vitaceae	***
Schlehe (Prunus spinosa)	Rosaceae	***
Hagebutte (Rosa canina)	Rosaceae	***

Tab. 4: Mycorrhizal status of perennial plants in parks and streets (Braunschweig, after Feldmann et al. 2000); DRC: Degree of root colonization (/ 0; * 1-33%, ** 34-66%; >66%); if not especially mentioned: AM Arbuscular Mycorrhiza, else: EM Ectomycorrhiza, EC Ericacious mycorrhiza. The sampling did not reveal representative results

German Name	Latin Name	Family	DRC
Eschen-Ahorn	Acer negundo	Sapindaceae	* EM
Kugelahorn	Acer platanoides \`Globosum´	Sapindaceae	* EM
Roter Spitz-Ahorn	Acer platanoides \`Schwedleri´	Sapindaceae	* EM
Silber-Ahorn	Acer saccharinum	Sapindaceae	* EM
Roßkastanie	Aesculus hippocastanum	Sapindaceae	* EM
Rote Roßkastanie	Aesculus x carnea \`Briotii´	Sapindaceae	/
Götterbaum	Ailanthus altissima	Simaroubaceae	* AM
Schwarzerle	Alnus glutinosa	Betulaceae	* EM
Berberitze	Berberis vulgaris	Berberidaceae	* AM
Sand-Birke	Betula pendula	Betulaceae	* EM
Gemeine Hainbuche	Carpinus betulus	Betulaceae	* EM
Kornelkirsche	Cornus mas	Cornaceae	* AM
Baum-Hasel	Corylus colurna	Betulaceae	* EM

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Eingriffliger Weißdorn	Crataegus monogyna	Rosaceae	* AM
Lavalles Weißdorn	Crataegus x lavallei	Rosaceae	* AM
Pflaumenblättriger Weißdorn	Crataegus x prunifolia	Rosaceae	* AM
Orient-Buche	Fagus orientalis	Fagaceae	* EM
Rotbuche	Fagus sylvatica	Fagaceae	* EM
Blutbuche	Fagus sylvatica \`Purpurea´	Fagaceae	* EM
Gemeine Esche	Fraxinus excelsior	Oleaceae	* AM
Gingkobaum	Gingko biloba	Gingkoaceae	* AM
Walnuß	Juglans regia	Juglandaceae	* EM
Europäische Lärche	Larix decidua	Pinaceae	* EM
Kirsch-Apfel	Malus prunifolia	Rosaceae	* AM
Mammutbaum	Metasequoia glyptostroboides	Cypressaceae	* AM
Gemeine Fichte	Picea abies	Pinaceae	* EM
Schwarz-Kiefer	Pinus nigra	Pinaceae	* EM
Weymouthkiefer	Pinus strobus	Pinaceae	* EM
Gemeine Kiefer	Pinus sylvestris	Pinaceae	* EM
Gewöhliche Platane	Platanus x acerifolia	Platanaceae	* AM
Weißpappel	Populus alba	Salicaceae	* AM
Pyramidenpappel	Populus nigra \`Italica´	Salicaceae	* EM
Hybrid-Pappel	Populus x hybrida	Salicaceae	/
Gefülltblühende Vogelkirsche	Prunus avium \`Plena´	Rosaceae	* AM
Traubenkirsche	Prunus padus	Rosaceae	* AM
Yoshino-Kirsche	Prunus x yedoensis	Rosaceae	* AM
Birne	Pyrus spec.	Rosaceae	* AM
Stiel-Eiche	Quercus robur	Fagaceae	* EM
Pyramideneiche	Quercus robur \`Fastigiata´	Fagaceae	/
Amerikanische Roteiche	Quercus rubra	Fagaceae	* EM
Immergrüne Eiche	Quercus X turneri \`Pseudoturneri´	Fagaceae	* EM
Kegel-Robinie	Robinia pseudoacacia \`Bessoniana´	Fabaceae	* AM
Einblättrige Robinie	Robinia pseudoacacia \`Monophylla´	Facaceae	* AM
Chinesische Hängeweide	Salix baylonica	Salicaceae	* AM
Gemeine Eberesche	Sorbus aucuparia	Rosaceae	* AM
Schwedische Mehlbeere	Sorbus intermedia	Rosaceae	* AM
Gemeine Eibe	Taxus baccata	Taxaceae	* EM
Winter Linde	Tilia cordata	Malvaceae	* EM
Winter-Linde	Tilia cordata \`Greenspire´	Malvaceae	/
Sommer-Linde	Tilia platyphyllos	Malvaceae	* EM
Silber-Linde	Tilia tomentosa	Malvaceae	* EM
Krim-Linde	Tilia x euchlora	Malvaceae	* EM

Persistent Identifier: urn:nbn:de:0294-sp-2013-ppphe-2

DISCUSSION

Substrates for roof tops and all substrates used for the production of vegetables, ornamentals or other seedlings and cuttings – whether professionally used or by hobby gardeners - normally are free from mycorrhizal fungi. It can be assumed that under conditions of horticultural plant production there is a latent deficiency in symbioses with the potential consequence of higher stress susceptibility of facultative or obligate mycorrhiza dependent host plants.

The inoculation of a huge amount of plant species like shown in this paper resulted in colonization of roots in spite of using a standard commercial inoculum with only three different AMF. In the most of the cases annual plants are endomycorrhizal, while perennial plant species are ectendo- or ectomycorrhizal or belonging to further special types of mycorrhiza. It has to be pointed out, that a consumer has to check which mycorrhiza-types the inoculum he is intending to use contains. Ectomycorrhizal inoculum does not work on endomycorrhizal hosts and so on, because the symbiotic fungi are completely different.

Some plant families like Brassicacea or Caryophyllaceae are known as non-mycorrhizal and where found in our studies non-mycorrhizal or slightly mycorrhizal, too.

We already knew that the diversity and effectiveness of naturally occurring mycorrhizal fungi in horticultural systems are low and that we have to design the inoculum to reach maximum effectiveness (Feldmann & Schneider, 2008). In urban areas, one of the most important factors was that isolation between green areas, gardens or parks and long-term conservation of artificial, man-made plant sociological formations what leads to AMF communities patchy distributed, of low diversity and low effectiveness (Feldmann 1997). Here, in the majority of the cases we found roots from urban areas or orchards mycorrhized but did not collect detailed information about taxonomy or effectiveness. But because of the fact that most of the useful plants in orchards and urban areas are mycorrhizal hosts, makes mycorrhizal management in production systems, urban parks or roadsides senseful and, of course, is already carried out (Feldmann *et al.* 2008).

Agricultural and horticultural practice, especially measures of integrated plant protection, are influencing mycorrhizal fungi, once inoculated to target plants, by favouring the fungal activity, but also by handicapping the development of the symbiosis. Plant management has some impact to mycorrhizal communities and their effectiveness: even in organic horticulture inadaquate rotation systems can lead to loss of effective AMF (Bethlenfalvay and Linderman 1992).

At horticultural sites which are often replanted by different plant species or varieties the planting sequence may negatively influence the survival of inoculated AMF if e.g. non-host like *Cruciferae* are planted for more than one year. Furthermore, permanent cultivation of a single host genotype may reduce the mycorrhizal effectiveness which can be avoided by co-cultivation with other mycorrhizal plant species (Feldmann & Boyle 1999). The low colonization specifity of AMF can be used for inoculation purpose if e.g. old trees have to be

inoculated. Co-cultivation of such trees with "donor plants" will result in higher densities of AMF structures in the soil and in a subsequent colonization of the target tree with the fungal symbiont. Whether AMF can be inoculated once and remain permanently effective in cases of such trees is not known for urban conditions in Germany. From the tropics we know, that the effectiveness of tree symbionts may be low if the tree is growing free from other hosts (Feldmann & Lieberei 1994).

Plant breeding severely influences the success of inoculations. There are differences between cultivars of one species which show a genotype specific expression of fungal effectiveness and plant responsiveness (Feldmann & Boyle 1998) to inoculations. Mostly, on the cultivar level the colonization behaviour but not the effectiveness of AMF is predictable (Feldmann *et al.* 2009).

High rates of mineral fertilizers often impede the colonization of target plants (Abbott & Robson 1984); more frequently given smaller doses are recommended in certain cases. Organic material, applicated as mulch increases the quantity of mycorrhization. Certainly, the type and quantity of fertilizers are selecting AMF strains and changing AMF communities. Disturbance of soil and tillage may select special AMF genotypes and has to be taken into account where sites are often replanted (Bethlenfalvay & Linderman 1992; Johnson & Pfleger 1992).

There are certain pathogenic antagonists, *Trichoderma* spp., *Gliocladium* spp., *Pseudomonas* spp., *Bacillus* spp. and PGPR which co-operate with mycorrhizal fungi in bio control of pathogens (Linderman 1992). It seems that the phytosanitary role of mycorrhizal fungi can be made even more effective when they are combined with other plant protection measures. Internet investigations looking for commercial products mixed with AMF show that the fungi are combined with humic acids, *Trichoderma* spp, bio stimulants, beneficial bacteria, soluble sea kelp, yucca plant extracts, amino acids, and vitamins to promote rapid and healthy root development. To reduce transplant stress and watering maintenance, and to slow release all soluble components of the formulation, water management gel is sometimes added to complete the packages.

Pesticide use often is shown to negatively influence mycorrhiza formation (Johnson & Pfleger 1992) Nevertheless, in own tests with active substances of plant protection products we never found a complete destruction of mycorrhiza in test plants. Especially already established mycorrhiza is not impeded or even favoured by use of these plant protection products (Feldmann, 2003).

Overall, it is obvious that the mycorrhizal technology easily may find its place in complex management protocols like formulated in integrated plant protection concepts. The pre-requisite, the knowledge about the mycorrhization potential of useful plants provided in this article gives basic input.

Persistent Identifier: urn:nbn:de:0294-sp-2013-ppphe-2

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Persistent Identifier: urn:nbn:de:0294-sp-2013-ppphe-2

In: Schneider C, Leifert C, Feldmann F (Eds), Endophytes for plant protection: the state of the art. © 2013 Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-11-2.

5-42 Testing the potential of three entomopathogenic fungi as endophytes for the biological control of the large pine weevil

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ABSTRACT

The large pine weevil, *Hylobius abietis* (Coleoptera : Curculionidae) is a major pest of coniferous trees reforestation in Europe. The impact of this pest can lead to 100% mortality in newly-planted seedlings. The juvenile stages are found inside stumps where they feed and develop. The emerged adults on the other hand feed on the bark and soft tissues of newly planted trees.

The use of entomopathogenic nematodes and entomopathogenic fungi against the developing stages is being investigated by our lab as a way to control this insect ecologically. Two entomopathogenic fungi from commercial sources have been tested in clearfelled sites : *Beauveria bassiana* and *Metarrhizium anisopliae*. During field trials, a third fungal species has been found around and inside coniferous stumps acting as an entomopathogen against *H. abietis*: *Beauveria caledonica*.

As the damage caused by this insect is to young trees, direct protection of these is desirable. Inoculating coniferous seeds of Sitka spruce (*Picea sitchensis*) and lodgepole pine (*Pinus contorta*), the two major species of coniferous trees in Ireland, with these entomopathogenic fungi as endophytes could lead to a a durable protection of these plants.

Sterilized spruce and pine seeds were treated with each of the three entomopathogenic fungi in various ways to maximise the chance of a direct contact between the seed and fungal material. The seeds were then sowed in peat soil and kept for at least two months before being assesed for endophytes. First results are shown in this poster.

Persistent Identifier: urn:nbn:de:0294-sp-2013-ppphe-2

Elsayed T, Grosch R, Smalla K, The effect of soil type on the abundance and diversity of plant associated rhizospheric and endophytic bacteria with antagonistic activity towards *Ralstonia solanacearum*. In: Schneider C, Leifert C, Feldmann F (Eds), Endophytes for plant protection: the state of the art, pp. 241-242. © 2013 Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-11-2.

5-43 The effect of soil type on the abundance and diversity of plant associated rhizospheric and endophytic bacteria with antagonistic activity towards *Ralstonia solanacearum*

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ABSTRACT

Ralstonia solanacearum is an epidemic phytopathogenic bacterium responsible for bacterial wilt in potato and many other plant species. This pathogen can survive in the soil for several years and can spread very fast via water streams and latent infections in the tubers are particularly problematic. Endophytic biocontrol agents might be a solution as they occupy the same niches as colonized by *R. solanacearum*. The aim of this study was to assess the diversity of bacterial antagonists of *Ralstonia solanacearum* in different soil types and potato plant spheres, and to estimate the effect of soil type on the abundance and diversity of antagonistic bacteria. The total bacterial communities from bulk soil, rhizosphere soil and from the endophytic compartments were compared by PCR-DGGE fingerprints of 16S rRNA genes amplified from total community DNA. Approximately 2000 bacterial isolates obtained from different spheres of potato plants grown in three different soils (Diluvial sand, Alluvial loam and Loess loam) were screened for in vitro antagonistic activity towards *R. solanacearum* and 200 isolates with antagonistic activity were further characterized. Using BOX-PCR fingerprints, the 200 antagonistic bacteria were compared. The biological control mode of action and the plant growth promoting potentials were investigated as well. The BOX-PCR fingerprint as well as the 16S rRNA sequences showed that some endophytic antagonistic isolates identified as *Pseudomonas umsongensis* colonized all potato plant ecto- and endo-spheres. The total bacterial communities denaturing gradient gel electrophoresis (DGGE) profile revealed distinct bacterial community structure associated with each plant

sphere, and within each plant sphere the microbial community composition was mainly shaped by the soil type. The effect of soil type decreased in the endophytic compartments, suggesting that this endophytic compartment harbored unique indigenous bacterial communities protected from surrounding environment even so some of the endophytic antagonistic bacteria isolated from different soil types shared the same BOX-PCR fingerprints. Currently we investigate under greenhouse conditions whether endophytic bacteria can more effectively antagonize *R. solanacearum* than rhizosphere colonizing bacteria.

Persistent Identifier: urn:nbn:de:0294-sp-2013-ppphe-2

SESSION 6

Dark Septate Endophyte-Workshop

6-1 The DSE-complex: An introduction

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Persistent Identifier: urn:nbn:de:0294-sp-2013-ppphe-2

Jumpponen A, Mandyam KG, Adoption and utility of an Arabidopsis model to dissect endophyte symbioses. In: Schneider C, Leifert C, Feldmann F (Eds), Endophytes for plant protection: the state of the art, pp.244-247. © 2013 Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-11-2.

6-2 Adoption and utility of an Arabidopsis model to dissect endophyte symbioses

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ABSTRACT

Despite the abundance of root-associated endophytic fungi, their function has remained elusive. During experiments to determine the host ranges of common endophytes isolated from a tallgrass prairie, at least two taxa (*Microdocchium* and *Periconia*) were observed to form functional mutualisms with *Arabidopsis thaliana* and increase the host's growth. Furthermore, these endophytes reduce *A. thaliana* susceptibility to a foliar pathogen suggesting induction of systemic plant defenses. *Arabidopsis thaliana* does not associate with mycorrhizal fungi and there are only limited examples of endophyte associations in *A. thaliana*. Accordingly, we have taken advantage of this unique and fortuitous mutualism to further our understanding of the root-associated symbioses. We argue that this system is broadly applicable and the wealth of information available for *A. thaliana* will allow advances in understanding mycorrhizal and non-mycorrhizal root symbioses alike. Data from unpublished and ongoing microarray (Affymetrix ATH1) experiments indicate that endophytes lead to upregulation of carbon metabolism as well as general plant defenses against potentially pathogenic fungi. Furthermore, metabolome level experiments indicate clear shifts on metabolic profiles. Together, these data indicate that both host transcriptome and metabolome are modulated by the endophyte symbionts.

INTRODUCTION

Field collected roots are frequently colonized by dark septate endophyte (DSE) fungi (Kageyama *et al*. 2008, Mandyam & Jumpponen 2008). This colonization is characterized by intracellular melanized hyphae that occupy healthy host roots (Jumpponen & Trappe 1998, Addy *et al*. 2005). While polyphyletic and ecologically and physiologically variable (Jumpponen & Trappe 1998, Addy *et al*. 2005, Kageyama *et al.* 2008, Newsham 2011), DSE fungi colonize hosts forming similar morphologies irrespective of the species (Jumpponen & Trappe 1998, Mandyam *et al*. 2012, Yu *et al*. 2001). The consistent colonization and

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variable host responses invite seeking a model system that would permit dissection of this symbiosis.

Isolations from roots of mixed plant communities have yielded a selection of common soil fungi including *Acremonium* sp., *Aspergillus* sp., *Cladosporium* sp., *Curvularia* sp. and *Fusarium* sp. (Mandyam *et al*. 2010). However, when inoculated on a common host (*Allium porrum* L.), these fungi produced no DSE structures. In contrast, *Microdochium* and *Periconia* formed DSE structures both in *Allium porrum* and *Andropogon gerardii*. Because *Microdochium* and *Periconia* were repeatedly recovered from field-collected plants, their suspected broad host range was empirically evaluated in additional laboratory studies. Six tested prairie grasses and forbs were colonized by these endophytes as indicated by inter- and intracellular melanized hyphae, microsclerotia or chlamydospores (Mandyam *et al*. 2012). Although all tested hosts were susceptible to colonization, both the rate of colonization and the host growth responses were highly variable (Mandyam *et al*. 2012). Interestingly, microscopic analyses indicated that grasses were more heavily colonized than forbs in the laboratory and field. While speculative, these observations tempt a co-evolutionary interpretation: if monocotyledonous species are more heavily colonized than their dicotyledonous counterparts, might they also derive a greater growth, fitness, or evolutionary benefit?

While assessing fitness benefits is difficult, the emerging hypotheses were supported to a degree: although highly variable, positive responses to colonization tended to be more frequent in grasses than in forbs (Mandyam *et al*. 2012). These studies indicated that the DSE fungi likely colonize a broad range of hosts, whose responses may be evolutionarily confined. However, despite the more common positive responses among grasses, there were examples of positive responses to DSE colonization in forbs as well. While these observations point towards genotype-level interplay between fungal strains and host plants, they provide minimal information on the mechanisms underlying this symbiosis. As a starting point, selection of host-fungus pairs that behave differentially in symbiosis may prove a valuable tool to further characterize the elusive DSE symbiosis.

ARABIDOPSIS MODEL FOR ENDOPHYTE SYMBIOSES

The broad host range of the common DSE fungi motivated exploration of their ability to colonize the non-mycorrhizal model plant *Arabidopsis thaliana*. Similarly to native plants, microscopic analyses confirmed that *A. thaliana* forms DSE symbioses in the field, greenhouse, and laboratory. *Arabidopsis thaliana* growth responses to inoculations with *Periconia* and *Microdochium* strains were variable and depended on the host ecotype. Although the negative growth responses were most common in *A. thaliana,* positive responses were also observed, permitting thus selection of isolates and host accessions that perform predictably in symbiosis. In sum, *A. thaliana* has potential to serve as a model for more detailed dissection of the DSE symbiosis – especially to improve our appreciation for the positive or variable host responses.

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The well-established model systems also serve to explore the fungal modulation of host responses to pathogens. In experiments, where endophyte-inoculated *A. thaliana* plants were consecutively inoculated with a foliar pathogen (*Botrytis cinerea*), *Periconia*-inoculated plants were more resistant against the pathogen suggesting an induction of systemic resistance. While such inoculation experiments are informative, *A. thaliana* model provides a number of additional benefits including well-annotated microarrays. *Arabidopsis thaliana* transcriptomic responses to *Microdochium* colonization were evaluated using ATH1 microarrays. The *Microdochium-* and mock-inoculated *Arabidopsis* differed mainly in expression of genes that were i) defense-, ii) metabolism-, iii) cell cycle-, and iv) transcription-related. Transcription factors (TFs) elicited by chitin and with a role in defense (myb; Libault *et al*. 2007), protein kinases, respiratory oxidative burst proteins involved in stress regulation, disease resistance-responsive genes, and fungus-elicited defense genes were among the induced genes. However, defense genes downstream of salicylic or jasmonic acid signaling commonly elicited by pathogens (bacterial or fungal; de Vos *et al*. 2005) were NOT induced. The confirmed positive growth responses were most likely related to induction of auxin-responsive, cytokinin-synthesis, and cell-wall-synthesis genes, a pattern similar to that observed for *A. thaliana*-PGPR (Plant Growth Promoting Rhizobacteria) symbiosis (Wang *et al*. 2005). Many metabolism and defense genes were also suppressed, also a pattern similar to the *A. thaliana*-PGPR symbiosis. An interesting suppressed defense gene was the ethylene-responsive element binding factor (ERF), a defense signaling protein usually expressed in response to pathogen attack or abiotic stress (Fujimoto *et al*. 2000, Oñate-Sánchez & Singh 2002). Although still awaiting metabolome level complementation, the analyses of *A. thaliana* transcriptome have provided some insight into the fundamental function of the DSE symbiosis. Whilst the fungi evade the commonly induced pathogen signaling, they induce some others. Furthermore, these analyses suggested also modulation of *A. thaliana* metabolism by DSE colonization. The coarse preliminary metabolome analyses concur and indicate clear alteration of metabolic profiles of inoculated plants when compared to mock-inoculated controls, albeit the altered metabolites remain presently unidentified.

CONCLUSIONS

While temporal, intra-, and interspecific variability may prohibit general statements about DSE symbiosis, the interplay between fungal strains and host genotypes permit coupling individuals to optimize the information value that can be derived from genomic experiments. The documented ability of DSE fungi to form functional symbiosis with the model plant *A. thaliana* opens the genomic toolbox and provides the advantages of a well-developed model organism for a deeper genomic, transcriptomic and metabolomic dissection of the DSE mutualism. The preliminary Affymetrix ATH1 microarray experiments provide insights that facilitate better understanding of the fundamental mechanistics of this symbiosis. Complementing such transcriptomic analyses with metabolomics and perhaps analyses of

Persistent Identifier: urn:nbn:de:0294-sp-2013-ppphe-2

specific mutant lines likely further deepens our appreciation for the mechanisms of the DSE symbiosis.

ACKNOWLEDGEMENTS

These studies were supported by the National Science Foundation (Grants 0344838, 0221489) and the Ecological Genomics Institute at Kansas State University. Konza Prairie Biological Research Station (KPBS) maintained the field sites and was supported by National Science Foundation Long Term Ecological Research (LTER) program.

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Persistent Identifier: urn:nbn:de:0294-sp-2013-ppphe-2

In: Schneider C, Leifert C, Feldmann F (Eds), Endophytes for plant protection: the state of the art. © 2013 Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-11-2.

6-3 The dominant DSE lineages of semiarid sandy areas of the Great Hungarian Plain - what can they point out?

