Graz University of Technology

Doctoral Thesis

Investigations on the suppression of fruit rot and bacteriosis of Styrian oil pumpkin

Dissertation zur Erlangung des akademischen Grades Doktor der Naturwissenschaften (Dr. rer. nat.) an der Fakultät für Technische Chemie, Verfahrenstechnik und Biotechnologie an der Technischen Universität Graz

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Abstract

Styrian oil pumpkin (*Cucurbita pepo* L. subsp. *pepo* var. *styriaca* Greb.) is a pumpkin cultivar originated from the Austrian district Styria. The unique characteristic of this Cucurbitaceae is the absence of an outer seed hull, facilitating the production of pumpkin seed oil, a very healthy supplement for gourmet cuisines. Styrian oil pumpkin is cultivated in Southern European and Northern African countries, China, Russia and the USA. However, especially in Styria, the cultivation of Styrian oil pumpkin and the production of pumpkin seed oil is of great economic and cultural importance.

Since 2004, heavy yield losses of oil pumpkin have been reported in Styria due to fruit rot. The fungus *Didymella bryoniae* was identified as the most important causal agent of fruit rot, provoking black rot of pumpkins. The ascomycete infests also vegetative parts of oil pumpkin plants, known as the gummy stem blight disease. Beside this fungus, the bacterial pathogens *Pectobacterium carotovorum*, *Pseudomonas viridiflava*, *Pseudomonas syringae*, and *Xanthomonas cucurbitae* affect oil pumpkin plants in Styria. *P. carotovorum* causes soft rot of pumpkins whereas the other bacterial species provoke leaf damage.

D. bryoniae strains from different field sites in Austria were investigated and it was shown that they exhibited a high morphological and low genotypic variability. The pathogenicity of *D. bryoniae* isolates, representing different morphological groups, and of strains of *P. carotovorum*, *P. viridiflava*, *P. syringae*, and *X. cucurbitae* was confirmed in greenhouse assays. The transport of the observed prokaryotic phytopathogens by the mycelium of *D. bryoniae* was shown, indicating co-infections by the fungus and the bacteria.

In order to biologically control the studied microbial pathogens by indigenous microorganisms of the oil pumpkin host plant, a multiphasic approach was performed. Community structures of *Bacillus* and *Pseudomonas* were analyzed, revealing differences in the constitution of populations of these two important bacterial genera with respect to the microhabitat and developmental stage of the host. Seed-borne, root-, flower-, and fruit-associated bacteria and fungi (predominantly endophytes) were isolated and tested for *in vitro* antagonism against *D. bryoniae* and three of the investigated bacterial pathogens. Out of 2320 screened isolates, 43 bacterial broad-spectrum antagonists were used for genotypic characterization, leading to the selection of 6 strains that were identified by DNA sequence analysis: *Lysobacter gummosus* L101, *Lysobacter antibioticus* L175, *Lysobacter antibioticus* L169, *Serratia plymuthica* S13, *Pseudomonas chlororaphis* P34 and *Paenibacillus polymyxa* PB71. Apart from *L. antibioticus* L175, all broad-spectrum antagonists synthesized volatile and/or soluble antifungal compounds *in vitro*. *L. gummosus* L101, *P. chlororaphis* P34, *S. plymuthica* S13, and *P. polymyxa* PB71 were analyzed for enhancement of plant health and growth of Styrian oil pumpkin in the course of field trials at different sites in Styria in two vegetation periods (2010 and 2011). Increases in seedling emergence up to 109% by *S. plymuthica* S13 as well as reproducible enhancements of harvest yields by *L. gummosus* L101, and *P. polymyxa* PB71 were observed. The latter plant growth promoting bacteria are aimed to be commerzialized as biological product for Styrian oil pumpkin for the integrated or organic agricultural market.

Zusammenfassung

Der Steirische Ölkürbis (*Cucurbita pepo* L. subsp. *pepo* var. *styriaca* Greb.) ist eine Kürbissorte, welche aus Steiermark, einem österreichischen Bundesland, stammt. Das einzigartige Merkmal des Cucurbitaceae-Vertreters ist die Abwesenheit einer Samenschale, wodurch die Herstellung von Kürbiskernöl, einer gesunden Nahrungsergänzung, welche in Gourmetküchen Verwendung findet, erleichtert. Der Steirische Ölkürbis wird in Südeuropa, Nordafrika, China, Russland und USA angebaut. Vor allem in Steiermark ist die Kultivierung des Ölkürbis und die Gewinnung von Kürbiskernöl ein wichtiger wirtschaftlicher und kultureller Faktor.
Seit 2004 wurden erhebliche Ernteeinbußen im steirischen Ölkürbisanbau durch Fruchtfäule verzeichnet. Der pilzliche Schwarzfäule-Erreger *Didymella bryoniae* wurde als Hauptverursacher der Fruchtfäule bestimmt. Der Ascomycet befällt auch vegetative Pflanzenteile des Steirischen Ölkürbis, was als Gummistängel-Krankheit bezeichnet wird. Abgesehen von dem Pilz befallen die bakteriellen Pathogene *Pectobacterium carotovorum*, *Pseudomonas viridiflava*, *Pseudomonas syringae* und *Xanthomonas cucurbitae* Ölkürbispflanzen in der Steiermark. *P. carotovorum* verursacht die Kürbis-Weichfäule, während die anderen Bakterienarten Blattkrankheiten hervorrufen.

D. bryoniae-Stämme von unterschiedlichen Feldern in Österreich wurden untersucht, und zeigten eine hohe morphologische und geringe genotypische Vielseitigkeit. Die Pathogenität von *D. bryoniae*-Isolaten, welche die verschiedenen morphologischen Gruppen repräsentieren, als auch von Stämmen von *P. carotovorum*, *P. viridiflava*, *P. syringae* und *X. cucurbitae*, wurde in Gewächshausversuchen bestätigt. Es konnte der Transport der untersuchten, prokaryotischen Phytopathogene durch das Myzel von *D. bryoniae* bewiesen werden, das auf Co-Infektionen durch den Pilz und den Bakterien schließen lässt.

Um die erforschten mikrobiellen Pathogene mittels biologischer Kontrolle durch autochthone Mikroorganismen der Ölkürbis-Wirtspflanze zu unterdrücken, wurde ein vielseitiger Forschungs-Ansatz entworfen. Die Analyse von *Bacillus*- und *Pseudomonas*-Gemeinschaften hat ergeben, dass die Populationszusammensetzungen dieser wichtigen bakteriellen Gattungen vom Mikrohabitat und dem Entwicklungsstadium der Wirtspflanze abhängen. Samenbürtige, Wurzel-, Blüten-, und Frucht-assoziierte Bakterien und Pilze (vorwiegend Endophyten) von verschiedenen Ölkürbiskultivaren wurden isoliert und auf *in vitro*-Antagonismus gegen *D. bryoniae* und drei der Bakterienpathogene getestet. Aus 2320 getesteten Isolaten wurden 43 bakterielle Breitband-Antagonisten einer genotypischen Charakterisierung unterzogen, wodurch 6 Stämme zur Identifizierung mittels DNS-Sequenzanalyse ausgesucht wurden: *Lysobacter gummosus* L101, *Lysobacter antibioticus* L175, *Lysobacter antibioticus* L169, *Serratia plymuthica* S13, *Pseudomonas chlororaphis* P34 und *Paenibacillus polymyxa* PB71. Abgesehen von *L. antibioticus* L175 gaben alle Breitband-Antagonisten flüchtige und/oder lösliche, fungizide Substanzen unter Laborbedingungen ab. *L. gummosus* L101, *P. chlororaphis* P34, *S. plymuthica* S13 und *P. polymyxa* PB71 wurden hinsichtlich der Steigerung von Gesundheit bzw. Wachstum des Steirischen Ölkürbis, im Zuge von Feldversuchen an verschiedenen Standorten in Steiermark und in zwei verschiedenen Wachstumsperioden (2010 und 2011), erforscht. Erhöhungen der Keimraten von bis zu 109 % durch *S. plymuthica* S13, als auch reproduzierbare Steigerungen der Ernteerträge durch *L. gummosus* L101 und *P. polymyxa* PB71, wurden beobachtet. Letztere, pflanzenwachstumsfördernde Bakterien sollen in ein biologisches Präparat für den integrierten bzw. biologischen Ölkürbisanbau einfließen.