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ABSTRACT

Dark septate endophytes (DSE) comprise a group of root-colonizing endophytic fungi that belong to a few orders of the phylum Ascomycota. Although it seems that DSE fungi are present in all major biome types and climate regions, furthermore relatively frequent in (semi)arid and harsh environments, studies on their presence and diversity are sporadic. Our knowledge about their role and function in ecosystems is sorely limited.

In this study, to approximate our knowledge on DSE fungi in semiarid areas we examined the dominant lineages using the approach, use of dominant generalist DSE lineages is crucial to understand functional role of DSE fungi in different ecosystems. Our aims were (i) to isolate and compare the DSE fungi of invasive and indigenous plants to find dominant generalist DSE fungi, (ii) to collect more isolates belongs to dominant clades from different hosts and microhabitats and (iii) to study the genetic variability of this strains.

Root samples were collected from woody and herbaceous plants of sandy areas of the Great Hungarian Plain. ITS sequence of nrDNA extracted from the 296 isolates clustered into 41 groups. We found that 14 of these 41 groups were DSE, representing approximately 60% of the isolates. Group-specific primer pairs targeting the ITS region were designed for dominant DSE groups (Cadophora sp., Rhizopycnis vagum, Periconia macrospinosa, Pleosporales sp.) to make accurate and fast diagnosis of the isolates possible. Because of a finer-scale genetic variability analysis may show up important correlations, inter sample sequence repeat (ISSR) analysis of the isolates was used.

Targeted sampling revealed, that different dominant DSE lineages can be specific to woody or herbaceous plants with scarcely found overlap. The variability analyses revealed unexpected variability of isolates masked by the similar ITS region.
The study was supported by the Hungarian Scientific Research Fund (OTKA K72776).

Persistent Identifier: urn:nbn:de:0294-sp-2013-ppphe-2

Zhang C et al., Evidence for biotrophic lifestyle and biocontrol potential of dark septate endophyte *Harpophora oryzae* to rice blast disease. In: Schneider C, Leifert C, Feldmann F (Eds), Endophytes for plant protection: the state of the art, pp. 249-257. © 2013 Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-11-2.

6-4 Evidence for biotrophic lifestyle and biocontrol potential of dark septate endophyte *Harpophora oryzae* to rice blast disease

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ABSTRACT

The mutualism pattern of the dark septate endophyte (DSE) fungus *H. oryzae* in rice roots and its biocontrol potential in rice blast disease caused by *Magnaporthe oryzae* were investigated. eGFP-tagged *H. oryzae* was used to monitor the colonization pattern. Hyphae invaded from the epidermis to the inner cortex, but not into the root stele. Fungal colonization increased with root tissue maturation, showing no colonization in the meristematic zone, slight colonization in the elongation zone, and heavy colonization in the differentiation zone. *H. oryzae* adopted a biotrophic lifestyle in roots accompanied by programmed cell death. *H. oryzae* protected rice from *M. oryzae* root invasion and induced systemic resistance against rice blast. The colonization pattern of *H. oryzae* was consistent with the typical characteristics of DSEs. *H. oryzae* enhanced local resistance and systemic resistance against rice blast.

INTRODUCTION

Fungal endophytes, an important component of plant-associated mycobionts, have attracted a great deal of attention due to their striking species and functional diversity. DSEs are representative Class 4 endophytes characterized by melanized hyphae and microsclerotia formed in cortical cells (Rodriguez *et al.* 2009). More than 600 plant species in 114 families with a wide geographic distribution have been shown to harbor DSEs. In some cases, DSEs are predominant compared to mycorrhizal fungi, indicating that they represent an equally important root fungal community (Knapp *et al.* 2012).

However, the biological effects of DSEs on plant growth and fitness remain elusive. Our findings will enhance our understanding of the mutualistic interaction between *H. oryzae* and rice.

MATERIALS AND METHODS

Endophytic, pathogenic fungi and plant materials

An eGFP-expressing transformant Ho31gfp of endophytic *H. oryzae* strain R5-6-1 (Yuan *et al.* 2010) was used. The eGFP-tagged *M. oryzae* strain Guy11 (Dong *et al.* 2009) was used as a pathogen.

The blast-susceptible rice cultivar CO-39 (*O. sativa*) was used as a compatible host plant for inoculation experiments.

Co-culture, root staining and microscopy

Surface-sterilized rice seeds were pre-planted in 1/2 Murashige & Skoog solid medium for 5 days, and inoculated with 3-day-old Ho31gfp spores on top of the root system (10^7 conidia/tube, 3 seedlings/tube). The fungal structures were observed under an Olympus fluorescence microscope BX51 (Tokyo, Japan). The root infection process was monitored by an LSM780 laser scanning confocal microscope (Carl Zeiss Inc., Jena, Germany). eGFP was excited with 488 nm and detected at 500-530 nm. Root cell wall autofluorescence was detected at 650-700 nm.

To test the viability of infected root cells hosting fungal structures, lipophilic endocytic dye FM4-64 was used to monitor endocytosis and endosome formation (Bolte *et al.* 2004).

Pathogen infection

For leaf infection, using plants pre-cultured with R5-6-1 for 10 days at the three-leaf stage (about 15 d.a.i.), each tube was inoculated by spraying 1 mL of a Guy11 conidial suspension (1×10^5 conidia/mL) onto the leaves using an airbrush. In another experiment, roots were inoculated with 2 mL of a Guy11 conidial suspension (1×10^5 conidia/mL). The disease severity was assessed at 6 days after leaf inoculation and 8 days after root inoculation. The diseased leaf area percentage (%DLA) was recorded to permit a more accurate evaluation of the disease area and severity (Chi *et al.* 2009). The percentages of disease severity and area were determined for at least 50 plants per replicate in at least three independent experiments.

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RESULTS

Dynamic development of *H. oryzae* in roots

Root colonization was initiated from conidia, which formed dark “runner hyphae” upon germination (Fig. 1a) following the grooves between the epidermal cells on the root surface. Runner hyphae formed appressorium-like infection structures hyphopodia (Fig. 1b, arrow) on the surface and penetrated the epidermal cells via penetration pegs (Fig. 1b, arrowhead). Fungal growth was visible in the epidermal and cortical cell layers of the root. The intracellular hyphae were thick, with neck-like constrictions where they crossed the plant cell walls (Fig. 1c, arrow). The intercellular hyphae expanded parallel to the main axis of the root and occasionally branched into the intercellular spaces between cell walls (Fig. 1d). In addition to runner hyphae, darkly pigmented pearl-shaped chlamydospores formed on the root surface (Fig. 1e) germinated with septate tubes and developed swollen structures (Fig. 1f). The intracellular chlamydospores germinated and then completely filled a single cell before invading adjacent cells (Fig. 1g). During intracellular colonization, clusters of inflated, chlamydospore-like, thick-walled cells (Fig. 1h) were compacted within the cortical cells, which seemed to be the prophase of microsclerotia. When observing DSEs colonization, it is necessary to allow adequate time (>15d.a.i.) for the development of intracellular microsclerotia (Fig. 1i), which are described as compact darkly pigmented irregularly lobed thick-walled hyphae. Later, fungal hyphae excessively occupied the epidermal and cortical cells, especially at the basal parts of the root hairs, in which branching hyphae formed a large number of chlamydospores and microsclerotia (Fig. 1j). Transversely, the fungus entered the root epidermis and later invaded from the outer cortex to the inner cortex (Fig. 2a, b). No hyphae approached the central part of the roots, resulting in their absence from the aerial parts of the plants. Concomitantly, a gradual increase of fungal colonization and proliferation generally associated with root maturation was observed (Fig. 2c, f). The root cap was slightly encompassed by hyphae, the root tip meristem zone showed no colonization, and the elongation zone showed mainly epidermal colonization with a few hyphae (Fig. 2e). In contrast, the differentiation zone was heavily occupied by intercellular and intracellular hyphae (Fig. 2d).

Biotrophic lifestyle of H. oryzae accompanied by host cell death

We investigated root cell vitality by FM4-64 staining. During early colonization (≤10 d.a.i.), *H. oryzae* hyphae penetrated and occupied root cells, in which internalization was observed in endomembrane structures (Fig. 3a, arrowheads) that formed in a similar fashion in non-invaded cells (Fig. 3a, arrows), indicating that fungal colonization did not affect cellular membrane dynamics or viability. However, in the late colonization stage (≥15 d.a.i.), in infected cells filled with plentiful hyphae and microsclerotia, endocytosis disappeared as

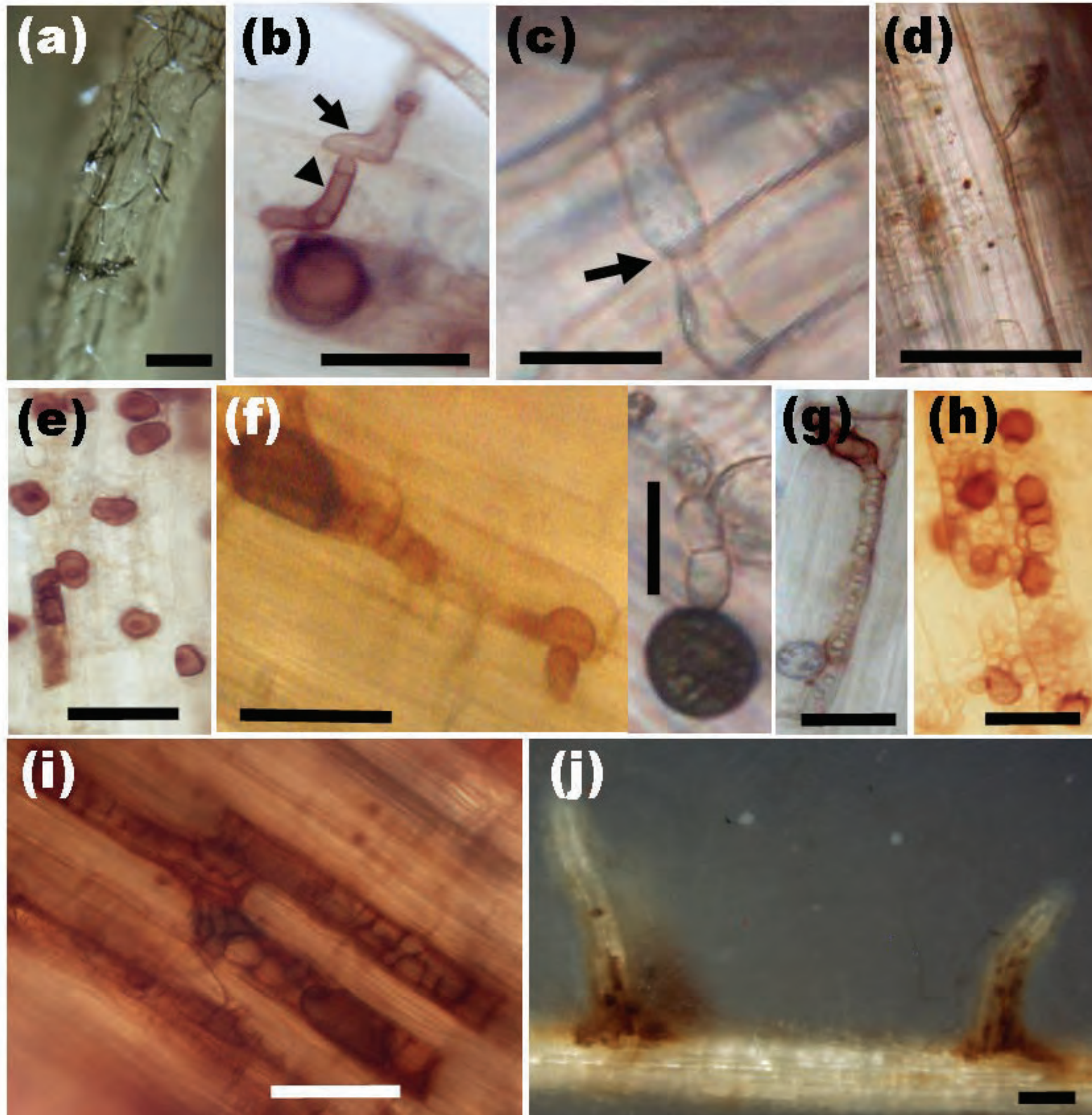

Figure 1 Fungal structures of *H. oryzae* during root infestation. (a) Dark runner hyphae enwrapping the root surface. Bar, 100 µm. (b) Light microscopy of hyphopodia (arrow) formed by runner hyphae as infection structures on the root surface and corresponding penetration peg (arrowhead). Bars, 20 µm. (c) Intracellular hyphae in cortical cells forming narrow neck-like constriction (arrow) where they cross the cell wall. Bar, 20 µm. (d) Intercellular hyphae expanding and branching occasionally in the cortical layer. Bar, 50 µm. (e) Darkly pigmented and thick-walled chlamydospores on the root surface. Bar, 20 µm. (f) Upon germination, chlamydospores producing septate germ tubes and appressorium-like bulges with corresponding infection pegs. Bars, 10 µm. (g) Germination of intracellular chlamydospores within epidermal cells. Bar, 20 µm. (h) Clusters of inflated, rounded, thick-walled cells compacted in the cortical cells before the formation of microsclerotia. Bar, 20 µm. (i) Light microscopy of darkly pigmented, irregularly lobed, thick-walled intracellular microsclerotia in the cortical cells. Bars, 50 µm. (j) Abundant hyphae and chlamydospores assembled at the basal parts of root hair cells. Bars, 500 µm.

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well as endosome formation (Fig. 3b, arrowhead), indicating the occurrence of host cell death. Whereas the adjacent root cells remained alive, as proved by the endosome formation (Fig. 3b, arrow). Therefore, *H. oryzae* adopted an intimate biotrophic relationship with the root cells accompanied by partial host cell death.

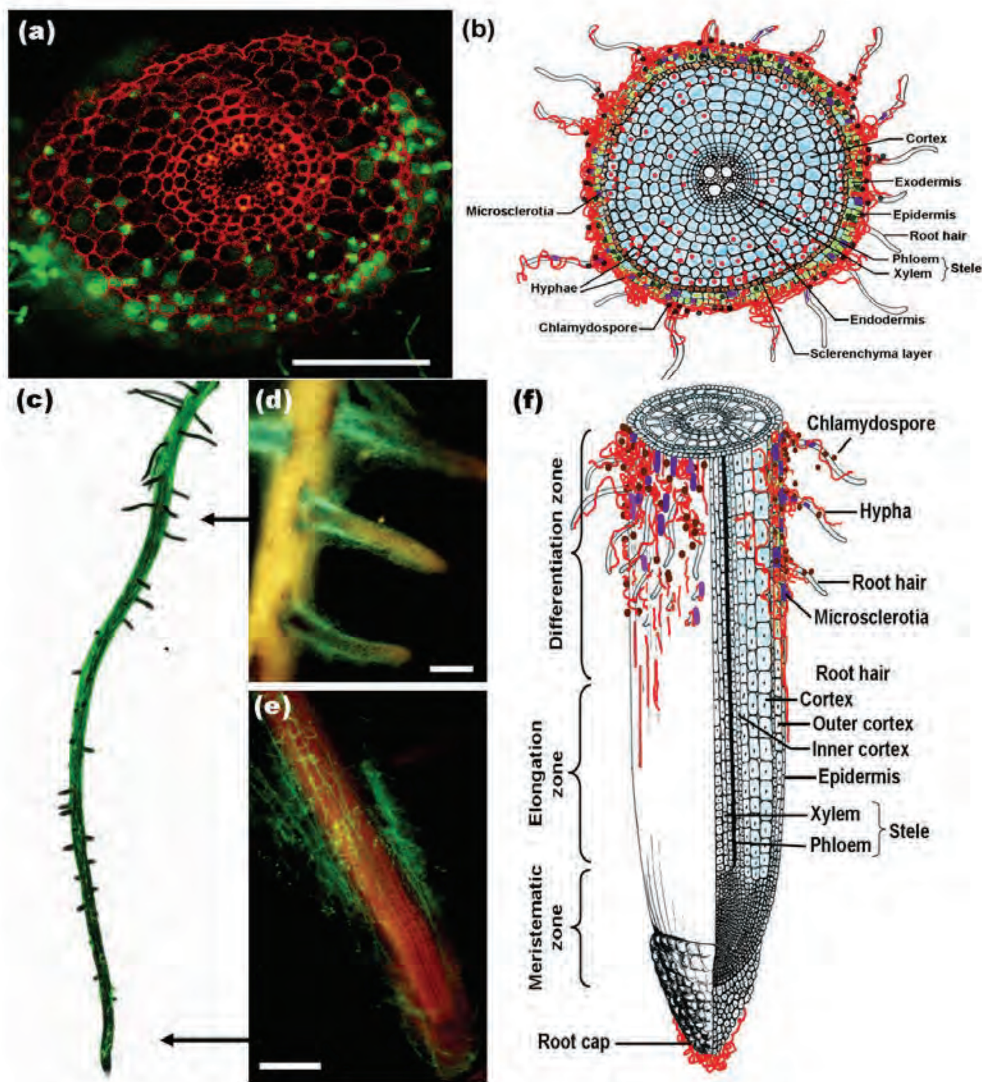

Figure 2 Colonization pattern of *H. oryzae* in rice roots. (a) In a root cross-section, eGFP-tagged hyphae gradually extended from the epidermis to the cortex without penetrating the stele. Bar, 200 µm. (c) A gradual increase in fungal colonization was associated with root maturation. Fungal colonization showing heavy colonization in the differentiation zone (d), slight colonization in the elongation zone, and no colonization in the meristematic zone (e). Bars, 500 µm. (b) and (f) Schematic representations of root colonization by *H. oryzae*. (b) The colonization pattern as seen in a transverse section. (f) Longitudinal section showing the association of fungal colonization with root maturation. Blue and green indicate living and dead cells, respectively. Red lines and dots: hyphae; black dots: chlamydospores; purple patches: microsclerotia.

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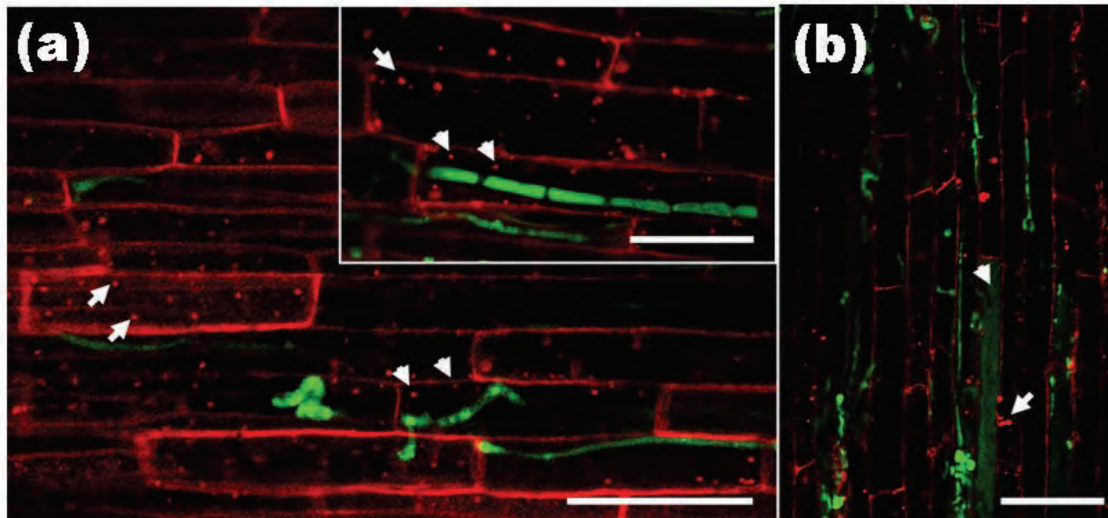

Figure 3 Vitality of *H. oryzae*-infected root cells as shown by staining with FM4-64. (a) The internalization of FM4-64 into endomembrane structures in fungus-infected cells (arrowheads) and non-invaded cells (arrows) during early colonization (≤10 d.a.i.). Bars, 100 µm. (b) At 15 d.a.i., endocytosis disappeared in the cells occupied by microsclerotia (arrowhead), but remained in the adjacent non-infected cells (arrow). Bar, 50 µm.

H. oryzae*-induced local resistance against root infection by *M. oryzae

We investigated whether root colonization by *H. oryzae* would protect rice from *M. oryzae* infection. Once inside the mock-inoculated root, eGFP-tagged *M. oryzae* was highly invasive and propagated from the cortical cells through the epidermis and into the stele (Fig. 4a). Furthermore, *M. oryzae* was seen to spread from the roots of rice plants to the aerial tissues (Fig. 4b), causing lesions on the leaves (Fig. 4c), roots (Fig. 4d) and diamond-shaped necrotic lesions at the base of the stem (Fig. 4e). In contrast, *M. oryzae* was not found in R5-6-1-inoculated roots (Fig. 4f) or aerial tissues (Fig. 4g), and the devastating effect of *M. oryzae* was markedly abolished with few necrotic lesions on the leaves and stems (Fig. 4h, i), suggesting that *M. oryzae* was unable to infect the roots of rice that had been co-cultured with R5-6-1 for 15 days. These observations indicated that root colonization by *H. oryzae* had a significantly positive effect in protecting rice from pathogen root invasion.

Systemic resistance induced by *H. oryzae*

Having established local resistance, we also examined whether *H. oryzae* would trigger a systemic defensive response in rice. Therefore, we examined the consequences of R5-6-1 colonization upon foliar infection by *M. oryzae*. Marked reductions in disease lesion areas and severity were observed in R5-6-1-infected plants compared to controls ($P<0.001$) (Fig. 5b). These results indicated that disease lesions were restricted to a few tiny spots in R5-6-1-infested plants after *M. oryzae* spray inoculation, in contrast to the susceptible-type, spreading lesions in mock-infested plants (Fig. 5a). The disease area was assessed by monitoring the %DLA. The %DLA of the mock-infected plants was 52.3±12%, whereas that

of R5-6-1-infested plants was extremely low, only 3±2% (Fig. 5b). Therefore, we concluded that colonization by *H. oryzae* indeed induced systemic resistance against rice blast.