Abbreviations

ACC	1-aminocyclopropane-1-carboxylate
AHL	Acyl-homoserine lactone
ANOVA	analysis of variance
ARDRA	Amplified Ribosomal DNA Restriction Analysis
BCA	biological control agent
BD	Brown & Dilworth (medium)
BLAST	basic local alignment search tool
bp	base pairs
cw	calendar week
CFU	colony forming unit
CLSM	confocal laser scanning microscopy
DCA	detrended correspondence analysis
ddH_2O	double distilled water
DNA	deoxyribonucleic acid
DNS	Desoxyribonukleinsäure
ds	disease severity
EDTA	ethylenediamintetraacetic acid
Fe	iron
FISH	fluorescence *in situ* hybridization
g	g-force
IAA	indole-3-acetic acid
ISR	induced systemic resistance
ITS	internal transcribed spacer
Kb	kilo base
LB	Luria-Bertani
LSD	least significant difference
MA	malt agar
N_2	atmospheric nitrogen
NB	nutrient broth
NCBI	National Center for Biotechnology Information
P	phosphorous

PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PDA	potato dextrose agar
PGP	plant growth promotion
PGPB	plant growth promoting bacteria
PGPR	plant growth promoting rhizobacteria
QPDA	quarter-strength potato-dextrose agar
QS	quorum sensing
rDNA	ribosomal deoxyribonucleic acid
rRNA	ribosomal ribonucleic acid
rpm	revolutions per minute
RT	room temperature
SD	standard deviation
SDS	sodium dodecyl sulphate
SSCP	single-strand conformation polymorphism
TBE	Tris/Borate/EDTA
TSB	tryptic soy broth
UV	ultra violet
VOCs	volatile organic compounds
w/v	weight per volume

Introduction

Styrian oil pumpkin

Styrian oil pumpkin belongs to the family of Cucurbitaceae. The specialty of this pumpkin variety is the special seed structure: due to a natural mutation in the first half of the 19th century, the wooden seed shell disappeared. This facilitated the production of pumpkin seed oil that has been used as a food supplement (especially for salad). The dark green oil contains unsaturated fatty acids, vitamins, minerals, phytosterols, and antioxidant phenolic compounds among other substances (Fruehwirth and Hermetter 2007). Pumpkin seed products are of medical importance as they are used for phytotherapy against benign prostate hyperplasia (BHP) (Dreikorn 2002) and contain cholesterol-lowering substances (Fruewirth and Hermetter 2007).

Fig. 1. Dark green oil pumpkin seeds (left), lacking a wooden seed shell in comparison to seeds of a seed shell bearing pumpkin variety (right).

Styrian oil pumpkin is a very important crop plant for the Austrian district Styria, nevertheless it is also grown in other European countries, China, Russia and the USA. Since 2004 heavy yield losses of oil pumpkin were observed in Styria mainly due to black rot, caused by the fungus *Didymella bryoniae* (Huss et al. 2007). The ascomycete is termed *Phoma cucurbitacearum* in its asexual stage that is frequently found on diseased oil pumpkin plants (Huss et al. 2007). Conidia and spores of the pathogen are transmitted by water (e.g. rain) and air, and infest fruits (black rot) as well as vegetative plant parts (gummy stem blight) of many Cucurbitaceae species beside oil pumpkin (e.g. cucumber) (Keinath et al. 1995). In addition, an increase in oil pumpkin diseases caused by bacterial pathogens has been noticed in Styria: *Pectobacterium carotovorum* causes soft rot of pumpkins, whereas *Pseudomonas viridiflava, P. syringae* and *Xanthomonas cucurbitae* lead to leaf diseases on Styrian oil pumpkin (Huss 2011). Conventional pest management methods like breeding, crop rotation and application of copper based fungicides are insufficient to control this multi-pathogen disease.

Plant-associated microorganisms

Ecology

Plants are colonized by microorganisms (bacteria and fungi) that inhabit different organs of their host. These microenvironments or microhabitats include seeds (spermosphere), roots (root surrounding soil: rhizosphere, root surface: rhizoplane, root interior: endorhiza), leaf surfaces (phyllosphere), flowers (anthosphere), stems (caulosphere) and fruits (carposphere). It was shown that different plant compartments harbor distinct microbial communities (Sessitsch et al. 2002; Berg et al. 2005; Fürnkranz et al. 2012). The most attractive plant-associated environment for microorganisms is the rhizosphere. This microhabitat is characterized by a high concentration of nutritional substances arising from the roots, called ´rhizodeposit`, attracting bacteria and fungi (Hartmann et al. 2008). Up to 21% of the carbon that is fixed by plants is released via the secretion of root exudates (Lugtenberg and Kamilova 2009), constituting a substantial rhizodeposit fraction. Substances like phenolic compounds (flavonoids), carboxylic acids, sugars, and amino acids that are secreted by roots act as chemoattractants and lead to efficient root colonization by microbes (Faure et al. 2009). Microbial community structures in the rhizosphere are highly influenced by rhizodeposit composition that again depends on the host plant genotype. In addition, soil quality, geography, climate, the plant developmental stage, plant health, and anthropogenic influences affect microbial population structures in the rhizosphere (Berg and Smalla 2009).

Microbial endophytes

Bacterial and fungal plant colonizers are either located on plant surfaces (ectosphere) or can be found inside plant tissues (endosphere). The latter are called endophytes [(*endon* (gr.): within; *phyton* (gr.): plant] and live in intercellular spaces or can even be found intracellularly (inside plant cells) (Gray and Smith 2005; Schulz and Boyle 2005). Microbes enter plants predominantly via roots (Compant et al. 2010), stomata and hydathodes (Gudesblat et al. 2009), wounds, such as broken trichomes, epidermal junctions (Senthilkumar et al. 2011), or the flower (de Neergaard 1989) to become endophytes. Furthermore, endophytes can be seed-borne and transmitted vertically (van Overbeek et al. 2011). According to the definition of Schulz and Boyle (2005), endophytes undergo relationships with their hosts that can range from virulent to

mutualistic. The type of interaction between endophyte and host plant can be variable and might change for example from neutral into parasitic as a response to biotic and abiotic environmental factors (e.g. stress conditions, plant developmental stage) (Schulz and Boyle 2005).

Promotion of plant growth and health by bacteria and fungi

Prominent examples for mutualistic host-endophyte interactions are endomycorrhizal fungi (especially arbuscular mycorrhiza) (Schulz and Boyle 2005), supporting the plant`s phosphorus (P) and water uptake. Bacteria belonging to the genera *Rhizobium*, *Mesorhizobium*, *Allorhizobium*, *Bradyrhizobium*, *Sinorhizobium*, and *Azorhizobium* undergo symbiosis with plant species belonging to Fabaceae, whereby the bacteria fix atmospheric nitrogen (N_2) in root nodules and convert it into plant available ammonium (NH_4) by the N_2-fixers (diazotroph`s) nitrogenase enzyme complex (Bloemberg and Lugtenberg 2001). Root nodulation is also known for symbionts belonging to the bacterial genus *Frankia* that interact with certain tree species (Normand et al. 2007). However, also non-symbiotic associations between plants and their microbial colonizers can lead to an increase in plant health and growth that can be based on N_2 fixation, solubilization of P, secretion of phytostimulators, and reduction of plant stress (Dart 1986; Bloemberg and Lugtenberg 2001; Gull et al. 2004; Glick 2005). Bacteria conferring these traits are termed ´plant growth promoting bacteria` (PGPB) (Bashan and Holguin 1998). Examples for free-living, plant-associated diazotrophs belong to the genera *Azospirillum*, *Herbaspirillum*, *Acetobacter*, *Azotobacter* (Bloemberg and Lugtenberg 2001). PGPB provide P to plants by solubilizing either organic or inorganic bound P. Organic bound P becomes plant available due to the secretion of phosphatases, phytases, phosphonatases, and C-P lyases whereas release of mineral phosphate is enabled by organic acids produced by P-solubilizers (Lugtenberg and Kamilova 2009). Bacterial phytostimulators have a broad-spectrum efficacy ranging from cofactors with antioxidant activity [pyrroloquinoline quinone (PQQ)] to different phytohormones and volatile substances (Lugtenberg and Kamilova 2009). Besides cytokinins, gibberellins and abscisic acid, auxin (indole-3-acetic acid, IAA) is the most prominent example for a bacterial derived phytohormone (Bloemberg and Lugtenberg 2001; Karadeniz et al. 2006). Non-plant phytohormone synthesis is also known for mycorrhizal fungi (Ho 1987). Apart from microbial synthesis of phytohormones, bacteria can regulate the host`s synthesis of messenger substances even by transfer of respective bacterial genes into the plant genome or alter phytohormone concentrations by degradation of these substances (Faure et al. 2009). 2.3-

butanediol belongs to gaseous phytostimulators of bacterial origin (Lugtenberg and Kamilova 2009). The reduction of plant stress by PGPB is attributed to degradation of 1-aminocyclopropane-1-carboxylate (ACC), a precursor of the plant stress hormone ethylene, via the enzyme ACC deaminase (Glick 2005).

Indirect paths for maintaining a healthy plant status are an effective plant colonization and competence against pathogens for nutrients and niches by e.g. synthesis of antimicrobial compounds and parasitism, inhibition of the pathogen`s cell communication by signal interference, and production of metabolites involved in systemic resistance of plants (Bar-Ness et al. 1991; O`Sullivan and O`Gara 1992; Bloemberg and Lugtenberg 2001; Lugtenberg and Kamilova 2009; Raaijmakers et al. 2009). These traits are expressed by antagonistic microbes, so called ´biological control agents` (BCA) (Chernin and Chet 2002). An efficient colonization of the plant tissue by BCA/PGPB goes along with biofilm formation as it was shown on root surfaces (Lugtenberg and Kamilova 2009). Extensive plant colonization is not only a prerequisite for the establishment of biocontrol and plant growth promotion traits by bacteria and fungi, but enables the elimination of pathogens from colonization sites, and is therefore described as a biocontrol feature per se (Lugtenberg and Kamilova 2009). Some rhizosphere BCA are able to bind iron highly effectively by synthesis of siderophores and deplete in this way the iron pool for pathogens in the soil (Lugtenberg and Kamilova 2009). Synthesis of antimicrobial substances belongs to microbial defense strategies and is commonly exerted by BCA to suppress phytopathogens. A broad range of respective liquid and volatile substances is known so far. Extracellular enzymes like β-1.3-, β-1.4- and β-1.6-glucanases, cellulases, chitinases, and proteases are produced by a wide range of microorganisms (Whipps 2001; Harman et al. 2004; Vinale et al. 2008). Beside extracellular enzymes, antibiotics are produced by many BCA. Phenazines, pyoluteorin, pyrrolnitrin, 2.4-Diacetylphloroglucinol (2.4-DAPG) are examples for Gram-negative bacterial antibiotics whereas streptomycin, tetracycline, zwittermycin, and kanosamine are antibiotics from Gram-positive bacteria (Lugtenberg and Kamilova 2009). *Trichoderma* spp. produce substances like viridiol, gliotoxin and gliovirin that are toxic for phytopathogens (Vinale et al. 2008). Well known volatile antimicrobial substances are hydrogen cyanide (HCN) and 2.3-butanediol (Lugtenberg and Kamilova 2009). Certain BCA like *Trichoderma* sp. (Harman et al. 2004) combine their inhibitory capacity, based on secretion of antibiotics and extracellular enzymes, with parasitism on phytopathogens. Another way, how bacterial antagonists interact with causal agents of plant diseases, is their influence on the

parasite`s cell communication. A well described mode of bacterial communication is quorum sensing (QS) (Waters and Bassler 2005). QS is a cell density-dependent phenomenon that controls many functions like horizontal transfer of plasmids, expression of rhizosphere competence traits (e.g. antibiotic and biofilm formation) and virulence factors (Waters and Bassler 2005; Faure et al. 2009). Acyl-homoserine lactones (AHLs) and oligopeptides serve as QS signal molecules for either Gram-negative or Gram-positive bacteria respectively. Interference of QS signaling of phytopathogens is based on the synthesis of QS-signal biomimics or AHL degradation by AHL lactonases (Faure et al. 2009). On the other hand, PGPB and BCA use QS to establish traits supporting plant growth and health (Müller et al. 2009). This underlines again the importance of an efficient root colonization by respective microorganisms as their expression of biocontrol- and plant growth promotion traits is often subjected to a certain quorum. The efficacy of the plant`s immune response during phytopathogenic infestation can be increased by rhizomicrobial-mediated induced systemic resistance (ISR). Substances like oligosaccharides, proteins, lipopolysaccharides (LPS), salicylic acid (SA), AHLs, 2.3-butanediol, and siderophores act as elicitors of jasmonic acid and ethylene signals that are released by the plant to initiate ISR (Harman et al. 2004, Lugtenberg and Kamilova 2009).

The interactions between beneficial plant-associated microbes, phytopathogens, and the host plant are summed up in Fig. 2.

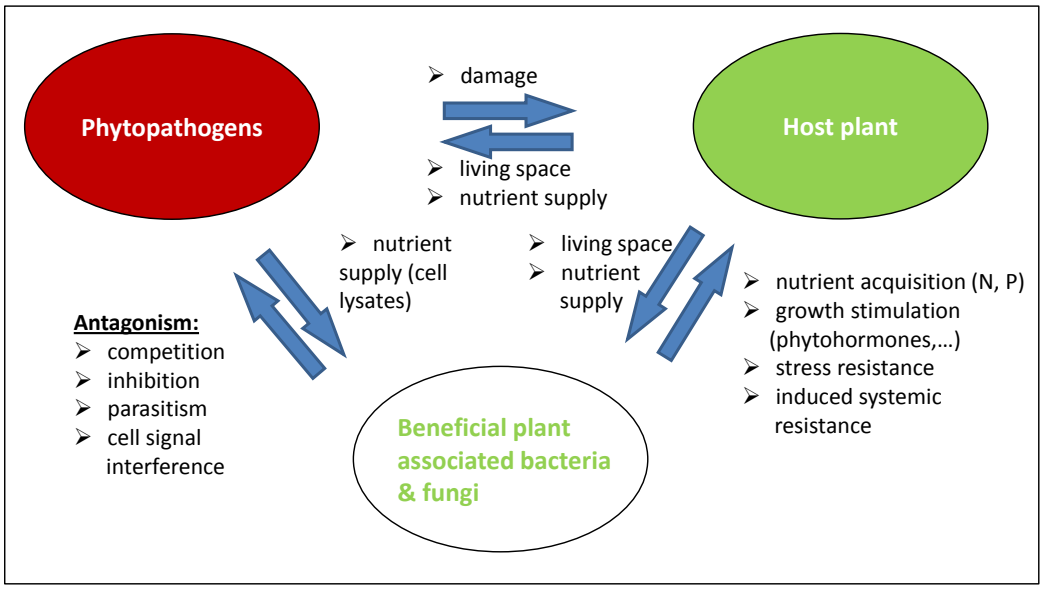

Fig. 2. Interactions between beneficial plant-associated microbes, phytopathogens, and the host plant.

Use of microbial inoculants for plant growth promotion and biocontrol of phytopathogens in agriculture

Microbial inoculants that are used in agriculture can be differentiated according to their mode of action. Biofertilizers, plant strengtheners, and phytostimulators consist of PGPB whereas biopesticides contain BCA (Berg 2009). For processing bacteria and fungi into biological products, a high stability and practical utilization of them have to be achieved. Different types of formulations exist for a good conservation and user-friendly application of PGPB respectively BCA: liquid-based formulations, wettable powders, water-dispersible granules and pellets (Berg 2009). Transferring promising inoculants into biological products is often a challenging undertaking. Especially from gram-negative bacteria it is often difficult to obtain a formulated product with a high shelf-life. Because of expensive and time-consuming registration procedures it is important to exclude potential pathogenic bacteria in an early step of the product development (Berg 2009). Different methods were established to recognize bacteria that could be harmful for the environment and the human health. To these methods count the affiliation of new PGPB/BCA to risk groups above two (http://www.dsmz.de), growth tests at 37°C, and the accomplishment of pathogenicity assays (Zachow et al. 2009). Furthermore, effects of the application of plant-beneficial bacteria and fungi on non-target microorganisms need to be considered (Scherwinski et al. 2008). Examples for bacterial and fungal inoculants that are manufactured as biological products are *Bacillus subtilis* FZB24, *Serratia plymuthica* HRO-C48, *Pseudomonas chlororaphis* MA 342, and *Trichoderma harzianum* T22 (Fravel 2005; Berg 2009).

Objective of this doctoral thesis

The aim of this doctoral thesis was the development of a biological product to enhance the plant health and growth of Styrian oil pumpkin (*Cucurbita pepo* L. subsp. *pepo* var. *styriaca* Greb.). In order to biologically control aforementioned oil pumkin pathogens and particularly *D. bryoniae,* strains of the ascomycete from different field sites in Austria were investigated regarding their genotypic variability and pathogenicity. Furthermore the bacterial oil pumpkin pathogens *Pseudomonas* spp., *P. carotovorum,* and *X. cucurbitae* were investigated with respect to their synergistic interactions with *D. bryoniae*. Bacterial communities associated with different oil pumkin varieties and microhabitats were in investigated at different plant developmental stages by cultivation-independent analysis. *In vitro* broad-spectrum antagonists

against respective oil pumpkin pathogens were obtained with focus on endophytes. Bacterial broad-spectrum antagonists were analyzed by genotypic fingerprinting methods in order to select strains that were subjected to taxonomic affiliation. Identified isolates were investigated for their mode of antagonistic action and efficacy for suppression of gummy stem blight on oil pumpkin under greenhouse conditions. Out of selected bacteria an alginate-based formulation was produced that was used for coating of the host plant`s seeds. In parallel, priming of the hull-less seeds in cell supensions of corresponding strains was performed. These inoculation treatments were tested with and without addition of a chemical stripper against respective controls (including an exclusive chemical treatment, used in conventional agriculture) in the course of different field trials performed at different sites in Styria and at different vegetation periods (2010 and 2011). Effects of the different treatments on oil pumpkin growth and health over time were thereby evaluated.