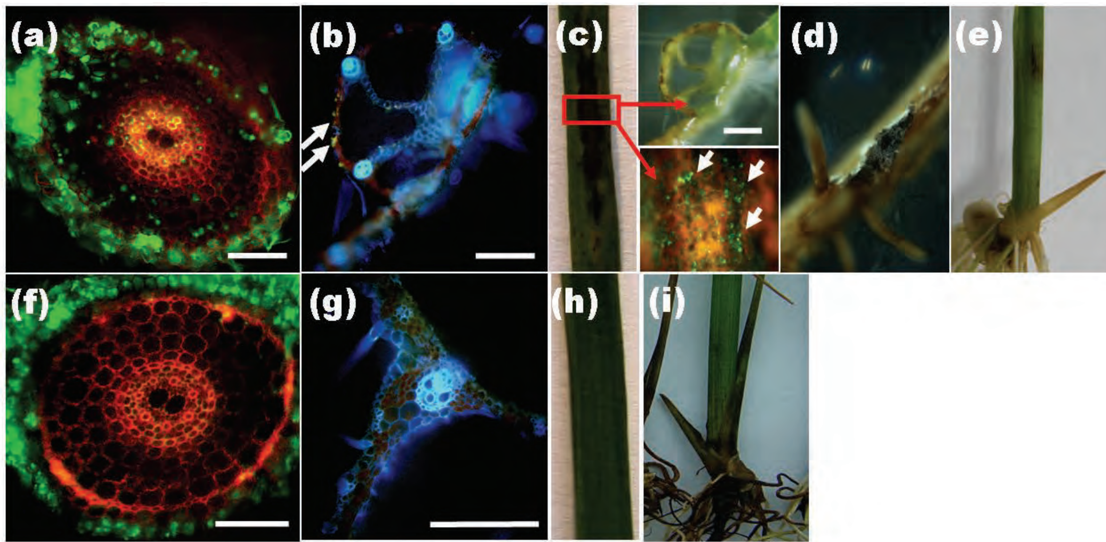

Figure 4 Effects of *H. oryzae* on root infection by *M. oryzae*. (a)–(e) In mock-infected roots, eGFP-tagged *M. oryzae* propagated in the stele (a) and spread systemically from root to leaf through the vascular tissue (b), causing typical blast symptoms in the leaves (c), roots (d) and stem (e). (f)–(i) In *H. oryzae*-infected roots, no eGFP-tagged *M. oryzae* hyphae emerged in the roots (f) and leaf vascular tissues (g), with the disappearance of blast disease on the leaves (h) and stem (i). Bars, 200 µm. Arrows indicate eGFP-tagged *M. oryzae*.

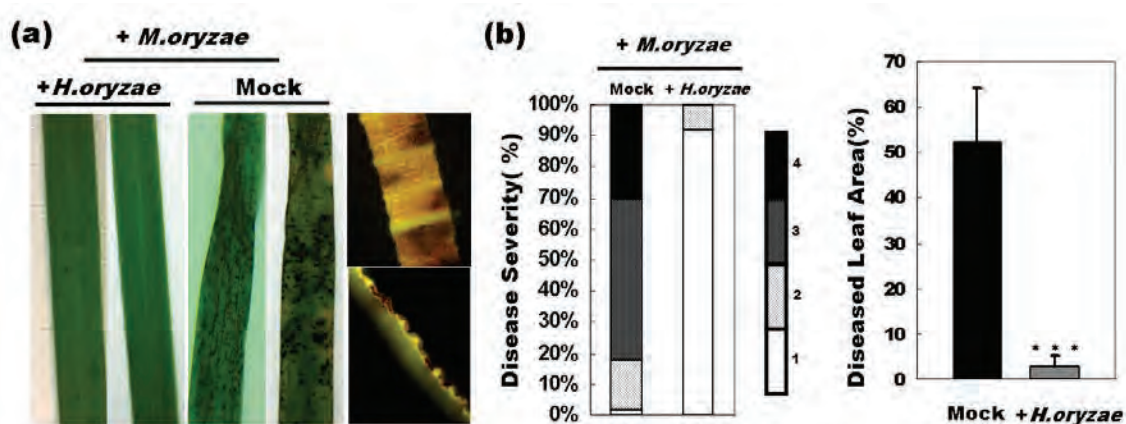

Figure 5 Systemic protection against rice blast by *H. oryzae*. (a) The alleviation of devastating symptoms on the leaves of *H. oryzae*-infected rice in contrast to mock-infected controls was observed. (b) Left panel, lesion severity was judged in four levels ranging from 1 (resistant) to 4 (highly susceptible) (each treatment, *n*=50). Bars indicate the percentage of lesions with each severity level. Right panel, the lesion area was assessed by the %DLA using an Axiovision image analyzer. The values are the means±SD from 50 leaves of *H. oryzae*-infected or mock-infected rice (***, $P<0.001$).

DISCUSSION

Root colonization pattern of *H. oryzae* in rice

The colonization pattern of *H. oryzae* consisted mainly of three consecutive spatiotemporal phases. First, extracellular colonization was initiated by runner hyphae predominantly at the basal part of root hairs, which were regarded as preferential sites, then extended to weave a loose fungal network on the root surface (≤3d.a.i.). Next, it progressed to a biotrophic proliferation phase with intercellular and intracellular hyphae growing along the main axis of the root with centripetal branching and subsequent reproduction of extracellular and intracellular chlamydospores (<10 d.a.i.), while the colonized epidermal and cortical cells remained alive. Finally, cell death-associated colonization was observed, in which most epidermal and partial cortical cells inhabited by plentiful hyphae and microsclerotia in response to halting of further penetration underwent cell death (≥15d.a.i.), while less infected adjacent cells remained alive. These observations indicated a close association between host cell death and abundant fungal hyphae. Longitudinally, fungal colonization increased gradually with tissue maturation, which was shown in *Piriformospora indica*-barley symbiosis (Deshmukh *et al.*2006).

Importance of DSEs for agricultural management

The present study established a new symbiotic system between rice and *H. oryzae*. Based on the protection of rice from the rice blast pathogen, *M. oryzae*, the mutualistic symbiosis of crop plants and DSEs has great potential for sustainable agriculture. From a long-term agronomical viewpoint, *H. oryzae* promises to confer positive effects on disease resistance and cereal yield. The exploitation and utilization of DSEs such as *H. oryzae* may, therefore, not only curtail the input of fungicides and fertilizers in crop production but also may be a novel resource for improving both disease resistance and grain yield.

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Persistent Identifier: urn:nbn:de:0294-sp-2013-ppphe-2

In: Schneider C, Leifert C, Feldmann F (Eds), Endophytes for plant protection: the state of the art. © 2013 Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-11-2.

6-5 Application of Dark Septate Endophyte (DSE) fungi in cultivation of vegetables

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ABSTRACT

Dark Septate Endophytes (DSE) represent a form-group of fungi colonizing plant roots. These endophytes are common and frequent members of root associated fungal communities of environments with strong abiotic stress. The main aim of the study presented here was to study if DSE fungi could be used in production of vegetable crops. This potential could have especial importance when non-mycorrhizal vegetable crops (e.g. members of the family Brassicaceae) are inoculated. Our long-term aim is to study how DSE inoculation could affect, beside other parameters, the water demand and how this might effect the irrigation of the crops. First we aimed to establish efficient inoculums and to test different inoculation technologies to find the most effective way to involve those endophytes to farming.

Persistent Identifier: urn:nbn:de:0294-sp-2013-ppphe-2

SESSION 7

Workshop on detection technologies in endophyte research

7-1 Applications of Fungal Endophytes in Agriculture

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Persistent Identifier: urn:nbn:de:0294-sp-2013-ppphe-2

König S et al, *Phytophthora* spp. Lab-on-a-Chip diagnostics – a useful tool for the identification of endophytes? In: Schneider C, Leifert C, Feldmann F (Eds), Endophytes for plant protection: the state of the art, pp. 260-261.
© 2013 Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-11-2.

7-2 *Phytophthora* spp. Lab-on-a-Chip diagnostics – a useful tool for the identification of endophytes?

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ABSTRACT

For *Phytophthora* species that are quarantine or regulated organisms highly specific and sensitive diagnostic tools are recommended for surveys and monitoring. These diagnostic techniques should give results within short time and should be not too expensive. The techniques currently used for routine diagnosis of *Phytophthora* species in plant tissue are mainly molecular techniques (conventional and real-time PCR) and direct isolation. While using PCR techniques in a single run enables the detection of a single species or a small species set, DNA microarray techniques as the Lab-on-a-Chip allow the detection of a broad range of species. Furthermore the study of more complex species communities by PCR needs cost and time intensive down stream applications as cloning or next generation sequencing. Within a finished three year project an initial prototype of a Lab-on-a-Chip system could be established (Julich *et al*. 2011) that enables PCR amplification and chip hybridization on a single miniaturized device. A current project comprises (i) the identification of a genomic region suitable for the generation of *Phytophthora*-species specific DNA-probes, (ii) the generation and specificity screen of the probes, and (iii) the adaption of different techniques for DNA-target amplification and chip hybridization. Due to the intended application of the technique by non-biologists directly on the infested site, we proved a couple of simple protocols for plant tissue homogenization, DNA-extraction, and DNA-target processing. Processing the DNA during their amplification has been shown as essential for running the Lab-on-a-Chip, because target DNA hybridization on chip bound probes depends on the availability of single strand DNA. Functioning of the Lab-on-a-Chip system and procedures for the probe and protocol improvement will be presented and limitations as well as the ecological significance of the method for plant endophyte studies discussed.

Persistent Identifier: urn:nbn:de:0294-sp-2013-ppphe-2

ACKNOWLEDGEMENT

Both projects have been funded by the German Federal Office for Agriculture and Food (BLE) based on a decision of the parliament of the Federal Republic of Germany. FNo. 28-1-54.081-10

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Persistent Identifier: urn:nbn:de:0294-sp-2013-ppphe-2

Ozaktan H, Isolation optimization of bacterial endophytes from cucumber plants and evaluation of their effects on growth promotion and biocontrol. In: Schneider C, Leifert C, Feldmann F (Eds), Endophytes for plant protection: the state of the art, pp. 262-268. © 2013 Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-11-2.

7-3 Isolation optimization of bacterial endophytes from cucumber plants and evaluation of their effects on growth promotion and biocontrol

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ABSTRACT

The aim of this study was to find, within cucumber plants, endophytic bacterial (EB) isolates that can provide significant biological control of Fusarial wilt of cucumber caused by *Fusarium oxysporum* f. sp. *cucumerinum* (FOC) and angular leaf spot of cucumber caused by *Pseudomonas syringae pv .lachrymans* (PSL) and that can enhance plant growth under *in vitro* conditions. The endophytic bacteria were isolated from the internal tissues of roots, leaves and stems of healthy cucumber plants, which were individually disinfected by soaking each in 5% sodium hypochlorite for 10min, followed by immersion in 70% ethanol for 5 min. They were then rinsed three times in sterile distilled water to clear them of alcohol before obtaining bacterial isolates by two different techniques such as trituration of leaves and imprinting of stem and root tissues onto Triptic Soy Agar (TSA). Surface sterility checks were carried out for each sample to monitor the efficiency of the disinfestation procedure during isolation. If no bacterial growth occurred in the sterility check, the recovered bacteria were considered to be endophytes. In this study, 104 EB strains were isolated from internal tissues of healthy cucumber plants which were grown in greenhouse and field in Turkey. It was determined several phenotypic properties and found approximately equal numbers of Gram-negative and Gram-positive isolates. These isolates were screened *in vitro* for their plant growth promoting traits like production of indol

3-eacetic acid (IAA), hydrogen cyanide (HCN), siderophore, phosphate solubilization and antagonistic activity against FOC and PSL. More than 30% of the EB strains produced detectable levels (20-125 µg ml $^{-1}$) of IAA in culture filtrates. Siderophore production of EB strains were exhibited by 46% isolates ranging from 3 to 19 mm zones. HCN production was more common trait of *Pseudomonas* strains (16%). Solubilization of phosphate was detected by 29% in the EB isolates. More than 53% of the isolates of EB inhibited the mycelial growth of FOC at the rate of 20% to 64% on PDA plates. Twenty three of tested 104 EB strains produced inhibition zones on TSA against PSL between 2 to 7 mm. Our study will continue for further evaluation of the EB isolates exhibiting biocontrol and multiple plant growth promoting (PGP) traits on soil–plant system.

INTRODUCTION

Greenhouse production is generally located on the coastal regions of Turkey. About 87% of the greenhouse production area in Turkey is concentrated in the Mediterranean region. According to 2011 statistics, total cucumber production in Turkey is about 1.751.504 tons (faostat, 2011). Turkey is in second order in terms of total cucumber production around the world.

FOC, which is a soil-borne fungus, causes wilting and death of cucumber plants grown in greenhouse. Fungus colonizes the xylem vessls of cucumber plants as systemically and causes vascular discoloration. On the other hand, angular leaf spot of cucumber caused by PSL is one of the most widespread and serious disease of cucumber plants in Turkey. It is difficult to control of FOC and PSL because these plant pathogens could cause systemic invasion and move in the cucumber plant tissues by xylem vessels. There is no resistant cucumber cultivars to FOC and PSL. In response to environmental and health concerns about extended use of pesticides, there is considerable interest in finding alternative control approaches for use in integrated pest management strategies for crop diseases.

Biological control has been considered as a potential method for controlling the plant diseases. The use of EB strains to control plant-pathogenic bacteria and fungi is receiving increasing attention as a sustainable alternative to synthetic pesticides. In order to reduce the input of pesticides and fertilizers and to make an eco-friendly agriculture, it will be important to develop inocula of biofertilizers, and biopesticides. EB strains, which live inter- and intracellularly in plants without inducing pathogenic symptoms, interact with the host biochemically and genetically. EB may play many important beneficial roles in the metabolism and physiology of the host plant, including fixing atmospheric nitrogen, sequestering iron from the soil, solubilizing phosphates, synthesizing plant – growth hormones, and suppressing of ethylene production by 1-aminocyclopropane-1-carboxylate (ACC) deaminase, degrading toxic compounds, inhibiting strong fungal activity and antagonizing bacterial pathogens (Hurek & Reinhold-Hurek 2003, Whipps 2001). The

internal plant tissues provide a protective environment for endophytic bacteria, which colonize an ecological niche similar to plant pathogens. The use of these EB to control plant-pathogenic bacteria and fungi is receiving increasing attention as a sustainable alternative to synthetic pesticides. In order to reduce the input of pesticides and fertilizers and to make an eco-friendly agriculture, it will be important to develop inocula of biofertilizers, and biopesticides.

The aim of this study was to find, within cucmber plants, endophytic bacterial (EB) isolates that can provide significant biological control of Fusarial wilt of cucumber caused by *Fusarium oxysporum* f. sp. c*ucumerinum* (FOC) and angular leaf spot of cucumber caused by *Pseudomonas syringae pv .lachrymans* (PSL) and that can enhance plant growth under *in vitro* conditions.

MATERIALS AND METHODS

Isolation of endophytic bacteria

The endophytic bacteria were isolated from the internal tissues of roots, leaves and stems of healthy cucumber plants, which were individually disinfected by soaking each in 5% sodium hypochlorite for 10min, followed by immersion in 70% ethanol for 5 min. They were then rinsed three times in sterile distilled water to clear them of alcohol before obtaining bacterial isolates by two different techniques such as trituration of leaves and imprinting of stem and root tissues onto Triptic Soy Agar (TSA). Surface sterility checks were carried out for each sample to monitor the efficiency of the disinfestation procedure during isolation. If no bacterial growth occurred in the sterility check, the recovered bacteria were considered to be endophytes. Single colonies were isolated and maintained in pure cultures at -80^{0}C in 15% (v/v) glycerol (Nejad & Johnson 2000).

Phenotypic characterization of bacterial isolates

Colonies of bacterial isolates were characterized for the following traits: color, form, elevation, margin, diameter, surface, opacity, and texture. The Gram reaction was performed by using a 3% KOH test (Suslow *et al.* 1982). Endophytic bacteria were tested for hypersensitive response (HR) on tobacco leaf. EB strains, which were resulted HR (+) on tobacco leaves were discarded from the study as possible plant pathogens (Lelliott & Stead 1987; Schaad *et al*. 2001).

Screening for antagonistic activity of EB strains in vitro

All EB isolates were screened for their biocontrol activity toward FOC and PSL *in vitro* using a dual-culture technique (Trivedi *et al.* 2008). The antifungal activity of the EB was assessed against FOC by dual culture technique on potato dextrose agar (PDA) plates inoculated with the pathogen alone were maintained as control. The mycelial disc (5 mm) from 7 days old culture of FOC was placed in one side of the Petri plate containing PDA

medium, and then EB strains were streaked on the opposite of the petri plate by the help of sterilized inoculation needle. Three replications were maintained for each treatment. The plates were incubated at room temperature for seven days. The inhibitory effects of EB strains on the linear growth of FOC were determined. The percent of inhibited FOC was calculated by compared with fungal growth in control plates with the fungus only. A suspension of PSL strain was spread over King's Medium B Agar surface and after drying EB colonies were spot inococulated onto agar surface, one spot at each of the four corners of the plate. After 2 days incubation at 28°C, inhibition zones in mm were measured.

Elucidation of in vitro plant growth promotion traits of EB

The EB strains were screened by in vitro assays for the production of the following functional traits: hydrogen cyanide,HCN (Bakker & Schippers 1987); phosphate solubilization (Pikovskaya 1948) and indole 3- acetic acid, IAA (Bric *et al.* 1991) for plant growth promotion and siderophore (Schwyn & Neilands 1987) for antifungal activity. All these assays were replicated twice for each of the isolates.

Effects on seed germination and vigor index of selected EB strains

EB strains, which were found as promising *in vitro* for plant growth promoting traits and bicontrol activities toward FOC and PSL were tested for seed emergence on cucumber seeds (cv. Gordion) and measured for Vigor Index (VI). For bacterization, seeds were surface sterilized with 1% sodium hypochlorite for 1 min and soaked in EB suspensions ammended with 1% carboxy methyl cellulose (CMC) After bacterization, the seeds were placed onto sterile filter paper moistened with sterile distilled water (SDW) in petri plates (three plates with 10 seeds/plate) and left to incubate at room temperature. Control plates were arranged in a similar way, except that they were treated with 1% CMC only. For each isolate, effects on seed germination were measured by counting the number of fully germinated seeds per plate and comparing that with that of the control plates. After 5 days, the vigor index for each treatment was calculated by using the formula as described by Abdul Baki & Anderson (1973): VI= Percent germination X (seedling length + root length).

RESULTS AND DISCUSSION

A total of 44 healthy cucurbit plant samples, consisting of 34 greenhouse and 10 field were obtained from different sampling areas in Turkey. Surface sterility checks were carried out for each sample to monitor the efficiency of the disinfestation procedure during isolation. If no bacterial growth occurred in the sterility check, the recovered bacteria were considered to be endophytes. In this study, 104 different endophytic colonizing bacterial strains were isolated from healthy cucumber plants grown in greenhouse and field. On the basis of some cultural, morphological and biochemical characteristics a total of 104 endophytic bacterial strains were grouped into fluorescent Pseudomonads, Gram (-) bacteria, and Gram (+) bacteria.

Persistent Identifier: urn:nbn:de:0294-sp-2013-ppphe-2

In vitro plant growth promoting traits

More than 30% of the EB strains produced detectable levels (20-125 µg ml $^{-1}$) of IAA in culture filtrates. IAA production was highest in the Pseudomonads followed by *Bacillus* isolates. Siderophore production of EB were exhibited by 46% isolates ranging from 3 to 19 mm zones on CAS agar. Solubilization of phosphate was detected by 29% in the EB isolates ranging from 1 to 9 mm zones. Most EB isolates were HCN negative on TSA, with or without glycine. Only three *Pseudomonas* isolates showed detectable cyanide production by changing color from yellow to brown around its colonies.

Effects on bacterial and fungal growth

Isolates of EB were tested for inhibition of mycelial growth of FOC and colonial development of PSL *in vitro*. More than 53% of EB isolates inhibited the mycelial growth of FOC at the rate of 20% to 64% on PDA plates, producing inhibition zones or showing cell wall lysis. Twenty three of tested 104 EB strains produced inhibition zones on TSA against PSL between 2 to 7 mm. Most of the tested EB isolates showed biocontrol activity toward both pathogens *in vitro*.

Effects on seed germination

Thirty two isolates out of 104 EB, which were promising for *in vitro* plant growth promoting traits and bicontrol activities toward FOC and PSL were tested for seed emergence on cucumber seeds and measured for VI. Some of our bacterial isolates had no apparent effects on seed germination and VI, whereas others, when applied individually, caused suppression of seed germination (*in vitro)* and seedling growth which was compared with that of the control plates. It was observed that 40% of tested EB isolates from cucurbit plants improved the VI of cucumber seedlings comparing to treated with CMC (1% w/v) only. Hence, growth-regulating substances might have been involved.

The specific objective of this study was to find naturally occurring EB isolates within the tissues of cucumber plants and to investigate their capacities to (a) enhance the growth promotion and (b) provide the biological control against cucumber pathogens FOC and PSL *in vitro*. EB isolates were isolated after surface sterilization of living roots, stems and leaves. It was observed that the inner tissues of healthy cucumber plants were very rich for EB colonization Trituration and imprinting of the plant tissues were the reliable and easy isolation techniques for EB (Hallmann *et al.* 1997).

Plant growth promotion mediated by endophytic bacteria may be exerted by several mechanisms, e.g. production of plant growth hormones, synthesis of siderophores, solubilisation of minerals such as phosphorous (Chernin & Chet 2002, Hurek & Reinhold-Hurek 2003, Whipps 2001;). In this study, it was proved that tested EB strains were found very promising in respect to *in vitro* plant growth promotion parameters.

Some of our bacterial isolates had no apparent effects on seed germination and VI, whereas others, when applied, caused suppression of seed germination (*in vitro)* and VI which was

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compared with that of the control plates. It was found that some bacterial endophytic isolates from healthy plants inhibited the growth of tomato seedlings in reinoculation assays, possibly through the production of certain metabolites (van Peer *et al.* 1990). On the other hand, approximately 54 % of tested EB isolates had a strong potential for promoting seed germination and VI comparing to control plates. Nejad & Johnson (2000) described that the isolates of endophytic bacteria significantly improved seed germination and plant growth of oilseed rape and tomato.

In this study, majority of EB isolates showed antibiosis or lysis against both cucumber pathogens, PSL and FOC *in vitro*, whereas most of them were HCN negative on TSA. Endophytic bacteria isolated from potato roots expressed high levels of hydrolytic enzymes such as cellulase, chitinase and glucanase (Krechel *et al.* 2002). Consistent with this result, our experiments concerning *in vitro* biocontrol indicated that most isolates that were effective against FOC and PSL produced inhibitory metabolites other than hydrogen cyanide.

Furthermore, the proportion of endophytes that not only were able to suppress pathogenic fungi and / or bacteria but could also improve seed germination and plant growth has been found. Our study will continue for further evaluation of the EB isolates exhibiting biocontrol and multiple plant growth promoting (PGP) traits on soil–plant system.

ACKNOWLEDGEMENTS

This study was financed by national grants from the Scientific and Technological Research Council of Turkey (TUBITAK-COST 111 O 505) and Ege University Science-Technology Research and Application Center (EBILTEM). The authors thank the contributions of the participants in the EU-COST Action FA1103.

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Persistent Identifier: urn:nbn:de:0294-sp-2013-ppphe-2

In: Schneider C, Leifert C, Feldmann F (Eds), Endophytes for plant protection: the state of the art. © 2013 Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-11-2.

7-4 Understanding of plant colonization by endophytic bacteria by tracking them inside plants

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ABSTRACT

Endophytes, are currently used to stimulate plant growth, health, as well as for increasing yield and to reduce pathogenic infections. Understanding of the colonization process, niches establishments of such microsymbionts have given important knowledge regarding how the beneficial microbes could make intimate association with their hosts, before to provide beneficial effects. Gaining knowledge on the microbial behavior of specific strains, as well as the traits required for colonization and establishment on and inside the plants, has also lead to the understanding of where could be present the beneficial microbes in different plant parts following application, i.e. as a consequence of inoculation. In this study we will provide the general way of colonization of these microbes as well as describe specific sources and niches of colonization of some colonizers by using different microscopic tools enabling to determine the behavior of specific taxa inside plants.

Persistent Identifier: urn:nbn:de:0294-sp-2013-ppphe-2

Suebphankoy C et al., Detection of heat-resistant endophytic Bacillus spp. associated with commercial and indigenous rice seeds in Thailand and its effect to rice seed germination and seedling growth. In: Schneider C, Leifert C, Feldmann F (Eds), Endophytes for plant protection: the state of the art, pp. 270-274. © 2013 Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-11-2.

7-5 Detection of heat-resistant endophytic *Bacillus* spp. associated with commercial and indigenous rice seeds in Thailand and its effect to rice seed germination and seedling growth

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ABSTRACT

Three indigenous rice cultivars (cultivars Jow Haw, Ni Kor and Roa Su Ya) from Chiang Mai province, in the North of Thailand and four commercial rice cultivars (cultivars Chai Nat, Suphan Buri, Reing Thong and RD 31) from Phetchaburi province, in Central Thailand were collected and used in this experiment. Fifty rice seeds from each cultivar were used for isolating both epiphytic and endophytic *Bacillus* spp. These 50 seeds were submerged in 5 mL sterile water in the test tube and were subjected to hot water in the water bath (at 80°C) for 30 minutes. For isolating the epiphytic *Bacillus* spp., an aliquot from these tubes was streaked onto the PDA medium. For isolating the endophytic *Bacillus* spp., fifty rice seeds from each cultivar were surface sterilized with 70% ethyl alcohol for 30 minutes, after which the rice seeds were de-husked. The remaining rice seeds were macerated with the mortar in 5 mL sterile water and were then subjected to the heat treatment as described above. The aliquot was streaked onto the medium for bacterial isolation. Pure cultures of the isolated bacteria were identified basing upon the base sequence of the 16S ribosomal RNA gene. *Bacillus licheniformis* (from cultivar Jow Haw), *B. amyloliquefaciens* (from cultivars Chai Nat and Suphan Buri), *B. subtilis* (from cultivars Chai Nat, Reing Thong, and Suphan Buri) and *B. thuringiensis* (from cultivar Ni Kor) were isolated from the de-husked seeds and were considered to be an endophytic species. On the other hand, *Alicyclobacillus pomorum* (from cultivar RD 31), *B. licheniformis* (from

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cultivar Ni Kor), *Brevibacillus brevis* (from cultivars Roa Su Ya and Reing Thong), and *Bacillus* sp. (from cultivar Chai Nat) were an epiphyte on the surface of the rice seeds. Further study was conducted to determine the effect of endophytic *B. amyloliquefaciens* and epiphytic *A. pomorum* on the germination of rice seed and growth of seedling of the four commercial rice cultivars. Endophytic *B. amyloliquefaciens* had high impact to cultivar RD 31, with respect to both seed germination and seedling growth.

INTRODUCTION

Rice (*Oryza sativa*) is important to Thailand both as export commodity and as staple for consumption. Although, the main commercial cultivars of rice, which have been cultivated nationwide, have been developed by the Department of Rice, there are various indegeneous rice cultivars which have been cultivated by the Thai farmers in small areas all over the country. For instances, farmers in the highland in the North of Thailand have cultivated indigenous rice cultivars which are adapted to mountianeous area, where water for irrigation is scarce. This type of rice cultivation system provides a unique ecological niche in which novel microbes, particularly endophytic bacteria, may thrive and may be available for isolation and utilization.

In ecological term, rice plants are reported to associate with various beneficial microbes which play a role in sustaining growth and development of rice. In practical term, these microorganisms can be utilized in increasing rice productivity. Plant-Growth-Promoting Rhizobacteria (PGPR) is the main group of bacteria which receive a lot of attention for research and the product of these bacteria has been commercialized for crop production in various countries (Schisler *et al*. 2004).

Endophytic bacteria also have emerged to be a subject of investigation by scientists worldwide, due to their contribution to plants in term of both ecology and physiology. This group of bacteria is reported to be associated with root of rice plants (Hardoim *et al*. 2011). Recently, seed of rice is reported to be a good source of both pathogenic and beneficial bacteria in The Philippines (Cottyn *et al*. 2009). The endophytic bacterium from rice plants, *Pantoea sp.*, may also play a role as natural biofertilizer and bioprotective against plant pathogen (Ruiza *et al*. 2011), making this bacterium the candidate for utilization in increasing rice production.

This preliminary research is conducted to detect the heat-resistant endophytic *Bacillus* spp. which has associated with commercial and indigenous rice seeds in Thailand. Its role to rice seed germination and seedling growth has been investigated, in comparison with the epiphytic bacteria isolated from the rice seeds.

Persistent Identifier: urn:nbn:de:0294-sp-2013-ppphe-2

MATERIALS AND METHODS

Rice seed samples collection

Three indigenous rice cultivars (cultivars Jow Haw, Ni Kor and Roa Su Ya), from Chiang Mai province in the North of Thailand, and four commercial rice cultivars (cultivars Chai Nat, Suphan Buri, Reing Thong and RD 31) from Phetchaburi province in Central Thailand, were collected and used in this experiment. The collected rice seed samples were then kept in a paper bags during transportation back to the Microbiology Laboratory at Faculty of Animal Science and Agricultural Technology, Silpakorn University, Phetchaburi IT campus, Cha-Am, Phetchaburi, Thailand.

Isolation of endophytic bacteria

Fifty rice seeds from each cultivar were used for isolating both epiphytic and endophytic *Bacillus* spp. These 50 seeds were submerged in 5 mL sterile water in the test tube and were subjected to hot water in the water bath (at 80° C) for 30 minutes. For isolating the epiphytic *Bacillus* spp., an aliquot from these tubes was streaked onto the PDA medium. For isolating the endophytic *Bacillus* spp., 50 rice seeds from each cultivar were surface sterilized with 70% ethyl alcohol for 30 minutes, after which the rice seeds were de-husked. The remaining rice seeds were macerated with the mortar in 5 mL sterile water and were then subjected to the heat treatment as described above. The aliquot was streaked onto the medium for bacterial isolation.

Bacterial identification

A pure culture of the isolated bacteria was subjected to DNA extraction and bacterial identification was carried out basing upon its 16S rRNA gene sequence at the Biotechnology Research and Development Office, Department of Agriculture, Patumthani, Thailand.

Evaulating the effect of selected bacteria to seed germination and seedling growth

The effect of endophytic *B. amyloliquefaciens* and epiphytic *A. pomorum* on the germination of rice seed and growth of seedling of the four commercial rice cultivars (cultivars Chai Nat, Suphan Buri, Reing Thong and RD 31) were evaluated in the laboratory. One hundred rice seeds of each cultivar (four replications with 100 seeds per replication) were soaked in the mixture of sterile water and bacterial cell suspension (at 50:1/v:v; at 10^{13} cfu/mL for *B. amyloliquefaciens* and at 10^{15} cfu/mL for *A. pomorum*) for 24 hours, after which the seeds were placed upon the sterile filter paper in the plastic box (8 x17 x 4 cm. dimension; width/length/height). These plastic boxes were incubated at room temperature (26-32°C) and the number of seed which had germinated was counted 2 days after plating. Effect of these endophytic bacteria to seedling growth was carried out 5 days later, in which only healthy seedlings with normal growth were counted. Seedling height was also measured (four replications with 20 healthy seedlings per replication).

RESULTS

Species of heat-resistant endophytic bacteria associated with rice seeds

Bacillus licheniformis (from cultivar Jow Haw), *B. amyloliquefaciens* (from cultivars Chai Nat and Suphan Buri), *B. subtilis* (from cultivars Chai Nat, Reing Thong, and Suphan Buri) and *B. thuringiensis* (from cultivar Ni Kor) were isolated from the de-husked seeds and were considered to be an endophytic species. On the other hand, *Alicyclobacillus pomorum* (from cultivar RD 31), *B. licheniformis* (from cultivar Ni Kor), *Brevibacillus brevis* (from cultivars Roa Su Ya and Reing Thong), and *Bacillus* sp. (from cultivar Chai Nat) were epiphytic bacteria, isolating from the surface of the rice seeds.

Effect of selected bacteria to seed germination and seedling growth

B. amyloliquefaciens was effective in enhacing rice seed germination (with mean of rice seed germination at 94.5%), comparing to *A. pomorum* and non-treated control (with means of seed germination at 91.3% and 90.4%, respectively). Both *B. amyloliquefaciens* and *A. pomorum* were also capable of promoting seedling growth (with means of seedling height at 6.9 cm. and 6.8 cm. respectively, comparing to non-treated control (with mean of seedling height at 5.0 cm.). Different rice cultivars responded differently to the bacteria with respect to the means of both seed germination and seedling height (data not shown). Seeds of cultivar RD 31 had the greatest different between non-treated control and treated with *B. amyloliquefaciens*, with respect to both seed germination and seedling height.

DISCUSSIONS

Rice seeds were reported to be a host of endophytic bacteria. This preliminary study has consolidated the results of the previous studies, by acquiring the heat-resistant isolates of endophytic bacteria from de-husked rice seeds. The detection of *B. thuringiensis*, an insecticidal bacterium which was isolated from cultivar Ni Kor, is very interesting. In ecological term, it is very challenging to investigate the contribution of this bacterium in preventing this rice cultivar against insect pests. More studies, however, should also be carried out to detect this bacterium from other rice tissues, such as leaf sheath and leaf blade. The detection of endophytic *B. thuringiensis* in these tissues may help to explain the reaction of this specific cultivar against insect pests (Ruiza *et al*. 2011).

B. amyloliquefaciens isolate NBRISN 13 was reported to promote growth of various plants, including rice even under salt stress (Nautiyal *et al*. 2013). The endophytic *B. amyloliquefaciens* isolated from rice in this experiment may also possess special characteristic, apart from its efficacy in enhancing both seed germination and seedling height. This, however, requires further study to elucidate its mechanisms as the effect of this bacterium to rice may be genotype-specific to some rice cultivars.

B. licheniformis, which is an endophytic bacterium isolated from seed of cultivar Jow Haw, should also be subjected to further studies. This bacterium was reported to have a capacity to enhance growth of various crops, such as tomato and pepper. It was reported to have considerable colonisation and competitive ability, making this bacterium to be used as a biofertiliser and a biological control agent (Gracia *et al*. 2004). The beneficial effect of *B. licheniformis* should be tested in monocotyledon plants, such as rice as well.

Heat-resistant endophytic bacteria, such as *B. thuringiensis*, *B. amyloliquefaciens* and *B. licheniformis* which were isolated from the de-husked rice seeds, should be subjected to further studies as they may possess enzymes and other secondary metabolites which are resistant to degrade at high temperature. The finding should broaden the venue for utilizing these bacteria.

ACKNOWLEDGEMENTS

We would like to thank Silpakorn University, Phetchaburi IT campus, Cha-Am, Phetchaburi, Thailand, for financial support for Assistant Professor Dr. Pantipa Na Chiangmai to attend and present this research in the International Symposium on Plant Protection and Plant Health in Europe (PPPHE) 2013, Berlin, Germany.

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Persistent Identifier: urn:nbn:de:0294-sp-2013-ppphe-2

SESSION 8

Workshop on physiological and morphological changes due to endophytes

Persistent Identifier: urn:nbn:de:0294-sp-2013-ppphe-2

Lohse R, Patel AV , Production of secondary metabolites with endophytes isolated from a tropical tree: first results. In: Schneider C, Leifert C, Feldmann F (Eds), Endophytes for plant protection: the state of the art, pp. 276-277. © 2013 Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-11-2.

8-1 Production of secondary metabolites with endophytes isolated from a tropical tree: first results

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INTRODUCTION

Endophytes are microorganisms that live at least parts of their life cycle more or less asymptomatically in plants. The estimated high species diversity of endophytes and their adaption to various plant habitats presumes a rich and almost untapped source of new secondary metabolites for pharmaceutical or agricultural applications (Bacon and White 2000, Pirttilä and Frank 2011). Today a lot of interesting compounds from plants and trees are obtained via complex extractions in low concentrations. It is hypothesized that some of these pharmaceutical and agricultural compounds are produced by endophytes.

All parts of a tropical tree show an array of negative effects on insects including ovipositor deterrent, anti-feedant and other inhibitory activities. That is why we want to find out if this tree contains endophytic microorganisms and if these produce bioinsecticides associated with the plant metabolism (Figure 1).

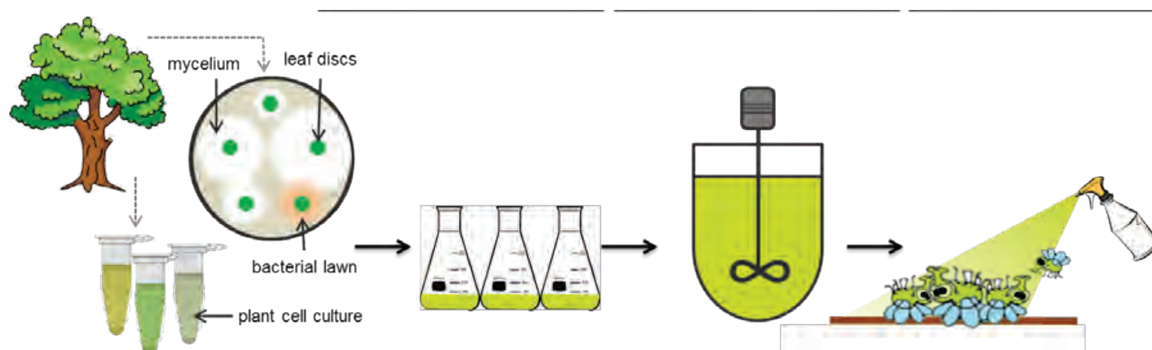

Figure 1 Isolation of endophytes, cultivation in shake flasks, scale-up of the process to a 2 L stirred tank reactor and efficacy test with insects.

MATERIAL AND METHODS

At first, plant material (seeds, leaves, stems) was collected. To eliminate epiphytic microorganisms, all the samples were surface-sterilized. The samples were immersed in 70% ethanol for 2 min and then sterilized with 5% sodium hypochlorite for 3 min and then rinsed again in 70 % ethanol for 2 min, before a final rinse in sterilized double-distilled water. Each sample was then surface dried under aseptic conditions. Segments of each sample were placed on SDA agar (Odds 1991) and were incubated for 7 days at 25°C. The isolated endophytes are cultivated in complex liquid media for 14 days at 25°C. The culture broths were analysed by HPLC-DAD.

RESULTS AND DISCUSSION

The isolation procedure resulted in 14 endophytes (one bacterium and 13 fungi) were isolated. All of them grew in shake flask cultures. Terpenoid secondary metabolites that so far have been isolated from the tree were detected in the culture broth of one bacterium and three fungi with HPLC-DAD (Figure 2).

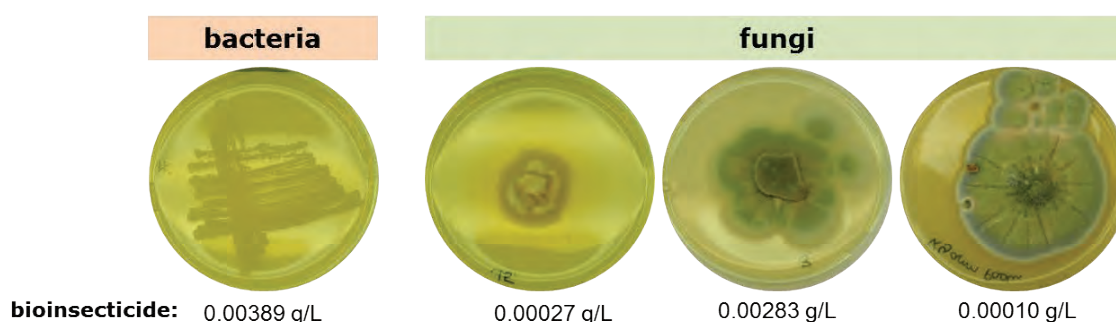

Figure 2 Cultivation of the endophytes on SDA and the concentration of the bioinsecticide in the culture broth 14 days after inoculation.

Further experiments will deal with research into genomics, transcriptomics and metabolomics in cooperation with partners, e.g. HPLC-MS/MS and MALDI-TOF-MS, and apply the findings to the development of low-cost culture media and mass-production of the secondary metabolites in a 2 L stirred tank reactor.

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Persistent Identifier: urn:nbn:de:0294-sp-2013-ppphe-2

In: Schneider C, Leifert C, Feldmann F (Eds), Endophytes for plant protection: the state of the art. © 2013 Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-11-2.

8-2 Wood inhabiting endophytic fungi as producers of bioactive metabolites

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ABSTRACT

The long living conifers and broadleaved trees are hosting a large range of endophytic microbes, especially fungi. The role of many of these species and taxonomically non-determined members has long been unclear. A part of the fungal endophytes are producers of known as well as to date unidentified secondary metabolites, which actively influence other biologically systems. One of the most studied groups of filamentous fungi living as saprophytes and endophytes in trees, being well known for their production of bioactive compounds, belong to the order of *Xylariales*.

The ability to identify/clarify the structure of these secondary metabolites by methods of mass spectroscopy enables a closer understanding of the interaction between endophytic microorganisms, their host tree and the environment.

Fungal strains were isolated from environmental sources of living or decaying woody material, roots, and needles. After isolation, replication of strains was carried out on nutrient-rich media. A standard method was developed to search for novel structures and to quantify known metabolites in these cultures. Without further separation, the whole agar plate was homogenized and aliquots were taken for extraction. After a clean-up procedure the sample was analyzed by LC-PDA-ESI-Q-TOF-MS (liquid chromatography coupled with a photodiode array detector and an electrospray ionization quadrupol/time-of-flight mass spectrometer).

In several members of the fungal strains various bioactive metabolites were identified. Our investigations support the theory that many of the endophytic fungi have a functional purpose for the defense mechanism of trees and other woody plants due to their specific metabolite formation.

Persistent Identifier: urn:nbn:de:0294-sp-2013-ppphe-2

Khianngam S et al., Isolation and screening of endophytic bacteria for hydrolytic enzymes from plant in mangrove forest at Pranburi, Prachuap Khiri Khan, Thailand. In: Schneider C, Leifert C, Feldmann F (Eds), Endophytes for plant protection: the state of the art, pp.279-284. © 2013 Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-11-2.

8-3 Isolation and screening of endophytic bacteria for hydrolytic enzymes from plant in mangrove forest at Pranburi, Prachuap Khiri Khan, Thailand

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ABSTRACT

A survey was conducted to collect plant samples in a mangrove forest at Pranburi, Prachuap Khiri Khan, Thailand. A total of 31 endophytic bacteria were isolated from different plant tissues such as root, inner bark, leave, flower and fruit of various mangrove plants. These bacteria were subsequently screened to detect their hydrolytic enzymatic activity. Twenty isolates showed promising enzymatic activity by creating a clear zone on an agar medium. They were identified based on their morphological, physiological and biochemical characteristics. The 16S rRNA gene sequence analysis of some isolates consolidated bacterial identification. Interestingly, the strain Rhf-2 isolated from a fruit of *Rhizophora mucronata* showed its ability to produce proteinase, lipase, amylase and cellulase activity. This strain was identified as *Bacillus safensis*, based on 99.3% 16S rRNA gene sequence similarity. Furthermore, the strains of *Curtobacterium citreum, Pseudomonas psychrotolerans, Bacillus safensis* and *Staphylococcus warneri*, which are the genera commonly associated with indigenous plants, were also detected. A novel *Paenibacillus* sp. was also isolated and has been subjected to physiological and biochemical studies.

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INTRODUCTION

Microbes are a good source of beneficial chemical substances which have been utilized in agriculture and industry. Recent studies reveal that endophytic microorganisms are among various microflora which have a capacity to produce secondary metabolites and various enzymes which can be used in various crucial industries, such as food, pharmaceutical products and textile. Many reports have indicated that plants, used for either medicinal or decorative purpose, are the host of endophytic bacteria which possess a wide range of enzymes (Carrim *et al.* 2006; Jalgaonwala & Mahajan 2011). These enzymes possess varied activities against many substrates, all of which have industrial significance.

Plants are adapted to various environmental conditions. Their growth, development and survival in these environments, particularly in the harsh and fluctuated conditions, such as mangrove forest in the bank of coastal area in Thailand, may be dependent upon other symbiotic microorganisms, particularly endophytic bacteria. In term of ecology, these endophytic bacteria may play a role in the life's cycle of plants in a mangrove environment. In practical term, novel endophytic bacteria which possess exotic characteristics should be subjected to in-depth studies with respect to their physiological and biochemical properties and the knowledge is utilized in both agriculture and industry.

In Thailand, plants in mangrove forest and other related ecological settings have not been subjected to studies with respect to endophytic bacteria yet. This preliminary research is thus conducted to collect plant samples in mangrove forest, to isolate endophytic bacteria and to screen their enzymatic activities.

MATERIALS AND METHODS

Collection of plant materials

Plant materials such as root, inner bark, leave, flower and fruit were collected from trees in the mangrove forest at Pranburi, Prachuap Khiri Khan, Thailand. Plant parts were cut from the trees and put in plastic bags. The collected samples were then kept in ice-box for transportation to the Microbiology Laboratory at the Faculty of Animal Sciences and Agricultural Technology, Silpakorn University, Cha-Am, Phetchaburi, Thailand.

Surface sterilization and isolation of endophytes

The different parts of plant samples were washed with a running tap water to remove soil particles and subsequently surface-sterilized by immersed in 70% ethanol for 5 minutes and 2% of sodium hypochlorite for 10 minutes. Each sample was rinsed 3 times with sterile distilled water to remove those chemical agents. The internal tissues of these plant parts were exposed with sterile surgical blade. The plant tissues of each plant parts were excised with this blade and placed in plates containing LB medium. These plates were incubated at 37°C for 2-7 days. Bacterial isolates were further purified and maintained on LB agar and LB agar slant in a test tube. One loop of the last rinsing liquid was also streaked on the LB agar to check the surface sterilization efficacy (Mei *et al*. 2008).