Summary of results

Studies on *D.* bryoniae and bacterial pathogens

Isolates of the pathogen were obtained from different field sites in Austria and grouped according their morphology. Representatives of the five phenotypic groups showed a high degree of pathogenicity on Styrian oil pumpkin in the course of a newly developed greenhouse assay. In contrast to the high morphologic versatility of the isolates a low degree of genotypic differences were assessed between the different strains. Furthermore it was found that the investigated bacterial pathogens *Pseudomonas* spp., *P. carotovorum*, and *X. cucurbitae* were translocated by *D. bryoniae*.

Oil pumpkin associated microbial communities

Bacillus and Pseudomonas community structures that originate from Styrian oil pumpkin showed a clear dependency by the microhabitat and the plant stage whereas the oil pumkin cultivar had no significant effect on population structures. Dominant members of the *Bacillus* community that were found to be associated with the Cucurbitaceae host are *B. weihenstephanensis, B. flexus, B. psychrodurans, B. siralis, B. subtilis, B. indicus, B. gibsonii* and *B. firmus*. The oil pumpkin *Pseudomonas* community consisted of the dominant species *P. oryzihabitans, P. putida, P. syringae, P. viridiflava* and *P. fluorescens*.

Abundances of oil pumpkin associated microorganisms and selection of broad-spectrum antagonists against oil pumpkin pathogens

In general, oil pumpkin associated fungi were under-represented in comparison to bacteria isolated from oil pumpkin plants. Highest microbial cell counts per g plant material were obtained from the female flower [up to 10^7 colony forming units (CFU) for bacteria and up to 1.2 x 10^3 for fungi]. Bacterial cell counts from the endorhiza and from the fruit were up to 1.6 x 10^4 and 3.8 x 10^3 respectively (fungi were under the detection limit).

In vitro antagonism tests revealed that 7% of tested microbial oil pumpkin associated isolates exhibited an inhibition effect on growth of *D. bryoniae*. Subsequent dual culture assays with potential *D. bryoniae* antagonists against bacterial oil pumpkin pathogens showed that out of 165 bacterial and fungal *D. bryoniae in vitro* antagonists, 43 prokaryotic strains demonstrated broad-spectrum antagonistic activity against at least two tested bacterial phytopathogens whereas none of the fungal isolates was tested positively. Genotypic fingerprinting of broad-spectrum antagonists led to the selection of six bacterial strains for further analyses: *Lysobacter antibioticus* L169, *L. antibioticus* L175, *Lysobacter gummosus* L101, *Paenibacillus polymyxa* PB71, *Pseudomonas chlororaphis* P34, *Serratia plymuthica* S13.

Characterization of selected broad-spectrum antagonists and performance *ad planta* in the greenhouse

Synthesis of soluble and/or volatile substances against *D. bryoniae* was noticed for all strains except *L. antibioticus* L175. A greenhouse experiment revealed significant decrease of disease severity (ds) on oil pumpkin plants after artificial infection with *D. bryoniae* when seeds were inoculated with *P. chlororaphis* P34 or a mix of *L. gummosus* L101, *P. polymyxa* PB71, *P. chlororaphis* P34 and *S. plymuthica* S13.

Efficacy of selected broad-spectrum antagonists regarding health and growth of Styrian oil pumpkin under field conditions

To evaluate performance of selected broad-spectrum antagonists under practical conditions, field trials with Styrian oil pumpkin were conducted in 2010 (one field site) and 2011 (two field sites). In comparison to control treatments, seed primings with suspensions of *L. gummosus*

L101 and *P. polymyxa* PB71 led to a decrease in mildew infestation in 2010, whereas no significant effects of bacterial treatments on fruit rot severity were observed in all field trials. *L. gummosus* L101 and *S. plymuthica* S13 conferred a higher desiccation tolerance especially at one field site in 2011. *S. plymuthica* S13 promoted the seedling emergence throughout all field sites, could compensate the effect of the chemical stripper at one field site and might be considered as a biological stripper for oil pumpkin. Applications of *L. gummosus* L101 as well as *P. polymyxa* PB71 resulted in reproducible increases in harvest yields across different field sites.

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Appendix

1. Compendium of publications

Publication I: Emerging multi-pathogen disease caused by *Didymella bryoniae* and pathogenic bacteria on Styrian oil pumpkin

Publication II: Microbial diversity inside pumpkins: microhabitat-specific communities display a high antagonistic potential against phytopathogens

Publication III: Promotion of growth, health and stress tolerance of Styrian oil pumpkins by bacterial endophytes

Additional publication I: Characterization of plant growth promoting bacteria from crops in Bolivia

Additional publication II: Using ecological knowledge and molecular tools to develop effective and safe biocontrol strategies – Strategy to control a multi-species disease in the Styrian oil pumpkin

Additional publication III: Multi-pathogen disease caused by *Didymella bryoniae* and bacteria on Styrian oil pumpkin: microbial ecology and biocontrol

Additional publication IV: Analysis of the antagonistic potential of microorganisms derived from the Styrian oil pumpkin against its pathogens

2. Acknowledgement

3. Curriculum vitae

4. Publication list

5. Statutory declaration

Publication I

Emerging multi-pathogen disease caused by *Didymella bryoniae* and pathogenic bacteria on Styrian oil pumpkin

Martin Grube, Michael Fürnkranz, Sabine Zitzenbacher, Herbert Huss, Gabriele Berg. Eur. J. Plant Pathol. (2011), DOI 10.1007/s10658-011-9829-8

Personal contribution: isolation and identification of *D. bryoniae* strains, infection studies with bacterial pathogens, support during infection studies with *D. bryoniae*, TCS analysis, support of the completion of the manuscript

Abstract

The Styrian oil pumpkin, *Cucurbita pepo* L. subsp. *pepo* var. *styriaca* Greb. is a crop of cultural, commercial, and medical importance. In the last decade, yield losses of pumpkins increased dramatically. The ascomycetous fungus *Didymella bryoniae* (Fuckel) Rehm was identified as main causal agent provoking gummy stem blight as well as black rot of pumpkins. We observed a remarkable phenotypic diversity of the fungal pathogen, which contrasted with a high genotypic similarity. Evidence of pathogenicity of *D. bryoniae* on Styrian oil pumpkin was demonstrated in a newly developed greenhouse assay. Isolates representing the five observed phenotypic groups fulfilled the Koch's postulates. In the field, the fungal disease was often associated with bacterial colonization by *Pectobacterium carotovorum*, *Pseudomonas viridiflava*, *Pseudomonas syringae* and *Xanthomonas cucurbitae*. The pathogenic behaviour of bacterial isolates on pumpkin was confirmed in the greenhouse assay. The high co-incidence of fungal and bacterial disease suggests mutualistic effects in pathogenesis. With a new assay, we found that bacteria can use the mycelium of *D. bryoniae* for translocation. We argue that the rapid rise of the multi-pathogen disease of pumpkins results from combined action of versatile pathogenic bacteria and the rapid translocation on a structurally versatile mycelium of the fungal pathogen.

Introduction

During the past decade, a dramatic increase of diseases in Styrian oil pumpkin (*Cucurbita pepo* L. subsp. *pepo* var. *styriaca* Greb.) has been observed; up to 80% yield losses were reported

(Huss 2007). The pumpkin cultivar ‘Styrian oil pumpkin’ is a phylogenetically young member of the Cucurbitaceae since the mutation leading to dark green seeds with stunted outer hulls arose in the 19th century (Frühwirth and Hermetter 2007). This unique cultivar has been grown for at least 100 years in Austria but today it has much broader growing areas, including several Southern European and African countries, China, Russia, and the USA. The seed is edible and used in the bakery industry and for the production of dark-coloured seed oil. Styrian pumpkin seed oil is a regional specialty (European Union protected designation of origin) and international gourmet oil. It has an intense nutty taste and is rich in polyunsaturated fatty acids but contains also vitamins, phytosterols, minerals and polyphenols (Frühwirth and Hermetter 2007). Studies suggest usefulness of the oil in the prevention and treatment of benign prostatic hyperplasia as well as to prevent arteriosclerosis and regulate cholesterol levels (Dreikorn 2002).