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Screening of enzyme activity

The production of cellulase, amylase, proteinase and lipase was analysed. Bacteria were inoculated on specific agar medium for each enzyme under investigation. The cultures were incubated at 37°C for 7 days, after which the enzymes were analyzed using specific methodologies (Carrim *et al.* 2006; Jalgaonwala & Mahajan 2011; Khianngam *et al*. 2012). Hydrolysis capacity (HC) value was calculated, based on the method of Gupta *et al*. 2012.

Bacterial identification

The phenotypic characteristics, including morphological, cultural, physiological and biochemical characteristics, were examined at 37°C for 2 days as described by Barrow & Feltham (1993). Hierarchical cluster analysis was performed by using SPSS for Windows version 11.5. The 16S rRNA gene of bacterial isolates was amplifed, and PCR product was purified and sequenced as described by Tanasupawat *et al.* (2004). The sequence bacterial isolates were aligned with selected sequences obtained from GenBank by using CLUSTAL_X version 1.83 (Thompson *et al.* 1997). The phylogenetic tree was constructed by using the neighbour-joining (Saitou & Nei 1987). The confidence values of branches of the phylogenetic trees were determined using bootstrap analyses (Felsenstein 1985), based on 1000 resamplings.

RESULTS AND DISCUSSION

Isolation of endophytes and enzyme activity

A total of 31 endophytic bacteria were isolated from different plant tissues such as root, inner bark, leave, flower and fruit of various mangrove plants at Pranburi, Prachuap Khiri Khan, Thailand (data not shown). Twenty isolates showed promising enzymatic activity by creating a clear zone on an agar medium as shown in Table 1. The results showed the diversity of endophytic bacteria of plant tissues of mangrove tree. These bacteria were capable of producing various hydrolytic enzymes to degrade specific substrate on a medium. Isolate Rhf-2, from a fruit of *Rhizophora mucronata* Poir, was capable of producing a combination of proteinase, lipase, amylase and cellulase activity. However, the majority of the isolated bacteria, had a lipolytic activity.

Bacterial identification

Bacterial isolates were clustered into two major groups (Group A and Group B), based on their phenotypic characteristics as shown in a dendogram (data not shown). Group A contained nine isolates of Gram-negative (Cdfl-1, Sul-2, Xml-1, Sul-1, Ctl-1, Acl-1, Ctf-1, Xml-2 and Tpl-1) and Group B composed of 11 isolates of Gram-positive (Cdf-1, Ctr-1, Ctr-5, Rhf-2, Avr-1, Xmr-1, Cdr-1, Ctr-4, Xmr-2, Avl-1 and Avl-2). Each major group could be subdivided into minor groups (data not shown) based on their phenotypic characteristics.

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Table 1. Enzyme activity of endophytic bacteria isolated from plants in mangrove forest

Host plant	Plant tissue	Isolate no.	Hydrolysis capacity (HC)			
			Cellulase	Amylase	Proteinase	Lipase
Xylocarpus moluccensis Roem.	Root	Xmr-1	4.30		1.23	1.71
	Root	Xmr-2		1.10		1.08
	Leave	Xml-1		1.14		
	Leave	Xml-2		1.44		
Avicennia marina (Forsk.) Vierh	Root	Avr-1	5.20		1.17	1.50
	Leave	Avl-1			2.82	6.14
	Leave	Avl-2	7.14		1.54	1.63
Rhizophora mucronata Poir.	Fruit	Rhf-2	3.36	1.27	1.42	1.36
Ceriops decandra Dirg Hou	Root	Cdr-1	2.61	1.25		1.50
	Fruit	Cdf-1				2.00
	Flower	Cdfl-1		2.00		2.00
Ceriops tagal (Perr) C.B. Rob.	Root	Ctr-1				3.29
	Root	Ctr-4	2.71	1.32		1.19
	Root	Ctr-5				4.00
	Leave	Ctl-1	2.77			
	Fruit	Ctf-1		1.09	1.56	1.23
Suaeda maritima (L.) Dumort	Leave	Sul-1		1.56	2.57	1.82
	Leave	Sul-2		1.88		1.43
Acrostichum aureum L.	Leave	Acl-1	3.33			
Thespesia populneoides (Roxb.) Kostel	Leave	Tpl-1	4.67	1.67		1.83

The phylogenetic tree, based on 16S rRNA gene sequence for the 7 representative isolates, were shown (Figure 1). In Group A, isolates Cdfl-1 (1,512 nt) and Sul-1 (1,437 nt) were closely related to *Curtobacterium citreum* DSM 20528^T (97.5%) and *Pseudomonas psychrotolerans* DSM 15758^T (99.4%), respectively. In Group B, isolates Ctf-1 (1,482 nt), Ctr-5 (1,471 nt), Rhf-2 (1,520 nt), Avl-1 (1,478 nt) and Avl-2 (1,585 nt) were closely related to *Bacillus infantis* JCM 13438^T (99.7%), *B. granadensis* N30135^T (98.8%), *B. safensis* NBRC 100820^T (99.3%), *Staphylococcus warneri* ATCC 27836^T (98.9%) and *Paenibacillus brasilensis* DSM 14914^T (95.7%), respectively (Table 2). Isolate Avl-2 should represent a novel species of the genus *Paenibacillus*, based on low 16S rRNA gene sequence similarity (less than 97%) (Stackebrandt & Goebel 1994).

CONCLUSIONS

In this study, 20 isolates showed proteinase, lipase, amylase and cellulase activity and they were clustered into two major groups, based on their phenotypic characteristics. On the basis of 16S rRNA gene sequence analyses, *C. citreum* isolated from *Ceriops decandra, P.*

Table 2. Species of endophyte bacteria associated with plants in mangrove forest

Group	Isolate no.	Closet species	%similarity	Identification	Host plant	Plant tissue
A	Cdfl-1	*C. citreum* DSM 20528^T	97.5	*C. citreum*	*C. decandra*	Flower
	Sul-1	*P. psychrotolerans* DSM 15758^T	99.4	*P. psychrotolerans*	*S. maritima*	Leave
B	Ctf-1	*B. infantis* JCM 13438^T	99.7	*B. infantis*	*C. tagal*	Fruit
	Ctr-5	*B. granadensis* N30135^T	98.8	*B. granadensis*	*C. tagal*	Root
	Rhf-2	*B. safensis* NBRC 100820^T	99.3	*B. safensis*	*R. mucronata*	Fruit
	Avl-1	*S. warneri* ATCC 27836^T	98.9	*S. warneri*	*A. marina*	Leave
	Avl-2	*P. brasilensis* DSM 14914^T	95.7	*Paenibacillus* sp.	*A. marina*	Leave

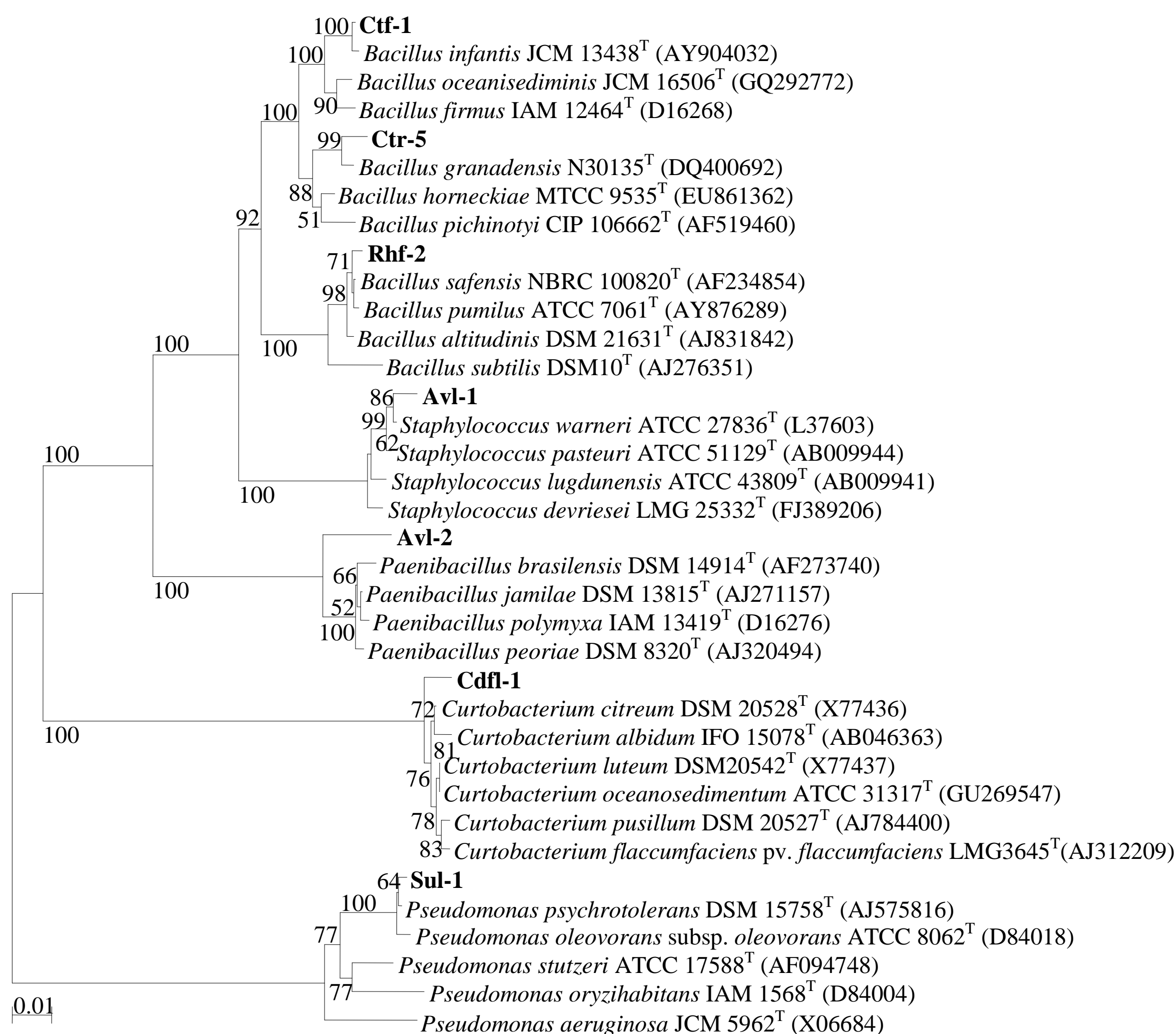

Figure 1. Neighbour-joining tree, based on 16S rRNA gene sequences, showed the phylogenetic relationships between representative isolates in each group. Bootstrap percentage above 50%, based on 1000 resamplings, was also shown. Bar 0.01 represented per nucleotide position.

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psychrotolerans from *Suaeda maritima, B. safensis* from *Rhizophora mucronata* and *S. warneri* from *Avicennia marina* are isolated. In addition, Avl-2 from *Avicennia marina* should represent a novel species of the genus *Paenibacillus.* This is the first report of the isolation of endophytic bacteria of plant tissues from host plants in mangrove forest at Pranburi, Prachuap Khiri Khan, Thailand with production of enzymatic activity. Plants in the mangrove forest are a good source of endophytic bacteria with applicable biotechnological potential.

ACKNOWLEDGEMENTS

We would like to thank Silpakorn University, Phetchaburi IT campus, Cha-Am, Phetchaburi, Thailand, for financial support to attend the International Symposium on Plant Protection and Plant Health in Europe (PPPHE) 2013 in Berlin, Germany.

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Persistent Identifier: urn:nbn:de:0294-sp-2013-ppphe-2

Pootaeng-on Y et al., Investigating the possible role of endophytic bacteria in influencing the level of phytase activity in seedling of maize (*Zea mays* L.) after germination. In: Schneider C, Leifert C, Feldmann F (Eds), Endophytes for plant protection: the state of the art, pp. 285-290. © 2013 Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-11-2.

8-4 Investigating the possible role of endophytic bacteria in influencing the level of phytase activity in seedling of maize (*Zea mays* L.) after germination

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ABSTRACT

Microbes, particularly bacterium, are good source of phytase, an enzyme capable of degrading phytate to *myo*-inositol phosphate intermediates. Seeds of two varieties of maize (*Zea mays* L.), one of which is Suwan 3 (Kasetsart University, Thailand) and the other is Pacific 999 (a commercial variety), were used in the preliminary investigation to determine their phytase activity, after germination. It was found that Suwan 3 had higher phytase activity than Pacific 999, assessing 11 days after germination. Endophytic bacteria were prevalent in the shoot of Suwan 3 at 6 and 11 days after germination. On the other hand, the endophytic bacteria were detected in the embryo of Pacific 999 at 6 and 11 days after germination as well. These isolates were also possessed comparatively high hydrolytic capacity. The spatio-temporal distribution of the endophytic bacteria and their hydrolytic capacity may thus be genotypic-specific.

INTRODUCTION

Maize (*Zea mays* L.) has been cultivated extensively in Thailand as the produce is an essential raw material in producing feedstuff for animal production. Both government agency and private company have played a role in a breeding program to produce maize varieties with required characteristics, such as high yield, resistance to pests and drought, and low phytate content (Weerathaoworn *et al*. 1992; Saengmaneetham *et al*. 2004; Na Chiangmai *et al*. 2011).

It is well established that plant parts are the good source of beneficial microorganisms, which associate with their host either externally or internally. The subject of endophytic organisms has been receiving more attention as these microbes have potential in producing beneficial secondary metabolites and other enzymes (Rai *et al*. 2007).

Endophytic bacteria, particularly *Bacillus* spp. and *Pseudomonas* spp., have been detected in a stem tissue of tropical maize (Figueiredo *et al*. 2009). *B. amyloliquefaciens*, a plant-growth-promoting rhizobacteria, has been reported to possess a capacity to degrade phytate through the activity of extracellular phytase, making this bacterium an important tool for promoting plant growth under the unavailability of phosphate (Idriss *et al*. 2002).

Isolates of *Bacillus* spp., which have been isolated from the inside tissue of various maize seedling, may also be capable of producing the phytase enzyme and it may play an important role in the growth and establishment of maize seedling in the field.

This study investigates the species of endophytic bacteria, associating with three parts of maize seedling (shoot, root and embryo) of both Suwan 3 (Kasetsart University, Thailand) and Pacific 999 (a commercial variety). The phytase activity of both maize varieties and the isolated bacteria is also determined.

MATERIALS AND METHODS

Maize samples used in the test

Seeds of Suwan 3 were obtained from The Inseechandrastitya Institute for Drops Research and Development (IICRD), Kasetsart Univeristy, Pakchong, Nakhonratchasima, Thailand, while those of Pacific 999 was purchased from the store at Tha-Yang, Phetchaburi, Thailand.

Germination of maize grains

Both Suwan 3 and Pacific 999 seeds were germinated in the laboratory. For surface sterilization, seeds were soaked in 2% NaOCl for 5 mins. After soaking, seeds were thoroughly rinsed with sterile water and soaked in sterile water for a period of 24 hours at room temperature (28±2°C) (Senna *et al*. 2006). The pre-treated seeds were placed on a sterile paper (used for germination testing) before sterile water was added to maintain appropriate humidity. The seeds were then allowed to germinate in disinfected dark cupboards at room temperature (28±2°C) for 1–11 days. The germinating seeds were moistened with sterile water at regular intervals. The seedlings were sampled at intervals of 1, 6 and 11 days. These seedlings were cut with sterile surgical blade into three parts, such as shoot, root and embryo for phytase assay, identification and isolation of endophytic bacteria.

Isolation of endophytic bacteria from parts of maize seedling

The internal tissues of these plant parts were exposed with sterile surgical blade after surface sterilization. The plant tissues of each plant part was then excised with this blade and placed in plates containing LB medium (Jalgaonwala & Mahajan 2011). These plates were

incubated at room temperature (28±2°C) for 2-7 days, in which the plates were inspected for the sign of bacterial growth. Bacterial isolates were further purified and maintained on LB agar and LB agar slant in a test tube.

Bacterial identification and detection of phytase production

Isolates of endophytic bacteria were tested for their phytase activity on PSM medium (Saairekha *et al*. 2012). The cultures were incubated at room temperature (28±2°C) for 7 days, after which the enzymes were analyzed using specific methodologies (Yanke *et al*. 1998). Hydrolysis capacity (HC) value was calculated, based on the method of Gupta *et al*. (2012).

Phytase assay in maize tissues

Tissues of three seedlings parts of both Suwan 3 and Pacific 999 varieties were also subjected to phytase analysis, using the methods as described by Senna *et al*. (2006) and Bae *et al.* (1999). The liberated inorganic phosphate (Pi) was quantified colometrically, using ammonium molybdate as described by Yanke *et al*. (1998). One unit (U) of phytase was defined as the amount of enzyme required to liberate 1 µmol inorganic phosphate per minute under test condition (pH 5.0, 55°C, 0.36 mM Na-phytate).

RESULTS AND DISCUSSIONS

Increasing activity of phytase enzyme in cereals was detected in seed and seedling tissues in the germination process and during the course of seedling growth (Senna *et al*. 2006). In this experiment, the pattern of phytase activity in seeds and seedling tissues (of both Pacific 999 and Suwan 3) in the germination process and during the course of seedling growth was similar to the report of Senna *et al*. (2006).

A value of phytase activity in each parts of maize seedling had no relationship with the presence or the absence of endophytic bacteria and the value of phytase activity as assessed by hydrolysis capacity (Table 1). For instance, phytase activity was quite high in the shoot of Pacific 999 (at 6 days after germination), while the endophytic bacteria was absence in the shoot of this maize.

In contrast, there was comparatively high phytase activity in embryo of Suwan 3 (at 11 days after germination), even though endophytic bacteria present in this embryo possessed weak hydrolysis capacity (Table 1). It is thus possible that there is a specific association of endophytic bacteria with each maize genotype. The spatio-temporal distribution of the endophytic bacteria and their hydrolytic capacity may also be genotypic-specific (Engelhard *et al.* 2000). The relationship of endophytic bacteria with plants is least understood (Sturz *et al.* 2000), so does their role in affecting plant in producing phytase enzyme.

Nonetheless, maize (Suwan 3), a composite variety, possessed the higher numbers of endophytic bacteria than Pacific 999; in which each isolate of bacteria associating with parts of Suwan 3 seedling had relatively high phytase activity (Table 1). It is thus plausible that

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maize Suwan 3 is a favorable habitat for endophytic bacteria, possessing relatively high phytase activity as assessed by hydrolysis capacity.

Table 1 Phytase activity of both maize seedlings (Pacific 999 and Suwan 3) and bacteria isolated from different parts of maize seedling at 1, 6 and 11 days after germination.

Day after germination		Phytase activity of seedling (U/kg DM)		Phytase activity from hydrolysis capacity (HC) of isolates			
		Pacific 999	Suwan 3	Paciffic 999		Suwan 3	
days	parts			Isolate no.	HC	Isolates no.	HC
1	embryo	35.5 ± 4.4	144.9 ± 24.7	Pef-1 Pef-2	w 3.00	Sef-1 Sef-2 Sef-3	1.33 1.71 2.40
6	shoot	407.6 ± 294.6	153.8 ± 75.2	-	-	Sps-1 Sps-2 Sps-3 Sps-4 Sps-5	2.00 4.00 1.67 3.00 3.40
	root	379.8 ± 114.9	251.2 ± 99.9	Prs-2	2.40	Srs-2 Srs-3	3.60 4.00
	embryo	167.8 ± 35.9	347.2 ± 25.6	Pes-1 Pes-2 Pes-4	3.11 1.89 4.60	Ses-3	3.50
11	shoot	419.8 ± 113.9	845.7 ± 64.3	-	-	Sle-1 Sle-2 Sle-3 Sle-4 Sle-5 Sle-6	w 3.25 1.67 1.75 1.83 1.71
	root	258.9 ± 94.8	683.7 ± 55.3	Pre-2 Pre-3	2.00 5.20	Sre-3 Sre-4	1.67 1.69
	embryo	251.9 ± 51.8	692.7 ± 146.8	Pee-1 Pee-3 Pee-4	1.33 3.00 2.80	See-1 See-3	1.31 w

w = weakly positive clearance zone

Persistent Identifier: urn:nbn:de:0294-sp-2013-ppphe-2

ACKNOWLEDGEMENTS

"We would like to thank Silpakorn University, Phetchaburi IT campus, Phetchaburi, Thailand, for financial support to attend the conference the International Symposium on Plant Protection and Plant Health in Europe (PPPHE) 2013 in Berlin, Germany. This work was support by Grant from Faculty of Animal Science and Agricultural Technology, Silpakorn University, Phetchaburi IT campus, Phetchaburi, Thailand. We are grateful to The Inseechandrastitya Institute for Drops Research and Development (IICRD), Kasetsart Univeristy, Nakhonratchasima, Thailand for providing seeds of maize (Suwan 3)."

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Persistent Identifier: urn:nbn:de:0294-sp-2013-ppphe-2

Nguyen H; Franco C; Vadakattu G, Interactions between selected endophytic actinobacteria and phosphate solubilizing Penicillium spp. for the growth of wheat. In: Schneider C, Leifert C, Feldmann F (Eds), Endophytes for plant protection: the state of the art, pp.291-292. © 2013 Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-11-2.

8-5 Interactions between selected endophytic actinobacteria and phosphate solubilizing *Penicillium* spp. for the growth of wheat.

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ABSTRACT

This study was carried out to investigate the interactions between two types of microorganism that are beneficial to agriculture-endophytic actinobacteria and phosphate solubilizing fungi on the growth of wheat plants. Each of the 6 wheat endophytes that are effective in controlling a range of fungal root pathogens in the field. (Streptomyces EN16, EN23, EN27 and EN28; *Microbispora* EN2 and *Nocardioides* EN46) were evaluated separately with two strains of phosphate solubilizing fungi- *Penicillium* P21 and P28- and the effects on the growth of wheat plants were recorded and analysed.

As soils with a Phosphorus (P) status tends to limit the production of arable crops, the application of soluble P fertilisers such as super-phosphate is widely used. However, much of the soluble P applied reacts with the soil and is converted to forms less available to plants. Phosphate-solubilising fungi are able to improve the P nutrition of plants and thus stimulate plant growth. In addition, actinobacteria are economically important microorganisms due to their ability to produce a range of secondary metabolites with biological activities. Our laboratory has identified endophytic actinobacteria that have the ability to control fungal root pathogens in cereal crops such as wheat (Conn & Franco, 2004).

Therefore, due to their antifungal activity it is hypothesized that the interaction of endophytic actinobacteria with *Penicillium* spp. decreases the phosphate solubilization leading to the decreased germination and growth of wheat plants.

In vitro analysis of the antagonism of the six actinobacteria versus two *Penicillium* spp. P21 and P28 showed that none of the actinobacteria inhibited fungal growth but that *Microbispora* sp. EN2 showed strongest inhibition of sporulation whereas *Streptomyces*

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EN16, EN27, and *Nocardioides albus* EN46 did not inhibit spore formation of either *Penicillium* spp. 2

The effect of different *Penicillium* concentrations on the growth of wheat was screened in two types of soils from different area in South Australia. Shoot and root weight were higher in plants receiving a low concentration of 107 CFU of *Penicillium* in both types of soils.

The interaction between beneficial isolates of endophytic actinobacteria, including *Streptomyces* spp. EN16, EN27 and *Nocardioides* sp.EN46 which showed no *in vitro* inhibition against *Penicillium*, and two *Penicillium* (P21 and P28) were studied in wheat (*Triticum aestivum* cv. Yitpi) in a pot experiment. Plant dried matter yield did not show any significant difference between treatments after four weeks of planting. However, after six weeks, the shoot dry weights increased significantly ($p<0.05$) by 22.62% and 10.05% in treatments with *Penicillium* P21 and P28, respectively. The actinobacterial treatments did have any significant effect on either shoot or root dry weight. However, root and shoot dry weights decreased in all plants at 6 weeks treated with the combination of *Penicillium* and actinobacteria when compared to the control plants treated with either *Penicillium* sp. alone. The total P concentrations in all plants co-inoculated with both types of beneficial microorganisms also decreased significantly. Of note, the percentage of root length colonization of Arbuscular Mycorrhizhae (AM) in treatments with both *Penicillium* strains was considerably higher than in the roots of control untreated plants and single treatments with actinobacteria. However, AM fungal colonisation was significantly higher in co-inoculation treatments of both *Penicllium* spp. and 2 of the actinobacteria, compared to either the untreated plants or the plants treated with the fungus alone.

Therefore, the results of these glasshouse studies indicate that the interactions of the different beneficial microorganisms appears to support the hypothesis that there will be a negative effect on plant growth. However, the mechanism involved may not be a direct inhibition of fungal growth and activity, but may be a more complex interaction. Moreover, the role of naturally occurring AM fungi in these plants adds a further complexity to this 3-way interaction.

In summary, in practical terms, we do not recommend the co-inoculation of biocontrol actinobacterial inocula with phosphate-solubilising fungi.

Persistent Identifier: urn:nbn:de:0294-sp-2013-ppphe-2

Sarjala T, Effect of endophytic root-associated fungi of Scots pine on seedling growth and polyamines. In: Schneider C, Leifert C, Feldmann F (Eds), Endophytes for plant protection: the state of the art, pp. 293-294. © 2013 Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-11-2.

8-6 Effect of endophytic root-associated fungi of Scots pine on seedling growth and polyamines

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ABSTRACT

Drained peatland forests represent a harsh environment for growth of Scots pine seedlings, which may be subjected to stress during their early development. Planted seedlings in drained peatland forest sites may face several varying abiotic stress conditions. Ectomycorrhizal (ECM) fungal symbionts are not abundantly available there for promoting early seedling growth or for stress protection. Therefore, our aim was to study whether there are endophytic root-associated fungi in Scots pine and what could be their role in the early development of pine seedlings under harsh conditions.

We isolated 191 root-associated fungal strains on pure culture from eight-year-old Scots pine trees grown on drained peatland and thereafter cultivated the fungi on agar plates for further studies and identification with DNA sequencing. Eleven fungal strains were used for inoculation of young, aseptically grown Scots pine seedlings. After one month of dual culture of the Scots pine seedlings with the fungi, the growth of the seedlings and polyamine (putrescine, spermidine, spermine) concentrations of the roots and needles were measured with HPLC. Specific changes in polyamine concentrations in the host plant have been suggested to be related to early stage growth induction by ECM fungi (Sarjala et al. 2010). On the other hand, polyamines are also known to be involved in defense responses against biotic and abiotic stresses in plants (Liu et al. 2008, Tang et al. 2007).

The inoculation with fungus mycelium did not significantly affect the number of root tips per seedling, the biomass of the roots or shoots, or the length of the seedlings. These results were in contrast to the effects observed earlier with ECM fungi inoculation even before a true mycorrhizal structure formation (Sarjala et al. 2010).

Inoculation had no effect on free putrescine or spermine concentrations and only two fungus strains increased free spermidine in the roots, whereas ECM fungi have been shown to

highly increase root polyamine concentrations. In the needles, only a few fungus strains either increased or decreased free polyamine concentrations, whereas with ECM species a significant increase in needle putrescine as well as significant decreases in spermidine and spermine could be seen soon after inoculation of the seedlings at the pre-mycorrhizal stage (Sarjala et al. 2010).

In conclusion, the endophytic root-associated fungi did not have similar effects as ECM on early growth or polyamine concentrations of Scots pine seedlings after inoculation in this study. The role of endophytic fungi during early growth of Scots pine seedlings seemed to differ from that of ECM, which have been demonstrated to promote the growth of seedlings immediately after inoculation at the pre-mycorrhizal stage. However, variation in host responses seen in polyamine concentrations between the inoculation treatments revealed the diversity in the species-specific effects on host physiology.

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Persistent Identifier: urn:nbn:de:0294-sp-2013-ppphe-2

SESSION 9

IUFRO Devision 7.02.04 – Phytoplasma and virus diseases of forest trees

Workshop: **Virus and phytoplasma diseases of forest and urban trees** - a satellite meeting within the 5th International Symposium on Plant Health in Europe

Date: 29. May 2013, 9:30-18:00

Organizer: DPG, ALVA, JKI, IAPPS, IUBS and COST

Section A:

Viruses affecting broad leafed urban and forest trees

Persistent Identifier: urn:nbn:de:0294-sp-2013-ppphe-2

Mühlbach H-P, The novel genus *Emaravirus*: Plant viruses with multipartite negative strand RNA genomes. In: Schneider C, Leifert C, Feldmann F (Eds), Endophytes for plant protection: the state of the art, pp. 296-297. © 2013 Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-11-2.

9-1 The novel genus *Emaravirus*: Plant viruses with multipartite negative strand RNA genomes

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INTRODUCTION

The genus *Emaravirus* harbours three virus species at present, the *European mountain ash ringspot-associated virus* (EMARaV), *Fig mosaic virus* (FMV) and *Rose rosette virus* (RRV). However, there are some more unassigned viruses discussed as putative members, such as raspberry leaf blotch virus (RLBV), pigeonpea sterility mosaic virus (PPSMV), maize red stripe virus (MRSV) or redbud yellow ringspot associated virus (Mielke-Ehret & Mühlbach 2012). EMARaV was the first member of the genus, which was established after sequencing four genomic RNA molecules (Mielke & Mühlbach 2007), based on dsRNA preparations from ringspot affected mountain ash leaves (Benthack *et al*. 2005). The genomes of Emaraviruses consist of at least four to seven single stranded RNA molecules of negative polarity. Each of these RNAs encodes in its complementary form a single polypeptide. Sequence analyses indicated for EMARaV, FMV, RRV, and RLBV that RNA 1 encodes the viral RNA dependent RNA polymerase, RNA 2 a glycoprotein precursor which is processed into two glycoproteins Gn and Gc, RNA 3 carries the nucleocapsid protein, and RNA 4 encodes a protein (P4) of yet undetermined function. In some cases, double membrane-bound virus-like particles of 80 to 200 nm in diameter were found in infected tissue, but detailed particle morphology has yet to be elucidated. Furthermore, evidence is accumulating that Emaraviruses and putative members of the genus are vectored by eriophyid mites. Some Emaraviruses such as FMV are worldwide distributed (Mielke-Ehret & Mühlbach 2012). In contrast, EMARaV itself was mostly found in Middle and Northern Europe (Robel *et al*. 2013).

Persistent Identifier: urn:nbn:de:0294-sp-2013-ppphe-2

MATERIAL AND METHODS

In order to address biological functions of EMARaV, *in vivo* and *in vitro* studies were undertaken. Temporal and spatial distribution of viral RNAs was studied by realtime RT-PCR. The subcellular localisation of recombinant viral proteins P2 and P3 was investigated by using isolated protoplasts from tobacco (*Nicotiana rustica*) leaves. The role of P4 was investigated in *Drosophila* S2 cells using the lacZ silencing system.

RESULTS AND DISCUSSION

Quantitative RT-PCR revealed the continuous presence of genomic RNAs of EMARaV in leaves, leaf buds and phloem with slight variations throughout the year. The amount of RNA 2 was always higher than the other RNAs. Copy numbers of viral genomic RNAs were higher than those of complementary (mRNA) molecules (Schlatermund 2008). In expression studies with the recombinant P2 of EMARaV in tobacco protoplasts the glycoproteins colocalized with the Golgi apparatus. Serological detection of Gn, Gc, P3, and P4 in leaves of *S. aucuparia* indicated accumulation of the glycoproteins and P4 in the lower epidermis, which suggests a role of these proteins in the vector transmission of the virus. The putative nucleocapsid protein P3 was found in almost all cell types in the leaf (Ikogho, unpublished). The use of the lacZ silencing system in insect cells suggested for the first time the role of P4 as silencing suppressor, but deserves further investigation (Klode, unpublished).

ACKNOWLEDGEMENTS

The technical assistance of Dagmar Svensson and Heidrun Meyer is gratefully acknowledged. Experimental studies were funded by the DFG through grants Mu 559/8-1 to 8-6.

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Persistent Identifier: urn:nbn:de:0294-sp-2013-ppphe-2

Robel J et al., Variability of the p3 and p4-coding genome region of *European mountain ash ringspot-associated virus* (EMARaV) in *Sorbus aucuparia* of different European regions. In: Schneider C, Leifert C, Feldmann F (Eds), Endophytes for plant protection: the state of the art, pp. 298-299. © 2013 Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-11-2.

9-2 Variability of the p3 and p4-coding genome region of *European mountain ash ringspot-associated virus* (EMARaV) in *Sorbus aucuparia* of different European regions

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INTRODUCTION

Virus-like symptoms on rowan trees (*Sorbus aucuparia* L.) such as chlorotic ringspots and mottling on leaves, were first described six decades ago (Kegler 1960). In 2007 the symptoms were associated with the *European mountain ash ringspot-associated virus* (EMARaV) (Mielke *et al*. 2007), the type-species of the newly established genus *Emaravirus* (Mühlbach & Mielke-Ehret 2011). The genome of EMARaV consists of four single stranded RNAs (RNA1-RNA4) of negative polarity. Each segment encodes a single protein (P1-P4) translated from the complementary strand. In previous studies, EMARaV variants from different parts of south Finland and Russia were analyzed with respect to the genetic variability of the nucleocapsid-coding region of the viral RNA3 (Kallinen *et al*. 2009, Valkonen & Rännäli 2010). However, these variants showed a low genetic variability within the coding region of the nucleocapsid protein (P3). In this study, genetic variability of P3 of EMARaV variants obtained from infected rowans from Germany, Sweden, Scotland and Finland was compared with the sequences of those samples from southern Finland and Russia. Further, sequence diversity of the non-structural viral protein P4 was investigated.

MATERIAL AND METHODS

Total RNA from symptomatic leaves sampled from infected rowans from Germany, Sweden, Scotland and Finland was isolated according to the protocol by Mielke *et al*. (2007). Viral

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RNA3 and RNA4 were amplified by RT-PCR with specific primer pairs. The amplification of three fragments from the RNA3 was performed according to Kallinen *et al*. (2009). The RNA4 encoded P4 protein was amplified with P4-specific primers. The PCR products amplified from RNA3 were directly sequenced. The P4 specific PCR products were cloned prior to sequencing. Neighbour-joining phylogenetic trees were generated using ClustalX 2.0 (Larkin *et al*. 2007).

RESULTS AND DISCUSSION

Amplicons of the expected size (588 bp, 665 bp & 878 bp, RNA3; 699 bp, RNA4) were generated from 18 EMARaV infected rowans of different stands and countries. After sequencing the variability of the non-structural protein P4 and the structural protein P3 were compared with respect to nucleotide and amino acid sequences. Similar results to Kallinen *et al*. (2009) were obtained by comparison of amino acid sequences of P3 from Germany, Finland and Sweden (99-100 %). The Scottish EMARaV variants showed lower identities between 97-99 %. and formed a distinct cluster in phylogenetic analyses. Comparison of the P4 coding-region also showed high sequence conservation (98-100 %). However, phylogenetic analysis did not result in an independent cluster of the Scottish variants.

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Persistent Identifier: urn:nbn:de:0294-sp-2013-ppphe-2

Eisold A-M et al., Characterisation of an unknown putative virus from *Ulmus laevis*. In: Schneider C, Leifert C, Feldmann F (Eds), Endophytes for plant protection: the state of the art, pp. 300-301. © 2013 Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-11-2.

9-3 Characterisation of an unknown putative virus from *Ulmus laevis*

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INTRODUCTION

Scientific findings indicate that viruses are widespread in trees of forests and urban green, and may cause losses of economic importance (Büttner *et al.* in press).

Over many years a monitoring was carried out with over 100 years old European White Elms (*Ulmus laevis* PALL.) in the park of Caputh near Berlin. During the last years, they showed leaf symptoms such as chlorotic ringspots, necroses and dieback, suggesting a virus as causal agent (Büttner & Führling 1993). Fungal and bacterial pathogens as causal agent of these symptoms could be excluded by microbiological assays. Also an infection by well studied viruses such as *Cherry leaf roll virus* (CLRV), *Elm mottle virus* (EMV), *Arabis mosaic virus* (ArMV) and *Tobacco ringspot virus* (TRSV) could not be confirmed. However, some facts point to a member of the genus *Carlavirus* (Bandte *et al.* 2004).

The aim of the present study is the isolation and characterization of a putative viral pathogen which is so far unknown. Based upon the assumption that the causal agent belongs to the genus *Carlavirus*, we apply different specific molecular biological and biochemical methods to identify the putative virus.

MATERIAL AND METHODS

Leaf material from 15 elms showing characteristic symptoms was collected in late spring over 3 years. The causal agent was transmitted mechanically from elm to *Chenopodium quinoa* by inoculation of leaves with homogenized bulbs from symptomatic elms. In order to scrutinize the hypothesis of a *Carlavirus* infection, nucleic acids were isolated from collected elm leaves as well as from inoculated *C. quinoa.* For cDNA synthesis i) random hexamers, ii) oligo-dT-primers or iii) *Carlavirus*-specific primers were used (Nie *et al.* 2008). Subsequently, RT-PCR was conducted with material from elm and *C. quinoa*

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applying *Carlavirus*-specific primers (Nie *et al*. 2008). Material infected with *Potato latent virus* (PotLV) and *Potato virus M* (PVM) provided by the Deutsche Sammlung Mikroorganismen und Zellkulturen (DSMZ) was used as positive control. For a partial virus purification symptomatic elm leaf material was used. To visualize the coat protein of the putative member of the *Carlavirus* aliquots of partial virus purification were electrophoretically separated by SDS-PAGE. Furthermore, RNA was isolated from the purification and used for RT-PCR as described above.

RESULTS AND OUTLOOK

Meanwhile the European White Elm belongs to the endangered woody species of Germany. Therefore is a need to investigate diseases on this species and viral infections play an important role in the degeneration process.

Using described methods, it was not possible to confirm the pathogen as a member of the genus *Carlavirus* by now. Successfully inoculated *C. quinoa* showed deformation, chloroses and necroses on leaves. By RT-PCR with material from elm and *C. quinoa* several fragments of different sizes were generated. After sequencing, they did not show accordance to published *Carlavirus* sequences.

Current and future studies will have a focus on dsRNA. Hence, dsRNA will be isolated and used for random PCR according to Froussard (1992). Amplicons will be cloned, sequenced and compared with sequence information from the NCBI-database. Furthermore, virus particles will be purified directly from symptomatic elm leaves as well as from inoculated *C. quinoa*. The morphology of the virus particles will be investigated by transmission electron microscopy.

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Valverde R A, Viral DSRNA: A Useful Reagent for the Identification and Characterization of Plant Viruses. In: Schneider C, Leifert C, Feldmann F (Eds), Endophytes for plant protection: the state of the art, pp. 302-303. © 2013 Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-11-2.

9-4 Viral DSRNA: A Useful Reagent for the Identification and Characterization of Plant Viruses

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ABSTRACT

Single-stranded RNA (ssRNA) viruses make up over 90% of all known plant viruses. During the replication of these viruses in plant cells, double-stranded ribonucleic acid (dsRNA) is produced as an intermediate product. This dsRNA is consistently present in plants infected with a ssRNA viruses, regardless of the host. DsRNAs that correspond to subgenomic RNAs are also produced. Morris and Dodds (1979) developed a method for the isolation and analysis of dsRNA from virus-infected plants. This method has been used in the detection, identification, and characterization of plant viruses. The method consists of the selective adsorption of dsRNA to fibrous cellulose at low ethanol concentrations and elution with ethanol free buffer (Figure 1). Detailed information regarding the applications of the dsRNA analysis to plant virus diagnosis has been published (Dodds, *et al 19*84; Valverde *et al*. 1986; 1990).

In general, different families of plant viruses have characteristic eletrophoretic dsRNA patterns that differentiate them from others and members of a genus tend to have similar dsRNA profiles (Valverde *et al.* 1986). Analysis of dsRNA extracted from tissues of a plant infected with a monopartite ssRNA genome virus should result in one dsRNA of twice the molecular weight of the ssRNA. Similarly, an infection with a virus with a bipartite genome should yield two dsRNA bands. In practice, however, other minor (less prominent) dsRNAs of lower molecular weight are obtained. It has been shown that some of these other dsRNAs are the replicative form of subgenomic RNAs. Other sources of dsRNAs from plants infected with RNA viruses are infections by satellite viruses (or satellite RNAs) and persistent dsRNA viruses (Roossinck, 2010; Valverde *et al*. 1986; 1990). Persistent dsRNA viruses include members of the *Chrysoviridae, Edornaviridae, Partitiviridae, and Totiviridae*. These viruses have not been shown to cause disease. Nevertheless, they are important because their banding profile could be confused with that of a ssRNA virus. In most cases these viruses are restricted to a particular cultivar of a plant species and are transmitted only vertically.

One advantage of extracting dsRNA from plants infected with RNA viruses is that one can detect mixed viral infections. Mixed infections often go undetected and can result in inadequate disease diagnoses. Unlike most other diagnostic techniques, dsRNA analysis is nonspecific and dsRNA is obtained regardless of the host or the RNA virus. Some ssRNA viruses are very unstable or infect hosts that contain excessive amounts of phenolic compounds and carbohydrates. Therefore, their purification for identification and characterization is not easy. One alternative is to utilize the dsRNA extraction method which could yield viral dsRNA that can be used as a reagent for RT- PCR, probe preparation, and /or molecular cloning.

The use of dsRNA analysis for virus identification or characterization has some limitations. Certain viral families, such as the *Potyviridae, Luteoviridae*, and *Rhabdoviridae*, yield very low quantities of dsRNA. Nevertheless, small amounts of viral dsRNA could be used for PCR or cloning.

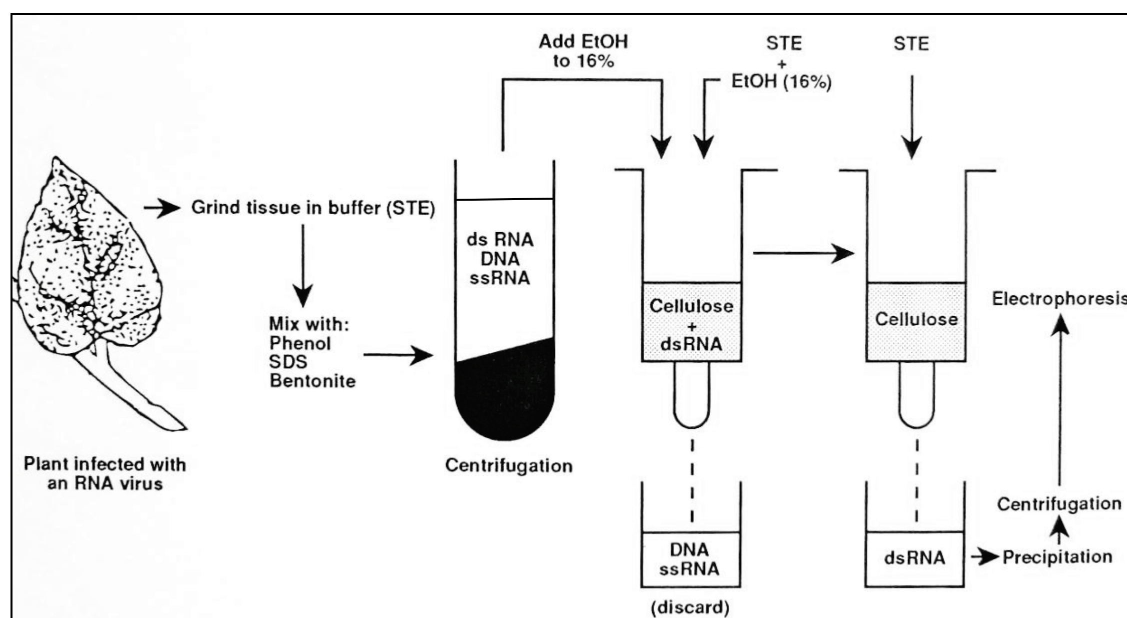

Figure 1. Schematic diagram of the dsRNA extraction method from plant tissues.

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Persistent Identifier: urn:nbn:de:0294-sp-2013-ppphe-2

IUFRO Devision 7.02.04 – Phytoplasma and virus diseases of forest trees

Workshop: Virus and phytoplasma diseases of forest and urban trees

Section B: Nepoviruses

Persistent Identifier: urn:nbn:de:0294-sp-2013-ppphe-2

Jalkanen R, von Bargen S, Büttner C, Frequency of the birch leaf-roll disease caused by *Cherry leaf roll virus* in Fennoscandia. In: Schneider C, Leifert C, Feldmann F (Eds), Endophytes for plant protection: the state of the art, pp. 305-306. © 2013 Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-11-2.

9-5 Frequency of the birch leaf-roll disease caused by *Cherry leaf roll virus* in Fennoscandia

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INTRODUCTION

Virus-related symptoms were increasingly found on birch leaves throughout northern Fennoscandia during the last ten years. Disease symptoms occurring abundant on downy (*Betula pubescens*) and silver birch (*B. pendula*) and many other native birch species and subspecies in Finland could be associated with an infection of *Cherry leaf roll virus* (CLRV) (Jalkanen *et al*. 2007; von Bargen *et al*. 2009). Symptomatic birch trees could be seen abundant in parks, street sides, roadsides and forests throughout the northern areas.

Although two silver birch saplings had been recorded to carry CLRV in Finland as early as in the 1980s (Bremer *et al*. 1991), the disease, hereafter birch leaf-roll disease, was described no earlier than in 2006 (Jalkanen *et al*. 2007). At the same time when the disease became common throughout the country in 2003–2006 (von Bargen *et al*. 2009), the disease spread dramatically also vertically so that some trees have strongly declined and even died due to the birch leaf-roll disease. As no data existed about the frequencies of symptomatic birches, we started to monitor first urban and later also forest areas countrywide.