As the main causal agent of black rot and gummy stem blight, *Didymella bryoniae*, (Auersw.) Rehm (anamorph *Phoma cucurbitacearum*) was identified. This fungus was first reported in Europe in 1869 from *Bryonia* (wild hops) and today it is found on six continents as pathogen on a broad range of *Cucurbitaceae* (Chiu and Walker 1949; Keinath 2010). Most information about diseases caused by *D. bryoniae* focused on melons or cucumber but not yet on pumpkins. The fungus can infect any stage of plants and shows a variety of symptoms according to the crop and stage concerned; furthermore it can be seed-borne, air-borne, or soil-borne (Lee et al. 1983; van Steekelenburg 1983; Bruton 1998; Keinath 2010). Black rot is an important pre- and post-harvest fruit rot of pumpkins which seriously reduces yield and quality (Zitter and Kyle 1992; Sitterly and Keinath 1996; Babadoost and Zitter 2009). Measures to suppress pathogens in oil pumpkin cultivation include application of chemical fungicides (pyrrole derivates, phenylamide), 3-years of crop rotations and breeding strategies for resistance against *D. bryoniae*. However, due to the fact that pumpkin seeds are always harvested in the field and all residues are incorporated in soil, there is an increasing pathogen pressure in fields.

Interestingly, in our region, Styrian oil pumpkins are frequently attacked also by bacterial soft rot caused by *Pectobacterium carotovorum*. Furthermore, bacteria such as *Pseudomonas viridiflava* and *Xanthomonas cucurbitae* can affect pumpkin plants in combination with *D. bryoniae* (Huss 2011). These co-infections of *D. bryoniae* and bacterial pathogens, which colonize and destroy the whole inner pumpkin pulp, led to extremely high yield losses. Most diagnostic processes cease with the detection of the first relevant pathogen but molecular methods often show a more diverse pathogen spectrum (Crous and Gams 2000¸ Grube and Berg

2009). Furthermore, synergistic pathogenic effects were already reported for *D. bryoniae* (Leben 1984; Zitter and Kyle 1992). To suppress these multi-pathogen diseases, all players and their interaction have to be analyzed.

The objective of the present work was to characterize different *D. bryoniae* isolates from *C. pepo* at the phenotypic and genotypic levels. Furthermore, collected isolates were tested for their pathogenicity on oil pumpkins using a newly developed assay. In addition, also a new experimental set-up was established to study the synergistic interaction between the fungus and pathogenic bacteria.

Materials and methods

Strain collection

In 2008, *D. bryoniae* isolates were collected from diseased oil pumpkins grown in fields located in Gleisdorf (47°6′14″N, 15°42′30″E) and the district St. Peter in Graz (47°3′18″N, 15°28′30″E) located in Styria. Infected tissue was placed on water agar (water, 15 g agar l^{-1}) supplemented with chloramphenicol (100 mg l^{-1}). Presumptive colonies of *D. bryoniae* were isolated and stored at −20°C. One additional isolate of *D. bryoniae* (A-220-2b) was included (isolated from pumpkins grown in an experimental station of Bundesanstalt für alpenländische Landwirtschaft Raumberg-Gumpenstein, Stadl-Paura, 48°5′2″N, 13°51′50″E). All bacterial pathogens, *P. carotovorum* ssp. *atrosepticum* 25–2, *X. cucurbitae* 6h4, *P. viridiflava* 2d1 and *P. syringae* 6g1 were originally isolated from Styrian oil pumpkins (Huss 2009; Göttinger Sammlung Phytopathogener Bakterien: GSPB, University of Göttingen, Germany). Before application, we confirmed identification by molecular analysis.

Phenotypic characterization of *D. bryoniae* isolates

Isolates of *D. bryoniae* were grown on malt agar (MA) at ambient temperature (23–25°C) in the dark for 3 days, before agar blocks from the isolates (5 mm diameter) were transferred to MA and quarter-strength potato-dextrose agar (QPDA; Roth GmbH, Karlsruhe, Germany) plates. MA plates were incubated in the dark for 7 days and the diameters of the colonies covering the agar plates were measured. Furthermore, the morphology and the color of the colonies were assessed. Isolates on QPDA plates were grown at ambient temperature and with a photoperiod of 12 h. The isolates were characterized morphologically for three replicates of each isolate.

Genotypic characterization of *D. bryoniae* isolates

To isolate fungal DNA, all *D. bryoniae* isolates were grown on QPDA for 5 days at ambient temperature. Cultures were scraped to remove mycelium and placed in 50 ml liquid medium containing 1 g of KH_2PO_4, 0.5 g of $MgSO_4$, 6.0 g of casein hydrolysate, 100 g of sucrose, 1 mg of $FeSO_4$, 0.15 mg of $CuSO_4$, 0.10 mg of $ZnSO_4$, and 0.10 mg of $NaMoO_3$ per litre of distilled water. The medium was adjusted to pH 4.9 with 0.1 M HCl and autoclaved before inoculation. The cultures were incubated for 7 days at 23°C, at 120 rpm in the dark. Liquid cultures were then filtered through a sterile filter paper, and the mycelium was washed with sterile distilled water. After removing as much liquid as possible, the mycelium was frozen and stored at −20°C. About 150 mg of the mycelia were transferred to 2 ml tubes containing glass beads (0.25–0.5 and 1.7–2.0 mm in diameter). After adding 500 µl lysing buffer (0.4 M Cetrimonium bromide (CTAB), 1 M NaCl, 7 mM Tris, 30 mM EDTA, pH 5.5), the tubes were agitated with a FastPrep^{TM} machine (Qbiogene BIO 101® systems, Carlsbad, CA). The tubes were incubated at 65°C for one h and mixed repeatedly. Then, 500 µl of chloroform:isoamyl alcohol (24:1) were added and vortexed briefly and centrifuged for 5 min at 13,000 × g. The supernatants were transferred to fresh tubes, and the chloroform:isoamyl alcohol step was repeated. One ml of precipitation puffer (14 mM CTAB, 40 mM NaCl) was added and incubated at room temperature for one hour. The tubes were centrifuged for 15 min at 13000 × g, supernatants were removed and pellets mixed with 350 µl 1.2 M NaCl. The treatment with chloroform:isoamyl alcohol was repeated twice. Supernatants were transferred to fresh tubes and 210 µl of isopropanol were added before samples were incubated at −20°C overnight. Afterwards tubes were incubated at room temperature for 5 min and subsequently centrifuged for 20 min at 13.000 × g. DNA was washed with 70% ethanol, dried, suspended in 40 µl PCR grade water and stored at −20°C.

To obtain DNA sequences of *D. bryoniae* for analysis of genetic relationship, ITS rRNA gene region was amplified and sequenced using primers ITS 1F (5′CTT GGT CAT TTA GAG GAA GTA A 3′) and ITS 4r (5′-TCC TCC GCT TAT TGA TAT GC-3′). PCR products were purified using the peqGOLD MicroSpin Cycle Pure Kit (Peqlab , Erlangen, Germany). Purified PCR products were sequenced using the genetic analyzer AB3730 and the ABI PRISM BigDye Terminator v3.1 Ready Reaction Cycle Sequencing Kit (Applied Biosystems, Vienna, Austria). Obtained data were subjected to BLAST search with the National Center for Biotechnology

Information (NCBI) database (http://www.ncbi.nlm.nih.gov) to confirm taxonomic affiliation to *D. bryoniae* (Altschul et al. 1997). All sequences were aligned using Molecular Evolutionary Genetics Analysis (MEGA) software (Version 4.0.2). A network of relationship was constructed with the TCS program v. 1.21.

BOX-PCRs were done as described by Rademaker and De Bruijn (1997) using the BOXA1R primer 5′-CTA CGG CAA GGC GAC GCT GAC G-3′. PCR amplification was performed with a Peltier Thermal Cycler PTC-200 (Biozym Diagnostic, Hessisch Oldendorf, Germany) using an initial denaturation step at 95°C for 6 min, and subsequently 35 cycles of denaturation at 94°C for 1 min, annealing at 53°C for 1 min. and extension at 65°C for 8 min. followed by final extension at 65°C for 16 min. A 10 µl aliquot of amplified PCR product was separated by gel electrophoresis on 1.5% agarose gels in 0.5 × TBE buffer for 6 h, stained with ethidium bromide, and photographed under UV transillumination. The reproducibility of the results was verified in at least two independent experiments.

Assay to test pathogenicity of *D. bryoniae* isolates

Oil pumpkins (*Cucurbita pepo* L. subsp. *pepo* var. *styriaca* Greb.) cultivar 'Gleisdorfer Diamant' (Saatzucht Gleisdorf, Austria) were seeded in 10 cm plastic pots filled with a soil mix containing clay (Gramoflor, Vechta, Germany). *D. bryoniae* strains SP2, SP6, SP12, GL1 and A-220-2b were grown on QPDA at ambient temperature and 12 h photoperiod until pycnidia were produced. Cultures were flooded with a solution of 0.1% (w/v) sucrose and 0.05% (w/v) casein and scraped to release conidia from pycnidia. The suspension was gently mixed on a vortexer and filtered to remove mycelium of the fungus. Spore concentration was determined by counting in a haemocytometer and adjusted to 10^5 conidia per ml. Two-weeks old plants were inoculated by injection of 50 µl spore suspension in the two first petioles. Plants inoculated with 0.1% sucrose/0.05% casein solution served as a control. The plants were covered with plastic bags for 11 days and kept at 20 ± 2°C and about 80% humidity with a 12 h photoperiod under greenhouse conditions. Plants were assessed for development of symptoms from the 6th to 11th day after infection. Infection experiments were carried out on 12 plants per *D. bryoniae* isolate. The presence of pycnidia was checked by microscopy. The experiment was repeated independently three times. From re-isolated *D. bryoniae* strains, DNA was extracted and used as template for BOX-PCR (as described above) to compare banding patterns with those from isolates that were used for infection.