MATERIAL AND METHODS

We monitored disease frequencies mainly in birch alleys of town centres in 2006–2012. For objectivity and if available, our main target in each town or village to be examined was to choose the street leading to the main church and named with Kirkkokatu or Kyrksgatan (= Church street) or similar, having a birch alley. Depending on the length of the alley and the number of birch trees along the alley, the street was entirely or from its most representative

part assessed for symptoms of the birch leaf-roll disease. In Rovaniemi and Kajaani, Finland, several locations were surveyed. It was repeated annually once to four times in some of the alleys in Rovaniemi, Kajaani and Nurmes. Two of the Rovaniemi sites represent forest environment. Disease frequency for the forest land was obtained by assessing one fifth of the permanent sample plots of the Finnish National Forest Inventory (NFI) for Finland in 2010. Total number of the surveyed birches was 4286 (1644 in towns and two forest road side locations) in Finland and 310 (six urban locations) in northern Sweden. The tree material consists of a mixture of downy (majority) and silver birch. Trees were monitored mainly between mid-July and mid-August to attain the best symptoms appearance in field assessment. A birch was assessed positive, i.e. symptomatic if it displayed some of the symptoms associated with a CLRV infection such as leaf roll, veinbanding, chlorotic ringspots, proliferation, reduced leaf size, necrotic lesions, and tip dieback.

RESULTS AND DISCUSSION

Average frequency of diseased trees associated with CLRV infection in town alleys was clearly lower in Sweden (15.5%) than Finland (28.3%). Street-based-values varied from zero to 71.4%. The frequency of symptomatic trees normally increased annually, with the feature that once symptomatic always symptomatic. However, it was recorded for the first time that several birches, which appeared very slightly symptomatic earlier, had no symptoms in the cool summer of 2012. More data about the disease in forests are needed because the frequencies of the two forest road side locations were very high, 40.8 and 45.7%, as compared to the NFI value of 0.23% only. In repeated surveys it became evident that personnel responsible for urban trees replace severely declined birches with a young specimen.

ACKNOWLEDGEMENTS

Research was partly funded by the DFG-grant BU890/15-1.

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Persistent Identifier: urn:nbn:de:0294-sp-2013-ppphe-2

von Bargen S, Langer J, Büttner C, Genome Organization of *Cherry Leaf Roll Virus* and Comparative Analyses of RNA2-Encoded Proteins. In: Schneider C, Leifert C, Feldmann F (Eds), Endophytes for plant protection: the state of the art, pp. 307-308. © 2013 Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-11-2.

9-6 Genome organization of *Cherry Leaf Roll Virus* and Comparative analyses of RNA2-encoded proteins

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INTRODUCTION

Cherry leaf roll virus (CLRV) infects many deciduous trees and shrubs including important species of European forests such as several birch species (*Betula* spp.), rowan, (*Sorbus aucuparia*), beech (*Fagus sylvatica*), ash (*Fraxinus excelsior*), elderberry (*Sambucus* spp.). Moreover, several stone fruits such as cherry (*Prunus avium*), walnut (*Juglans* spp.), olive (*Olea europaea*) are affected by the virus (Büttner *et al.* 2011, 2013). Recently, the complete genome was determined for a rhubarb isolate (von Bargen *et al*. 2012) and a cherry strain (Eastwell *et al*. 2012) revealing the genome organization of the bipartite positive stranded plant RNA virus (*Secoviridae*, *Nepovirus*).

MATERIAL AND METHODS

CLRV isolates originating from rhubarb (Bornheim, Germany) and sweet cherry (Bonn, Germany) were propagated in *Chenopodium quinoa*. A combination of methods was employed for generation of full length sequences of virus isolates. Either random primed cDNA libraries were produced from virus purifications according to Froussard (1992) or purified viral RNA was subjected to illumina´s sequencing by synthesis protocol. Short reads with an average size of 35 b were assembled and internal gaps were closed by sequencing of RT-PCR products employing virus specific primers applying total RNA prepared from *C. quinoa* infected with the respective virus isolate. The 3´ untranslated regions were amplified using RNA1 or RNA2 specific forward primers in combination with M4 primer and a M4T-primed cDNA targeting the polyA-tail as described by Chen *et al.* (2001). 5´ termini were determined by inverse PCR (Ochman *et al.* 1990).

Persistent Identifier: urn:nbn:de:0294-sp-2013-ppphe-2

RESULTS AND DISCUSSION

RNA1 encodes a polyprotein (P1) containing domains characteristic for a proteinase cofactor (PCo), a nucleotide-binding helicase (Hel), a genome-linked protein (VPg), a proteinase (Pro), and a RNA dependent RNA polymerase (Pol). The RNA2-encoded polyprotein (P2) comprises the putative movement protein (MP) and the coat protein (CP) of CLRV. The genome region upstream of the MP has a coding capacity of 77 kDa, however the function of the encoded peptide is unclear. Comparative sequence analyses of CLRV isolates from rhubarb and cherry revealed that the RNA2 is less conserved than the RNA1 of the virus. Among isolates the region encoding the putative X4 protein on RNA2 shows maximal 44 % identity on amino acid level. The putative X4 and MP encoded by the cherry isolates also differ in size from the rhubarb strain. In contrast to the rhubarb isolate, both genomic segments of cherry isolates contain a 2nd in frame ATG located 69 nucleotides downstream of the 1st ATG.

ACKNOWLEDGEMENTS

We thank Dr. Joachim Hamacher for providing virus isolates. Research was funded by DFG-grants BU890/14-1 and BA3961/2-1.

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Dierker L, von Bargen S, Büttner C, Identification of protein-protein-Interactions in the host-pathogen-system *Arabidopsis thaliana/Cherry leaf roll virus* (CLRV). In: Schneider C, Leifert C, Feldmann F (Eds), Endophytes for plant protection: the state of the art, pp. 309-310. © 2013 Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-11-2.

9-7 Identification of protein-protein-Interactions in the host-pathogen-system *Arabidopsis thaliana/Cherry leaf roll virus* (CLRV)

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INTRODUCTION

Cherry leaf roll virus (CLRV) is a worldwide distributed *Nepovirus* (family *Secoviridae*) that infects a wide range of herbaceous and woody plants (Büttner *et al*. 2011). In regard to appearance, symptom severity and crop losses an infection by CLRV has a high economic relevance as it is known from fruit trees such as cherry (*Prunus* spp.) or English walnut (*Juglans regia* L). CLRV is naturally transmitted by pollen and seed, but knowledge on mechanisms is still missing. In this study, specific interactions between the CLRV-movement protein (MP, 385 aa, 42 kDa), the coat protein (CP, 512 aa, 56 kDa) and plant proteins were investigated applying the yeast two-hybrid system (YTHS). Systemic infection of the host plant including the reproductive organs by the virus is achieved by cell-to-cell movement via plasmodesmata and long distance transport through the vascular system. Members of the family *Secoviridae* are transported as virions, thus requiring the coat protein. Further, the viral movement protein inducing tubular structures by multimerization within plasmodesmata is necessary for passage of virus particles to adjacent cells.

MATERIAL AND METHODS

Protein-protein interaction experiments were performed in *Saccharomyces cerevisiae* strain Y190 (Harper *et al*. 1993) applying the Matchmaker Two-Hybrid system (Clontech). Total RNA was isolated from *Chenopodium quinoa* leaves systemically infected with a CLRV isolate from rhubarb. CLRV-MP and -CP sequences were amplified by RT-PCR and cloned into the GAL4 DNA binding domain (BD) vector pAS2 and the GAL4 DNA activation domain (AD) vector pACT2. Using the lithium acetate method *S. cerevisiae* Y190 cells were

Persistent Identifier: urn:nbn:de:0294-sp-2013-ppphe-2

co-transformed with pAS2- and pACT2-fusion plasmids. Selection of positive transformants was performed by combination of the reporter genes *his3* and *lacZ*.

RESULTS AND DISCUSSION

The yeast two-hybrid assay revealed dimerization of the viral movement protein and the coat protein of CLRV by expression of both reporter genes. Self-interaction of CLRV-CP was expected since it is a prerequisite for encapsidation of the two CLRV RNAs by CP subunits (Jones & Mayo 1972; Walkey *et al*. 1973). Likewise, dimerization of the viral MP was detectable in the YTHS corroborating the essential multimerization of the movement protein into tubular structures. Consequently, specific interaction of the CP with the MP could be confirmed in YTHS supporting the hypothesis that CLRV is also transported as a virion along MP-tubules through plasmodesmata. Strong binding of the CLRV-MP to the plant protein At-4/1 could be detected in YTH assay. This plant protein has been shown to interact with the movement protein of *Tomato spotted wilt virus* (TSWV) and sequence alignment revealed a relation of At-4/1 to myosin- and kinesin-like proteins participating in cellular motility (von Bargen *et al*. 2001). Localization of the protein at the plasmodesmata supports its role in directed cellular movement processes (Paape *et al*. 2006). This suggests that CLRV and TSWV are not only sharing the tubule-guided mechanism but utilizing the same cell-to-cell transport in their host plants.

ACKNOWLEDGEMENTS

The work was supported by a grant from the DFG (BU 890/14-1).

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9-8 Heterologous expression of the viral proteinase of *Cherry leaf roll virus* (CLRV)

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INTRODUCTION

Cherry leaf roll virus (CLRV), a subgroup C-*Nepovirus*, belongs to the family of the *Secoviridae* (Sanfacon *et al.* 2009). The bipartite genome consists of positively orientated single-stranded RNA, which encodes for two polyproteins (P1 and P2). P1 harbors characteristic domains for a proteinase-cofactor (PCo), a helicase (Hel), a genome-linked protein (VPg), a proteinase (Pro), and an RNA-depending polymerase (Pol). P2 includes the movement protein (MP), the coat protein (CP) and a region at the 5`-end, that has not been functionally assigned by now (von Bargen *et al.* 2012). The polyproteins are processed to their functional units by the viral proteinase posttranslationally. *In-silico*-analysis of the full-length sequence revealed several putative processing-sites similar to already experimentally proven processing sites of related proteinases of nepoviruses like *Tomato ringspot virus* (ToRSV, Wang *et al.* 1999, Wang & Sanfacon 2000) and *Arabis mosaic virus* (ArMV, Wetzel *et al.* 2008). A prerequisite for the functional characterisation of viral gene-products is the elucidation of their processing to the mature subunits. Aim of this project is therefore the identification of the processing sites of the CLRV-proteinase after their heterologous expression in *E. coli* and native purification.

MATERIAL AND METHODS

In order to functionally characterize the proteinase of CLRV, it was expressed in *E. coli*. As the presence of the VPg was shown to affect the activity of the proteinase (Chisholm *et al.* 2001), the putative coding region of the proteinase, and a construct comprising the VPg and the proteinase were cloned into the expression vector pET28a (Novagen). *E. coli* expression strain BL21 DE3 was transformed and the proteins were heterologously expressed after induction with IPTG. Subsequently the proteins were purified under native conditions by Ni-NTA-agarose-affinity chromatography.

Persistent Identifier: urn:nbn:de:0294-sp-2013-ppphe-2

RESULTS AND OUTLOOK

The CLRV-Proteinease, as well as the construct consisting of the VPg and the proteinase were successfully expressed in *E. coli* and could be detected by SDS-PAGE and western blotting. Analogously, the genome-regions comprising the putative processing sites from both P1 (X1/PCo, PCo/Hel, Hel/VPg, VPg/Pro, Pro/Pol), and P2 (X3/X4, X4/MP, MP/CP) will be amplified from viral RNA via RT-PCR. After cloning and expression in *E. coli,* the proteins will be purified and subsequently subjected to *in vitro*-activity-assays as substrates for the proteinase. As a proof of principle, methods are presently being established using a construct consisting of the C-terminal part of the MP and the N-terminal part of the CP, including the putative processing site. The assumed proteolytic cleavage will be monitored by visualization of the processed substrate via SDS-PAGE and western blotting using the N- and C-terminal HIS-tags.

ACKNOWLEGEMENTS

We thank the DFG for funding this projekt (BA 3961/2-1).

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9-9 Vector transmission of *Cherry leaf roll virus*? Candidate insect species infesting *Betula* spp.

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INTRODUCTION

Natural occurrence of *Cherry leaf roll virus* (CLRV) has been documented worldwide in a multitude of deciduous, fruit, and ornamental trees and shrubs as well as in a variety of herbaceous plants (Büttner *et al.* 2011). The most recorded natural hosts of CLRV are birch species (*Betula* spp.), black elderberry (*Sambucus nigra*), English walnut (*Juglans regia*) and sweet cherry (*Prunus avium*). To date, the host range comprises in total 26 plant genera. Known transmission pathways by seeds, pollen, water, or mechanically for instance, by roots intergrowth are considered as interacting factors for CLRV dispersal. Involvement of biological vectors in the epidemiology of CLRV has not been resolved by previous sporadic studies. So far, evidence of nematode´s attendance in CLRV transmission has not been supported (Jones *et al.* 1981; Wang *et al*. 2002). Also *Myzus persicae* (Sulzer) has been excluded as a vector of a CLRV-elm isolate by transmission experiments in previous studies 40 years ago (Ford *et al.* 1972). Yet, a hypothesis on insect transmission of CLRV was constituted by several proofs of CLRV-contaminated individuals from different insect species in Germany, such as the birch seed-feeding bug *Kleidocerys resedae* and aphids (Werner *et al.* 1997), *Polydrusus* sp. (leaf weevil; Rebenstorf 2005) from CLRV-infected birches, and aphids sampled from CLRV-infected black elderberry in a field study in 2006 (Langer, not published). Therefore, we target on elucidating the putative vector transmission by systematic monitoring of the invertebrate fauna of birches and elderberry in Finland and Germany to find potential CLRV vectors and study them in detail.

MATERIAL AND METHODS

A first case study (Bandte *et al*. 2011) was conducted to comprise the spectrum of potential vector insects in CLRV-infected *B. pendula* in Berlin-Dahlem. As sap sucking insects are the most likely group of potential vectors, aphids, bugs and cicadas (*Hemiptera*) were selected from the vast of arthropods (approx. 2400 individuals) which were captured by tapping the birches. Individuals of potential vector species were analyzed for CLRV contamination by

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Immunocapture-RT-PCR using two antibodies (developed against CLRV isolates of two different serogroups) in parallel (Werner *et al.* 1997). Additionally, aphids (*Euceraphis betulae*) were collected in 2008 and 2009 from CLRV-infected *Betula pubescens* in Northern Finland. Since a specific antibody against Finnish CLRV isolates is not available yet, diagnosis was carried out by RT-PCR after total RNA extraction from pooled aphid samples.

RESULTS AND DISCUSSION

The largest group of target insects on *Betula pendula* in Berlin was represented by bugs (*Heteroptera,* 43%) with *Kleidocerys resedae* as the most frequent species. In total, ten bug species, eight cicada species, and nine aphid species were determined in CLRV-infected birches. Positive CLRV detection in *Kleidocerys resedae* (14/122) and the cicada *Kybos lindbergi* (1/6) by IC-RT-PCR substantiated previous findings. CLRV was not detected in any of the 26 tested aphid samples from Finland. The results obtained so far represent only a spot sample of experimental sites that will be systematically monitored for the next three vegetation periods. Insects will be tested for CLRV as described by (IC)-RT-PCR, additionally providing CLRV sequence fragments for phylogenetic profiling of CLRV variants gained from insects and their originating trees.

ACKNOWLEDGEMENTS

Research is funded by the DFG-grant BU890/23-1.

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In: Schneider C, Leifert C, Feldmann F (Eds), Endophytes for plant protection: the state of the art. © 2013 Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-11-2.

9-10 Distribution of symptom determinants on the *Arabis Mosaic* nepovirus genomic RNA2

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ABSTRACT

Arabis mosaic virus (ArMV) belongs to the plant virus genus Nepovirus of the family *Secoviridae*. In the wine producing areas southwest of Germany, including Neustadt an der Weinstrasse (NW), ArMV is, along with the Grapevine fanleaf virus (GFLV) and the Raspberry ringspot virus (RpRSV), two other nepoviruses, a causative agent of the grapevine fanleaf disease, one of the most widespread and damaging virus diseases affecting grapevine. ArMV is transmitted by the nematode vector *Xiphinema diversicaudatum*, and has a wide natural host range. Nepoviruses have two single-stranded positive sense genomic RNAs, which are linked to a VPg at their 5' ends, and polyadenylated at their 3'ends. While ArMV-NW produces very mild or no symptoms on *Chenopodium quinoa*, ArMV–Lv, isolated from privet (*Ligustrum vulgare*), produces extremely severe necrotic symptoms, eventually leading to the death of the plant. Full-length infectious clones of ArMV-NW (under the control of a double 35S promoter) were generated, and when mechanically inoculated onto *Chenopodium quinoa*, they produced a systemic infection with very mild or no symptoms, like the native ArMV-NW. These clones were used to generate chimeric constructs between ArMV-NW and ArMV-Lv, in order to map symptom determinants on the ArMV genome.

Fragments of the ArMV-Lv genome were amplified by RT/PCR from total RNAs extracted from ArMV-Lv-infected *Chenopodium quinoa*. The resulting PCR product was digested with restriction enzymes which were unique in the sequence of the ArMV-NW infectious clones, and the corresponding fragments exchanged. Plasmids corresponding to the different constructions were mechanically inoculated on *Chenopodium quinoa* at a concentration of 5µg/plasmid/plant. The different chimeric clones were assessed for their infectivity and the development of symptoms monitored. The inoculated plants were analysed by ELISA 14 days post-inoculation. Total RNAs were also extracted for Northern blot analysis. The results with the chimeric constructs resulting from the exchange of fragments between the RNAs 2 of ArMV-NW and –Lv seemed to indicate that the N-terminal region of the 2A gene, but also the movement protein, are involved in the development of symptoms on *Chenopodium quinoa*. However, these symptoms do not match in severity those of the native isolate ArMV-Lv, indicating that other regions on the viral genome, probably located on the RNA 1, may also be involved in symptom development.

Persistent Identifier: urn:nbn:de:0294-sp-2013-ppphe-2

IUFRO Devision 7.02.04 – Phytoplasma and virus diseases of forest trees

Workshop: Virus and phytoplasma diseases of forest and urban trees

Section C: Phytoplasma Diseases

Persistent Identifier: urn:nbn:de:0294-sp-2013-ppphe-2

Kube M et al., Impact of genome analyses on phytoplasma research. In: Schneider C, Leifert C, Feldmann F (Eds), Endophytes for plant protection: the state of the art, pp. 317-318. © 2013 Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-11-2.

9-11 Impact of genome analyses on phytoplasma research

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INTRODUCTION

Phytoplasmas are associated with diseases of several hundred-plant species including many important crops. These phytopathogenic bacteria are grouped in the provisional genus '*Candidatus* Phytoplasma'. Today, the majority of our knowledge on phytoplasmas is directly connected or derived from genome research, as a cell-free cultivation of phytoplasmas was unavailable for a long time. Only four genomes were completely determined so far (reviewed in Kube *et al.*, 2012) as there are '*Ca*. P. asteris' strains OY-M and AY-WB, '*Ca*. P. australiense,' and '*Ca*. P. mali'. Chromosome condensation and decreased G + C content are characteristical for these genomes. Sequences provided information for design of diagnostic markers as well as subsequent experiments on key proteins interacting with the plant host or insect vector.

Several key questions have to be answered in the future. They aim to extend the knowledge of so far uncovered '*Ca*. Phytoplasma species' providing information on their metabolism, membrane proteins, effectors and virulence genes. A promising starting point is the comparative analysis of complete genome sequences or phytoplasma draft sequences obtained from metagenomic data. Hence, several studies are in progress worldwide. An overview is provided on the core metabolism of phytoplasmas and the strategies currently used in our own studies. Benefits and limits of draft sequences are discussed.

MATERIAL AND METHODS

The genetic core of the four complete phytoplasma genomes was compared (Kube *et al.*, 2012) with the results of phytoplasma draft sequences recently published (Saccarrdo *et al.*, 2012). Limits of phytoplasma drafts obtained from short reads were estimated by *in silico* experiments. Complete phytoplasma chromosomes were used to calculate Illumina read data sets of various read length and number using the ARTtool (Huang *et al.*, 2012). Reads were *de novo* assembled using the CLC Genomic Workbench and compared to published results.

Persistent Identifier: urn:nbn:de:0294-sp-2013-ppphe-2

RESULTS AND DISCUSSION

Data analyses indicate an evolutionary adaptation resulting in obligate parasitism of phytoplasmas. Effectors and prominent membrane proteins manipulating vector and host are evolved in '*Ca*. Phytoplasma species'. This stands in contrast to the shared evolutionary adaption on the nutrient-rich environments corresponding to a common repertoire of metabolic features, which may undergo further condensation in some phylogenetic groups. Results of genomic drafts support this estimation. Different strategies for extraction of phytoplasma drafts from metagenomic data can be summarized as positive selection or initial negative selection approaches. Positive selection approaches uses reads or contigs assigned to known phytoplasma sequences for subsequent processing. Initial negative selection approach starts by the identification and removal of the contigs assigned to host/vector background. Remaining reads were assembled and resulting contigs undergo a subsequent positive selection of contigs after assignment to taxonomical groups.

An initial enrichment of phytoplasma DNA is not performed for most sequencing projects today. Deep sequencing compensates this step. Assemblies obtained from simulated single or paired-end reads with a length of 36-120 b frequently result in misassemblies of repeat regions and/or rejection of reads during the assembly because of conflicts. In consequence, phytoplasma drafts cannot cover the complete genome. Analysis of the shared gene content should be limited to identified features. Statements on the absence of genes in comparative analysis or genome size need additional verification.

ACKNOWLEDGEMENTS

The Deutsche Forschungsgemeinschaft supports this work (KU 2679/2-1 and BU 890/21-1).

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Persistent Identifier: urn:nbn:de:0294-sp-2013-ppphe-2

Siewert C, Büttner C, Kube M, *Sec*-dependent protein export in Phytoplasmas and *Acholeplasma laidlawii*. In: Schneider C, Leifert C, Feldmann F (Eds), Endophytes for plant protection: the state of the art, pp. 319-320. © 2013 Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-11-2.

9-12 *Sec*-dependent protein export in Phytoplasmas and *Acholeplasma laidlawii*

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INTRODUCTION

Phytoplasmas are obligate bacterial parasites of plants. The closest known relatives are the acholeplasmas, which do not contain primary pathogens. Species of this genus are characterized by a saprophytic lifestyle. Type strains were isolated from the environment including the surface of plants but also from several animals.