Assay to test pathogenicity of bacterial pathogens

Pathogenicity of bacterial isolates (Huss 2009) was confirmed by artificial infections of oil pumpkin plants 'GL Opal' and subsequent re-isolation of the bacterial strains: bacterial strains were grown in LB medium (Roth GmbH, Karlsruhe, Germany) at 30°C under agitation. Cells were transferred into sterile 0.15 M NaCl and counted to adjust a concentration of 1×10^8 cells ml^{-1}. Leaves were injured with a grater and suspensions as well as sterile 0.15 M NaCl (serving as a control treatment) were sprayed on the leaves. Plants were covered with plastic bags, kept at 26°C with a 12 h photoperiod and observed until 11 days after infection. Infested plant parts were homogenized with sterile NaCl (0.15 M); these suspensions were diluted and plated onto water and LB agar. Colonies from these plates were picked, streaked out and DNA was isolated from strains by cooking cells in PCR grade water for 10 min. Isolated DNAs from potential as well as applied pathogens were used for BOX-PCR (as described above). The obtained molecular fingerprints were compared with the patterns from the inocula.

Fungal-bacterial interactions on agar medium

The system developed by Kohlmeier et al. (2005) was modified for our study on *D. bryoniae*-bacteria interaction. One *D. bryoniae* isolate per morphogroup was grown on a disk (1.4 cm diam.) of potato dextrose agar (PDA) for 2 days. Bacterial pathogens *P. carotovorum* ssp. *atrosepticum* 25–2, *X. cucurbitae* 6h4, *P. viridiflava* 2d1 and *P. syringae* 6g1 were grown O/N in Tryptic Soy Broth (TSB) (Roth GmbH, Karlsruhe, Germany) at 30°C, 120 rpm. Bacterial suspensions were centrifuged for 5 min. at 7.500 × g and resuspended in 0.15 M NaCl. Bacterial cell concentrations were determined by counting in a haemocytometer and adjusted to approximately 10^8 cells ml^{-1}. *D. bryoniae* strains grown on PDA disks were inoculated with 10 µl of bacterial suspensions and placed in 0.5 - 1 mm distance from a strip of QPDA (minimal medium agar; 1×3 cm) as shown in Fig. 5. The gap between the media pieces prevented swarming of motile bacteria from one agar piece to the other, whereas *D. bryoniae* could easily bridge the gap with growing hyphae. The QPDA strips colonized by the strains after 12 days of incubation were sliced and placed in 2 ml tubes. One ml of 0.15 M NaCl was added and vortexed several times to separate bacteria attached to fungal hyphae. The suspensions were plated on LB agar and incubated at 30°C for several days. The identity of bacterial strains was checked by their individual BOX-PCR patterns.

Statistical analysis

Analysis of Variance (ANOVA) in addition with Games-Howell post hoc test ($P < 0.05$) was performed with Predictive Analysis SoftWare (PASW, Version 18.0.0) to compare diameters of *D. bryoniae* colonies.

Nucleotide sequence accession numbers

Obtained DNA sequences of *D. bryoniae* were deposited in GenBank under accession numbers HQ684024 to HQ684032 and DNA sequences from phytopathogenic bacteria were deposited under JF906521 to JF906524.

Results

Phenotypic diversity of pumpkin-associated *D. bryoniae* isolates

Altogether nine *D. bryoniae* strains were isolated from infested pumpkins in Austria during 2008 (Table 1). Based on their morphological traits developed within 7 days in culture, these isolates could be distinguished. On MA plates, isolates SP4, SP12 and GL1 formed beige (young growth zone) and black (older zone) substrate mycelia and white hairy aerial mycelia (Fig. 1). Isolates SP1 and SP6 formed beige to reddish-brown (young growth zone), or black substrate mycelia and the same aerial mycelia as the first group. Isolates SP2, SP9 and SP3 formed beige substrate mycelia with only a small part of black admixture; their aerial mycelia were white and fluffy-hairy. Isolate A-220-2b produced beige substrate mycelium and white aerial mycelium. On the basis of the observed differences, the isolates were grouped into four morphological groups; group I: SP4, SP12 and GL1; group II: SP1 and SP6; group III: SP2, SP9 and SP3; group IV: A-220-2b. When isolates grew more than 2 weeks, phenotypic characteristics became more similar on MA-plates. Growing on QPDA plates during a 12 h light/dark photoperiod, all isolates produced dark green to black substrate mycelia and white aerial mycelia. Pycnidia were produced in concentric rings. The daily growth had a range between 8.5 and 13.1 mm and all *D. bryoniae* isolates reached the edges of the petri dishes within 7 days.

Table 1. Growth and morphology of different isolates of *D. bryoniae* grown on malt agar at 22°C.

Isolate	Origin	Colony diameter (mm)[a]	Fungal colony morphology	Morpho-group
SP1	St. Peter/Graz/Styria	13.1 (a)	beige to reddish brown and black substrate mycelium, white hairy aerial mycelium	II
SP2	St. Peter/Graz/Styria	11.2 (b)	beige substrate mycelium, white fluffy hairy aerial mycelium	III
SP3	St. Peter/Graz/Styria	11.1 (abc)	beige substrate mycelium, white fluffy hairy aerial mycelium	III
SP4	St. Peter/Graz/Styria	11.7 (abc)	beige to black substrate mycelium, white hairy aerial mycelium	I
SP6	St. Peter/Graz/Styria	11.8 (ab)	beige to reddish brown and black substrate mycelium, white hairy aerial mycelium	II
SP9	St. Peter/Graz/Styria	11.0 (ab)	beige substrate mycelium, white fluffy hairy aerial mycelium	III
SP12	St. Peter/Graz/Styria	8.5 (c)	beige to black substrate mycelium, white hairy aerial mycelium	I
GL1	Gleisdorf/Styria	11.0 (ab)	beige to black substrate mycelium, white hairy aerial mycelium	I
A-220-2b	Stadl Paura/Upper Austria	12.6 (ab)	beige substrate mycelium, white aerial mycelium	IV

[a]mean values of three replicates; different letters signify significant differences (ANOVA; Games-Howell $P < 0.05$).

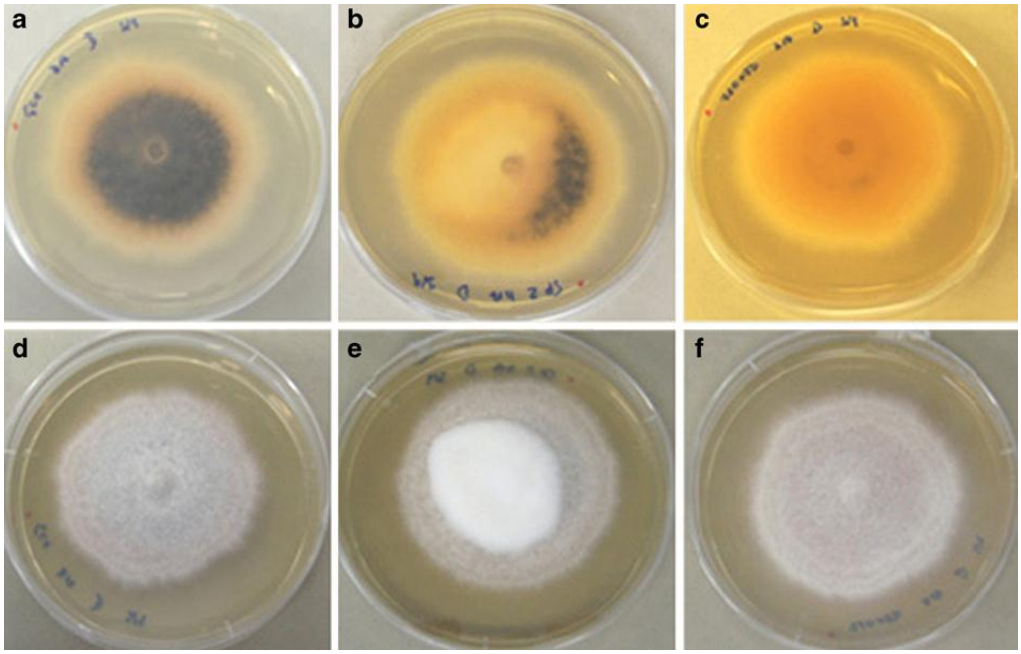

Fig. 1 Morphology of *D. bryoniae* isolates; Isolates grown on MA agar; a–c: view of cultures from below; d–f: view of cultures from top; a and d: isolate GL1 (Morphogroup I); b and e: isolate SP2 (Morphogroup II); c and f: isolate A-220-2b (Morphogroup IV).

Genotypic diversity of pumpkin-associated *D. bryoniae* isolates

ITS rRNA gene sequence analysis of the isolates confirmed their taxonomic affiliation to *D. bryoniae*. Differences between our sequences and *D. bryoniae* sequences from the database were analyzed with TCS program which reveal their high similarity (Fig. 2): sequences were identical with the exception of isolate SP1, which differed at two nucleotide positions. Only an isolate from grapevine EU030365.1 is distinct by 12 mismatches.

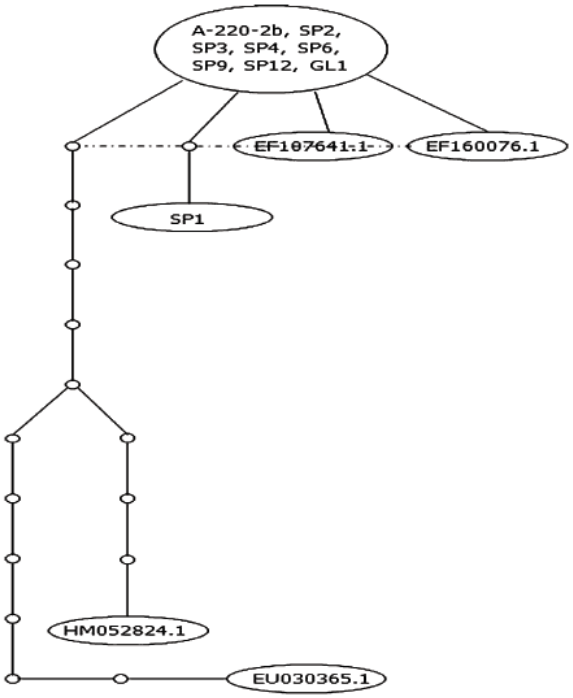

Fig. 2 TCS-analysis with obtained and reference DNA sequences of *D. bryoniae* (encoding ribosomal ITS regions, 384 bp) indicating number of non-matching bases between sequences.

To differentiate collected isolates at the genotypic level, all isolates were further analyzed by BOX-PCR fingerprints. The obtained BOX-PCR patterns again demonstrated a high similarity between the nine studied isolates (Fig. 3). Based on the presence/absence of an approximately 550 bp DNA fragment, they could be separated into two groups. The isolates SP1, SP2, SP3 and SP4 represent one, and SP6, SP9, SP12, GL1 and A-220-2b the other group.

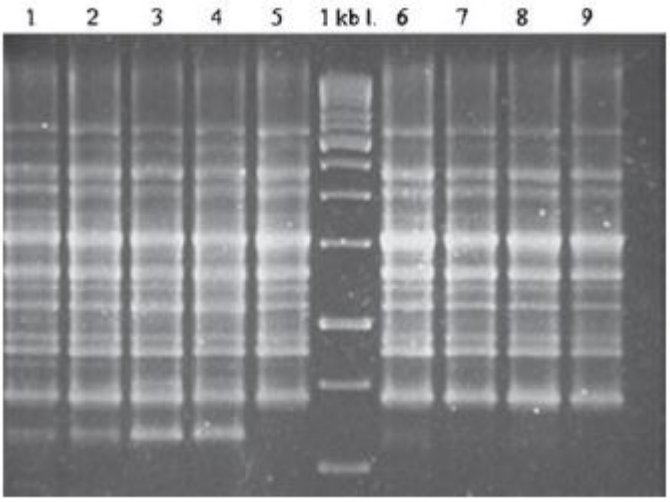

Fig. 3 BOX-PCR fingerprints of *D. bryoniae* isolates: lane 1, SP1; lane 2, SP2; lane 3, SP3; lane 4, SP4; lane 5, SP6; lane 6, SP9; lane 7, SP12; lane 8, A-220-2b; lane 9, GL1; DNA marker: 1 kb DNA-ladder; BOX-PCR products were loaded on a 1.5% (w/v) agarose gel.

Assays to test pathogenicity of *D. bryoniae* and bacterial phytopathogens on Styrian oil pumpkin

Five representatives (SP2, SP6, SP12, GL1, A-220-2b), selected according to their phenotypic and genotypic diversity as well as their production of pycnidia, were used for pathogenicity tests on Styrian oil pumpkin plants. All tested isolates were found to be pathogenic; they produced typical symptoms on the first true leaves. Five days after the infection the first symptoms were visible (Fig. 4). The early symptoms found on leaf stems were water-soaked lesions. These lesions rotted and the decay extended to the whole leaf. Thereafter grey-green leaf spots were formed in irregular patterns, which later changed their color to become dark brown. Furthermore, pycnidia on stems and leaves were noticed 11 days after infection. All tested isolates were re-isolated from infected tissues. Their identity was confirmed by morphological observation on QPDA plates and molecular BOX-fingerprint analysis.

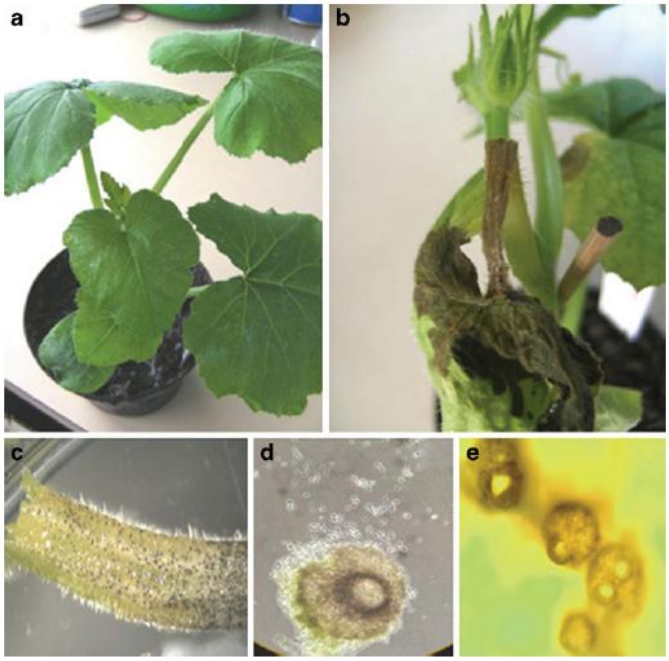

Fig. 4 Gummy stem blight of Styrian oil pumpkin caused after artificial infection with *D. bryoniae*: an uninfected plant; b infected plant with gummy stem blight lesion; c infected stem with pycnidia of *P. cucurbitacearum* (anamorph of *D. bryoniae*); d pycnidia with conidia; e pycnidia.

To verify pathogenicity of bacterial isolates *P. virdiflava* 2d1, *P. carotovorum* ssp. *atrosepticum* 25–2, *P. syringae* 6g1 and *X. cucurbitae* 6h4 on oil pumpkin, infection studies were performed. In all treated plants, 4 days after infections rotten lesions on leaves were visible, which increased rapidly within the next days (data not shown). From affected plant tissues tested bacteria were successfully re-isolated and their identity was confirmed by molecular fingerprints performed by BOX-PCR (data not shown).

Mycelium of *D. bryoniae* translocates pathogenic bacteria

The system used for assessing translocation of pathogenic bacteria by *D. bryoniae* is illustrated in Fig. 5. We selected four isolates of *D. bryoniae* (A-220-2b, GL1, SP2 and SP6) belonging to different morphotypes (Table 1) and bacterial phytopathogens *P. carotovorum* ssp. *atrosepticum* 25–2, *X. cucurbitae* 6h4, *P. viridiflava* 2d1 and *P. syringae* 6g1. Results of the translocation experiments are summarized in Table 2. In case of SP2, SP6 and GL1, all four bacteria were detected on the surface of the QPDA gel slice suggesting their translocation via the fungal hyphae. In contrast, this was not observed for isolate *D. bryoniae* A-220-2b. In the negative controls, all four bacteria were only detected at the inoculated slice (PDA).