Phytoplasmas are suggested to export proteins via the *Sec*-dependent system (Kakizawa *et al*., 2001) and are known to manipulate the phenotype of their host plants to their benefit by the secretion of effector proteins. For example, *tengu* encodes an effector of '*Ca*. P. asteris' strain OY-M (Hoshi *et al*., 2009). Its expression causes witches'-broom formation and dwarfism. Other effectors were identified for '*Ca*. P. asteris' strain AY-WB. SAP11 causes crinkled leaves and witches'-broom formations and SAP54 is responsible for the formation of leaf-like flowers (Sugio *et al*., 2011). Here we present a comparative analysis of the genetic repertoire of the *Sec*-dependent system and the prediction of exported proteins of the complete genomes of '*Ca*. P. asteris', '*Ca*. P. australiense', '*Ca*. P. mali' and *A. laidlawii*.

MATERIALS AND METHODS

Protein data of the four phytoplasma genomes (CP000061.1, CP000061.2, CP000062.1, CP000063.1, CP000064.1, CP000065.1, AP006628.1, AM422018.1, CU469464.1) and *A. laidlawii* (CP000896.1) were used to reconstruct the *Sec*-dependent system and to obtain deduced peptide sequences. Annotation of proteins was validated by InterPro and BlastP analysis. Proteins carrying a signal peptide but lacking a transmembrane region were predicted by Phobius (Käll *et al*., 2004) and compared by BlastP. Putative exported proteins were compared by BlastP.

RESULTS AND DISCUSSION

Both genera probably use the *Sec*-dependent system for protein export. All five analysed genomes encode *ffh*, *ftsY*, *secA*, *secE* and *secY*. *A. laidlawii* differs by encoding *secG* in addition. SecY, SecE and SecG are known to form the membrane complex. One may speculate, if phytoplasmas lost *secG* during genome condensation. This protein is essential for survival below 20 °C in *Escherichia coli* (Nishiyama *et al*. 1994). Phytoplasmas are protected from strong temperature shifts due to the colonization of plant sieve cells. In contrast, SecG may increase the fitness of the ubiquitous distributed *A. laidlawii*, which is frequently exposed to temperature variations.

Furthermore, all five genomes encode the YidC protein, which acts separately or together with the *Sec*-dependent system. This protein is involved in secretion and processing of immature membrane proteins (Dalbey *et al*., 2004).

The prediction of secreted proteins shows no shared protein content between the genera. This might be interpreted with respect to the different function of these proteins taking part in manipulation of host/vector for phytoplasmas or degradation of biomass for *A. laidlawii*.

ACKNOWLEDGEMENTS

The Deutsche Forschungsgemeinschaft supports this work (KU 2679/2-1 and BU 890/21-1).

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Persistent Identifier: urn:nbn:de:0294-sp-2013-ppphe-2

In: Schneider C, Leifert C, Feldmann F (Eds), Endophytes for plant protection: the state of the art. © 2013 Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-11-2.

9-13 Promising approaches to control apple proliferation disease: Plant resistance and cross protection using suppressive mild phytoplasma strains

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ABSTRACT

Phytoplasmas are insect-transmitted plant pathogenic prokaryotes of the class *Mollicutes* (‘mycoplasmas’). They cause systemic and persistent infections and affect mainly perennial plants including fruit trees and shrubs, grapevine and a range of forest and landscape trees. The diseases are difficult to control. In agricultural crops, limited success is currently achieved by insecticide application against the insect vectors and some phytosanitary measures. Since the beginning of phytoplasma research, it has been observed that certain genotypes of various plant species differ considerably in their susceptibility to phytoplasma infection. Most data on resistance are available from the genus *Malus* (apple), where in hybrids of *M. sieboldii* and *M.* x *domestica* the concentration of the apple proliferation agent (*Candidatus* Phytoplasma mali) remain at a very low level and symptoms do not develop. For this reason, a rootstock breeding program was initiated in which several genotypes were obtained on which commercial scion cultivars are not affected from infection.

Indication of another promising approach to control phytoplasmal diseases was obtained by examining phytoplasma populations in infected trees. Using single-strand conformation polymorphism (SSCP) and sequence analysis of genes encoding AAA+ proteins, it could be shown that mixed infections of distinct strains of *Ca*. P. mali are common. Application of virulence-associated gene markers revealed that the mixed infections consist of strains that differ considerably in virulence. In newly diseased symptomatic trees, severe strains are predominant. After a few year of disease, recovery may occur that is associated with a shift in the population leading to the predominance of mild strains. In further investigations, suppressive action on severe strains was identified in several mild strains. In the sequence of the gene encoding AAA+ protein AP460, suppressive strains differ significantly from nonsuppressive strains. However, before cross protection can be recommended to control phytoplasmoses of fruit trees and other persistent plants, further research is required to elucidate whether cross protection is a safe and efficient method.

Persistent Identifier: urn:nbn:de:0294-sp-2013-ppphe-2

von Bargen S et al., Sequence diversity of *Cherry leaf roll virus* makes a difference in infected birches in Finland. In: Schneider C, Leifert C, Feldmann F (Eds), Endophytes for plant protection: the state of the art, pp. 322-323. © 2013 Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-11-2.

9-14 Sequence diversity of *Cherry leaf roll virus* makes a difference in infected birches in Finland

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INTRODUCTION

Virus-related symptoms were increasingly found on birch leaves throughout northern Fennoscandia. Disease symptoms occurring on downy birch (*Betula pubescens*), silver birch (*B. pendula*), dwarf birch (*B. nana*), Kiilopää birch (*B. pubescens* var. *appressa*), mountain birch (*B. pubescens* ssp. *czerepanovii*), and curly birch (*B. pendula* var. *carelica*) in Finland could be associated with an infection of *Cherry leaf roll virus* (CLRV). The virus is a typical member of the *Secoviridae* family, genus *Nepovirus* and infects primarily deciduous trees and shrubs. The complete genomic sequence of the CLRV has been determined (von Bargen *et al*. 2012). The bipartite, isometric particles contain positive stranded RNA. Disease symptoms in birch trees are spreading since their first record in 2002 and a countrywide incidence of CLRV could be shown in affected-birch trees tested since 2006 (Jalkanen *et al*. 2007; von Bargen *et al*. 2009). Symptoms are observed in roadside and urban areas, but are also found in forests. However, the mode of virus dispersal in Fennoscandia is unclear.

MATERIAL AND METHODS

Diseased birch trees all over Finland were inspected for symptoms associated with a CLRV infection such as chlorotic vein banding, leaf roll and necrotic lesions. Total RNA was extracted from symptomatic leaf material according to Boom *et al*. (1990). CLRV was detected by RT-PCR using random hexamers for cDNA synthesis and virus specific primer pairs targeting regions encoding the RNA-dependent-RNA polymerase (RdRp) on RNA1, the coat protein (CP)-coding region on RNA2, and the 3´ untranslated region (3` UTR), the

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latter being highly conserved among isolates and is present on both genomic segments. Amplified PCR products were sequenced from both directions by Sanger sequencing. Obtained sequences were analyzed and compared to CLRV isolates characterized by Rebenstorf *et al.* (2006) applying ClustalX 2.0 using the incorporated neighbour-joining method for phylogenetic tree construction.

RESULTS AND DISCUSSION

Symptoms observed in CLRV infected birches in northern Europe are more prominent and differ from virus-affected birches from other European countries. It was not possible to obtain a CLRV isolate from a Finnish site by rub inoculation so far. Phylogenetic analyses of a conserved RdRp-sequence fragment showed that grouping of CLRV variants obtained from Finnish birches correspond with host-dependent classification determined by Rebenstorf *et al*. (2006). Partial sequences obtained from the RNA2 encoding the CP as well as the ultimate 3´ untranslated regions showed divergence on the nucleotide level in comparison with CLRV isolates from different hosts. This indicates towards the presence of a distinct population of CLRV in Finnish birches.

ACKNOWLEDGEMENTS

Research was funded by the DFG-grants BU890/15-1 and BA3961/2-1.

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Persistent Identifier: urn:nbn:de:0294-sp-2013-ppphe-2

von Bargen S, Bandte M, Büttner C, Virus-induced symptoms in leaves of woody plants. In: Schneider C, Leifert C, Feldmann F (Eds), Endophytes for plant protection: the state of the art, pp. 324-325. © 2013 Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-11-2.

9-15 Virus-induced symptoms in leaves of woody plants

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INTRODUCTION

A lack of knowledge of the presence and frequency of occurrence leads to the impression that viral diseases in forest trees are rare and therefore unimportant. The opposite is true. Virus-infections of broad leaved trees are widespread in forests as well as in urban areas. They are of economic impact, because stress tolerance of infected trees is considerably reduced (Nienhaus 1985, Nienhaus & Castello 1989, Büttner *et al*. 2013).

MATERIAL AND METHODS

Viruses have been identified to infect different broad-leaved trees and shrubs including the genera *Acer*, *Betula*, *Fagus*, *Fraxinus*, *Populus*, *Prunus*, *Quercus*, *Sambucus*, *Sorbus*, *Ribes*, *Rubus*, and *Ulmus*. In this study we are documenting virus-like symptoms we found in these important woody hosts, which were cultivated in forests, parks and as road-side trees.

RESULTS AND DISCUSSION

Virus-induced symptoms in leaves of deciduous trees display a large variety of discoloration, deformation, or decline. Important leaf symptoms are vein-netting, veinbanding, chlorotic spots, ringspots and line patterns, which are sometimes displayed as oak-leaf line pattern. Mosaic symptoms are characterized by distinct areas of at least three different colors and can thereby be distinguished from diffuse mottle and large chloroses. Common deformations induced by viruses are little leaf, leaf narrowing or fanleaf, leafroll, distortion or blistering. Cell death is often induced by virus-infections leading to tissue damage visible as necroses of leaves, dieback of twigs, and finally decline of the whole tree.

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Virus-induced symptoms may be confused with other biotic or abiotic factors, depending on developmental stage of plants as well as the vegetation period. However, virus-induced aberrations are usually uneven distributed in affected plants and areas (Bandte & Büttner 2004). Detailed expertise is a prerequisite to distinguish symptoms typically induced by viruses from other factors, allowing recognition and estimation of virus abundance in deciduous trees in order to maintain the health status of forest and urban trees.

ACKNOWLEDGEMENTS

Research was funded by the DFG-grants BU890/14-1, BU890/15-1 and BA3961/2-1.

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Breuhahn M et al., Transmission of *Cherry leaf roll virus* (CLRV) variants from German and Finnish birches by grafting.
In: Schneider C, Leifert C, Feldmann F (Eds), Endophytes for plant protection: the state of the art, pp. 326-327. © 2013
Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-11-2.

9-16 Transmission of *Cherry leaf roll virus* (CLRV) variants from German and Finnish birches by grafting

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INTRODUCTION

Cherry leaf roll virus (CLRV) is a worldwide distributed *Nepovirus* within the family *Secoviridae* affecting primarily decidious trees and shrubs (Büttner *et al*. 2011). The broad host range includes major forest tree species including silver birch (*Betula pendula*) and downy birch which are the most important broadleaved tree species in Northern and Eastern Europe (Hynynen *et al*. 2010). In northern Scandinavia and Finland the “birch leaf-roll disease” has emerged in the region since its first appearance ten years ago. The disease could be associated with a CLRV infection (Jalkanen *et al*. 2007). Investigations regarding genetic diversity demonstrated that CLRV variants from Finnish birches show atypical phylogenetic relations (von Bargen *et al*. 2009). Therefore, grafting trials were carried out to compare biological characteristics of CLRV variants from Finnish birches with virus variants occurring in *Betula* species in Germany.

MATERIAL AND METHODS

In spring of 2011 scions were obtained from different CLRV-infected downy birches (5 trees, Rovaniemi, Finland) and silver birches (5 trees, Berlin, Germany) and were grafted by triangulation onto 2-year old seedlings (*B. pubescens* and *B. pendula*). The 200 grafted scions (n = 20) were cultivated under identical conditions and were regularly inspected for symptoms associated with a CLRV-infection during the vegetation periods of 2011 and 2012. In parallel, leaf material was collected for CLRV detection by serological and molecular methods. Sampled *B. pendula* scions of German origin were tested by CLRV-

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specific IC-RT-PCR (Werner *et al.* 1997) and DAS-ELISA. Total RNA was extracted from *B. pubescens* rootstocks grafted with CLRV-infected scions of Finnish origin and subjected to RT-PCR applying three different primer sets targeting the replicase-coding region, the coat protein-coding region, and the 3´ untranslated region of CLRV.

RESULTS AND DISCUSSION

Seventy of 100 grafted CLRV-infected silver birch scions, obtained from German stands survived and grew vigourously in 2011 and 2012, whereas CLRV-infected scions originating from Finnish *B. pubescens* only survived for five months suggesting an effect caused by the atypical Finnish CLRV variants. However, graft transmission of CLRV variants affecting Finnish trees succeeded, because *B. pubescens* seedlings developed symptoms such as leaf roll, veinbanding, chlorotic ringspots, proliferation and CLRV was detected in 7 out of 20 investigated survived rootstocks. In June 2012 twenty out of 70 *B. pendula* scions showed virus-like symptoms and the virus could be detected in 24 silver birches including asymptomatic seedlings. Further investigations are necessary to determine biological and genetical traits of the CLRV variants present in grafted trees.

ACKNOWLEDGEMENTS

We thank Gerald Eckert for triangulation and maintenance of grafted birches. Research was funded by the DFG-grant BU890/15-1.

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Persistent Identifier: urn:nbn:de:0294-sp-2013-ppphe-2

Dieckmann L et al., Detection of the *European mountain ash ringspot associated virus* (EMARaV) in several European countries. In: Schneider C, Leifert C, Feldmann F (Eds), Endophytes for plant protection: the state of the art, pp. 328-329.
© 2013 Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-11-2.

9-17 Detection of the *European mountain ash ringspot associated virus* (EMARaV) in several European countries

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INTRODUCTION

The mountain ash tree (*Sorbus aucuparia* synonym rowan) is a popular species in Europe and is grown both in public greens and forests due to its robustness and decorative properties. Since more than 50 years there have been reports from various locations about disease symptoms such as chloroticr ingspots and mottling of leaves. The nature of the disease causing agent remained unknown until the observation of virus-like particles in the electron microscope (Ebrahim-Nesbat & Izadpanah 1992) and its transmissibility to healthy trees by grafting (Büttner & Führling 1995), which suggested a viral pathogen. This hypothesis was verified when dsRNA was successfully extracted from the inner bark and leaves of diseased trees (Benthack *et al.* 2005). The observed symptoms could be related to a virus now denominated *European mountain ash ringspot associated virus* (EMARaV) (Mielke & Mühlbach 2007), the typemember of the new genus *Emaravirus*, whose members display a minimum of four genomic (-)ssRNA segments (Mühlbach & Mielke-Ehret 2011).

Sorbus aucupaia is to date the only known host of EMARaV. The transfer to healthy trees was conducted successfully only within the species. A putative vector is the gall mite *Phytoptus pyri*, which has been found in diseased trees and in which the virus was successfully detected (Mielke-Ehretet al. 2010).

To acquire more information about the virus distribution across Europe, rowan trees with characteristic symptoms from several European countries were tested for an infection with EMARaV.

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MATERIAL AND METHODS

Leaf material from rowan trees was collected in Sweden, Finland, Scotland, Germany and Italy in 2011. Twentyfour of 29 samples displayed EMARaV specific disease symptoms such as chlorotic ringspots and mottling. Also galls were observed on several samples. Total RNA was extracted from sampled leaflets and transcribed into cDNA by reverse transcription with random hexamers. The four genome segments were detected in a PCR with specific primers (Mielke *et al.* 2008).

RESULTS AND DISCUSSION

The virus was detected in 60 % of the symptomatic trees. EMARaV was neither detectable in mountain ash showing veinbanding symptoms sampled in Italy, nor in asymptomatic trees from Germany. The RNA 3 was detected in 14 positively tested samples confirming an EMARaV infection of trees expressing chlorotic ringspots and mottle. Additonally, an RNA2 specific fragment was amplified from twelve analysed leaf samples by RT-PCR. All four viral RNAs were detetable in eight sampled trees originating from Sweden, Finland, Germany and Scotland. The virus was confirmed for the first time in five trees in Scotland showing characteristic chlorotic ringspots.

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Persistent Identifier: urn:nbn:de:0294-sp-2013-ppphe-2

Bütow U, Landgraf M, Büttner C, Detection of *Cherry leaf roll virus* in birch pollen by an improved IC-RT-PCR. In: Schneider C, Leifert C, Feldmann F (Eds), Endophytes for plant protection: the state of the art, pp. 330-331. © 2013 Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-11-2.

9-18 Detection of *Cherry leaf roll virus* in birch pollen by an improved IC-RT-PCR

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INTRODUCTION

Cherry leaf roll virus (CLRV) is a globally spread Nepovirus of the family Secoviridae and one of the most important viruses infecting *Betula* spp. (Bandte et al. 2009). Natural transmission occurs vertically by seed and horizontally via pollen. Detection of CLRV in different tissues of woody hosts is routinely carried out applying an IC-RT-PCR using a primer set targeting the partial 3´ untranslated region (Werner et al. 1997, Gentkow et al. 2007) which is the most conserved genomic region of the virus. However, the method has been found to be of limited suitability for CLRV detection in pollen samples of *Betula* spp..This work focussed on the establishment of a more sensitive detection method of CLRV in birch pollen.

MATERIAL AND METHODS

69 samples of pollen from 48 birch trees (*Betula pendula*, *Betula pubescens* and hybrids of both species) were collected at four different locations in Berlin, Germany in spring of 2011 and 2012. Most of the sampled trees showed disease symptoms such as chlorotic vein banding, diffuse chlorotic leaf pattern and leaf rolling associated with a CLRV infection. Also pollen was sampled from some asymptomatic birches in the immediate neighbourhood of the symptomatic birches. Initially, the presence of RT-PCR inhibitors in pollen extracts was examined. Equal amounts of plant material from CLRV-infected *Chenopodium quinoa* was mixed with dilutions of aqueous pollen extracts (10-1-10-7) and subjected to IC-RT-PCR applying universal CLRV-primers (Werner et al. 1997). Sensitivity of RT-PCR after RNA isolation and IC-RT-PCR was compared by use of dilution series of sample material. In the process also a new forward primer (mod RW2) was used to test 69 pollen samples with IC-RT-PCR for a CLRV infection.

Persistent Identifier: urn:nbn:de:0294-sp-2013-ppphe-2

RESULTS AND DISCUSSION

Comparison of IC-RT-PCR and RT-PCR after RNA isolation revealed no difference in sensitivity as in both cases CLRV was detectable until dilution 10-6. However, it could be demonstrated that substances present in the birch pollen extracts inhibited IC-RT-PCR. No CLRV was detectable in IC-RT-PCR when using pollen dilutions of 10-1, but applying at least a dilution of 10-2 overcomes this problem. Combination of the new universal CLRV primer mod RW2 together with RW1 in RT-PCR and IC-RT-PCR led to an at least 10fold higher sensitivity compared to the primer pair RW1/RW2. With the conventional IC-RT-PCR protocol (Gentkow et al. 2007) CLRV was detectable in 10 out of 69 investigated pollen samples. Testing the same samples with the modified IC-RT-PCR applying pollen extracts in a 10-2 dilution in combination with the primer modRW2/RW1 resulted in 31 CLRV positives. It could be demonstrated that 23 trees out of 48 were CLRV infected. The virus was detectable in pollen collected from symptomatic as well as asymptomatic *B. pendula, B. pubescens* and hybrids of both species at all four locations. The established modifications of the CLRV-specific IC-RT-PCR are a prerequisite for future studies depending on a sensitive and reliable method for CLRV detection in pollen of *Betula* spp..

ACKNOWLEDGEMENTS

This work was supported by grants from the Deutsche Forschungsgemeinschaft (DFG) (BU890/14-1, BU890/23-1).

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Persistent Identifier: urn:nbn:de:0294-sp-2013-ppphe-2

In: Schneider C, Leifert C, Feldmann F (Eds), Endophytes for plant protection: the state of the art. © 2013 Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-11-2.

9-19 Molecular Analysis of a German Isolate of Apple Stem Pitting Foveavirus

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ABSTRACT

The Apple stem grooving virus (ASGV, Capillovirus) and Apple stem pitting virus (ASPV, Foveavirus) are widely spread in apple growing regions. As they cause no symptoms on most cultivated apple varieties and rootstocks, they are considered as latent in *Malus* x *domestica*. Recently, *M. sieboldii* and its hybrids have gained new interest in Europe as they confer resistance to apple proliferation (AP) disease caused by *Candidatus* Phytoplasma mali. A new breeding program aiming to develop AP-resistant rootstocks of agronomic value reported unexpected tree decline which was found to be associated with ASGV and/or ASPV. As little information is available on the variability of these latent virus isolates, the complete genome of a German isolate of ASPV associated with tree decline was cloned and sequenced.

The comparison between the sequence of the german isolate and those of other ASPV isolates from the databases revealed a high degree of variability between these isolates, which is spread over the entire genome. Overall, the most conserved gene was the first gene from the triple gene block (between 89 and 95% identity between isolates), the most variable one being the coat protein gene (up to 61% diversity between isolates). The replicase gene showed overall identity levels of 65-95% between isolates. The distribution of this variability in the replicase gene was however uneven, some regions being conserved, and others showing a higher degree of variability.

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In: Schneider C, Leifert C, Feldmann F (Eds), Endophytes for plant protection: the state of the art. © 2013 Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-11-2.

9-20 Extraction and mechanical transmission of viruses from forest trees to herbaceous hosts and back transmission to seedlings of trees

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ABSTRACT

When forest trees develop symptoms characteristic for virus diseases like chlorotic patterns, dwarfing and deformation of leaves, withering and dieback of branches, other causes than virus-infections may be the reason and thus may lead to false conclusions. To clarify the viral etiology of these changes, extraction and transmission of the suspected viral agent is mandatory. In many cases this is a rather challenging task, because the distribution of those pathogens may be inhomogeneous within the plant and their concentration can be very low. Furthermore the time window for a successful extraction is often restricted to a few weeks. Therefore an approach has been developed to optimize the extraction procedure. This worked well for viruses in several deciduous tree species. The procedure comprises labeling of symptomatic branches in summer, sampling of those branches in the following early spring, and leafing out of the buds under greenhouse conditions. Extraction was carried out by pulverising of the leaf tissue in liquid nitrogen, uptake of the powder in buffer containing antioxidants followed by low speed centrifugation and ultracentrifugation of the supernatant. The resulting sediment was then resuspended and transmitted to herbaceous test plants kept in the dark before inoculation for 24 h. Back transmission to seedlings of the respective tree-species was performed by stem slash inoculation with concentrated inoculum. Some of the inoculated seedlings showed the expected symptoms and reacted positively in ELISA.