Table 2. Fungus-mediated mobilization of bacteria

Table 2 Fungus-mediated mobilization of bacteria

D. bryoniae isolates	Bacterial isolates								Control
	P. carotovorum, ssp. *atrosepticum* 25-2		*P. viridiflava* 2d1		*X. cucurbitae* 6h4		*P. syringae* 6g1		without bacteria
	PDA	QPDA	PDA	QPDA	PDA	QPDA	PDA	QPDA	QPDA
A-220-2b	+	−	+	−	+	−	+	−	−
GL1	+	+	+	+	+	+	+	+	−
SP2	+	+	+	+	+	+	+	+	−
SP6	+	+	+	+	+	+	+	+	−
no fungus	+	−	+	−	+	−	+	−	

+bacteria were detected, −bacteria were not detectable

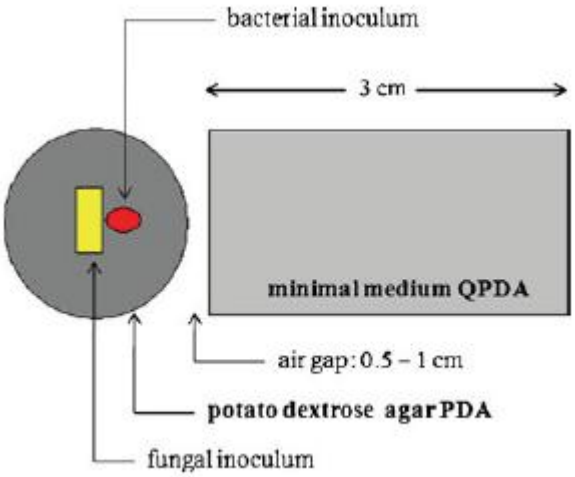

Fig. 5 Experimental design of interaction studies between *D. bryoniae* and pathogenic bacteria, carried out in a sterile petri dish.

Discussion

The fungus *D. bryoniae* was identified as main causal agent of high yield losses of Styrian oil pumpkin. Furthermore, *D. bryoniae* was found often associated with bacterial pathogens *P. carotovorum*, *P. viridiflava*, *P. syringae* and *X. cucurbitae*, which also contribute substantially to these yield losses. *D. bryoniae* hyphae were able to transport bacterial isolates, which was shown *in vitro*. These observations and the high coincidence of fungal and bacterial disease in the field suggest mutualistic effects in pathogenesis.

Based on morphological characterization in culture and by sequencing, all fungal isolates

from oil pumpkins affected by black rot were classified as *D. bryoniae* (Keinath et al. 1995). *D. bryoniae* produces white aerial mycelium and olivaceous-green to black substrate mycelium. *D. bryoniae* developed considerable morphological variation. We assigned these growth variants to five groups. Because growth conditions were similar and genetic diversity of the isolates was low, we suspect that epigenetic variation could be responsible for the morphological variation.

Further, we tested for the first time *D. bryoniae* isolates for their pathogenicity against oil pumpkin in a greenhouse assay. With the new assay - a modified version of the method described by Keinath et al. (1995) and Shim et al. (2006) - we showed that all tested isolates were significantly virulent to pumpkin. Five days after injection of conidial suspensions of the pathogen into leaf stems, and under conditions conducive to development of gummy stem blight, typical symptoms were observed (Lee et al. 1983), including the production of pycnidia on infected tissues. Re-isolated fungal strains from infected tissues were identified as *D. bryoniae*, which again confirmed the role in pathogenesis.

In the field, black rot was often detected together with symptoms of bacterial pathogens. We therefore suspected that some kind of interactions could take place in the development of pumpkin disease. Specific interactions of fungi with bacteria have been described in context with plant pathogenicity, in which bacteria are not independently acting as pathogens (Marpues et al. 2008). Kohlmeier et al. (2005) noticed that bacteria are mobilized by fungi and that apical growth of the fungi was not directly involved in the displacement of bacteria. Trifonova et al. (2009) reported that bacteria are able to migrate along fungal hyphae. This movement may involve bacterial growth along the hyphae or active migration on the surface of the hyphae. Continuous liquid films on the fungal surface seem to be important for translocation of bacteria (Furuno et al. 2010). Fungal hyphae mostly grow at the hyphal apices and regularly branch in regions of active growth (Wicket al. 2007). In our study, we tested the hypothesis whether a synergistic interaction between *D. bryoniae* and pathogenic bacteria *P. carotovorum* ssp. *atrosepticum* 25–2, *X. cucurbitae* 6h4, *P. viridiflava* 2d1 and *P. syringae* 6g1 exist. Using our new experimental setup to study hyphal-mediated bacterial translocation, we tested the *D. bryoniae* isolates GL1, SP6, SP2 and A-220-2b, each representing one morphological group. All tested isolates except A-220-2b could mediate the translocation of bacteria. Strain-specific differences are well known for several traits such as pathogenicity or antagonism, and these could be important in the virulence of the pumpkin diseases. This research demonstrates that *D.*

bryoniae promotes the translocation of different pathogenic bacteria in a fungal strain-specific manner. We also suggest that black rot management should start with effective control of gummy stem blight in the field as reported by Babadoost and Zitter (2009) and should be synchronized with control of diseases caused by bacterial pathogens.

The joint presence of fungal and bacterial pathogens on pumpkins is representative for the emerging incidence of multi-pathogen diseases of crops. Multifungal diseases have been known for some time, with Esca representing the most prominent example of a grapevine disease. Esca is induced by a complex of xylem-inhabiting fungi (Crous and Gams 2000), and there is evidence for early causal agents, which allow access to saprophytic opportunists. *D. bryoniae* often occurs with different *Phoma* species on cucurbit seedlings (Somai and Keinath 2002) or with powdery mildew (Zitter and Kyle 1992). Here we show that fungi may pave the way for rapid access for bacteria to plant tissue. The rapid rise of newly emerging plant diseases in various parts of the world could be promoted by climatic perturbations. More studies on multiple disease-contributing microorganisms are necessary.

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Publication II

Microbial diversity inside pumpkins: microhabitat-specific communities display a high antagonistic potential against phytopathogens

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Personal contribution: accomplishment of the underlying practical work at the field, in the greenhouse and the laboratory except FISH-CLSM analysis, and preparation of the manuscript

Abstract

Recent and substantial yield losses of Styrian oil pumpkin (*Cucurbita pepo* L. subsp. *pepo* var. *styriaca* Greb.) are primarily caused by the ascomycetous fungus *Didymella bryoniae* but bacterial pathogens are frequently involved as well. The diversity of endophytic microbial communities from seeds (spermosphere), roots (endorhiza), flowers (anthosphere), and fruits (carposphere), of three different pumpkin cultivars were studied to develop a biocontrol strategy. A multiphasic approach combining molecular, microscopic and cultivation techniques was applied to select a consortium of endophytes for biocontrol. Specific community structures for *Pseudomonas* and *Bacillus*, two important plant-associated genera, were found for each microenvironment by fingerprinting of 16S rRNA genes. All microenvironments were dominated by bacteria; fungi were less abundant. Of 2,320 microbial isolates analyzed in dual culture assays, 165 (7%) were tested positively for *in vitro* antagonism against *D. bryoniae*. Out of these, 43 isolates inhibited the growth of bacterial pumpkin pathogens (*Pectobacterium carotovorum*, *Pseudomonas viridiflava*, *Xanthomonas cucurbitae*); here only bacteria were selected. Microenvironment-specific antagonists were found, and the spermosphere and anthosphere were revealed as underexplored reservoirs for antagonists. In the latter, a potential role of pollen grains as bacterial vectors between flowers was recognized. Six broad-spectrum antagonists selected according to their activity, genotypic diversity and occurrence were evaluated under greenhouse conditions. Disease severity on pumpkins of *D. bryoniae* was significantly reduced by *Pseudomonas chlororaphis* treatment and by a combined treatment of

strains (*Lysobacter gummosus*, *P. chlororaphis*, *Paenibacillus polymyxa* and *Serratia plymuthica)*. This result provides a promising prospect to biologically control pumpkin diseases.

Introduction

Styrian oil pumpkin (*Cucurbita pepo* L. subsp. *pepo* var. *styriaca* Greb.) is a relatively new cultivar of Cucurbitaceae which arose in the 19th century: a specific mutation found in Styria (Austria) leads to characteristic dark green seeds with stunted outer hulls [1]. This unique cultivar is important not only in Austria, meanwhile it has much wider growing areas, including several Southern European and African countries, China, Russia, and the USA. In the past decades, the dark-colored Styrian pumpkin seed oil with its intense nutty taste became internationally popular in gourmet cuisine. It is rich in polyunsaturated fatty acids and also contains vitamins, phytosterols, minerals and polyphenols [1]. Studies suggest usefulness of the oil in the prevention and treatment of benign prostatic hyperplasia, in prevention of arteriosclerosis and regulation of cholesterol level [2].

During the last decade, a dramatic increase of black rot in Styrian oil pumpkin has been observed [3]. The causal agent of black rot is *Didymella bryoniae* (Fuckel) Rehm, which is known to cause diseases on cucurbits all over the world [4, 5]. The ascomycetous fungus can infect any stage of the host plants and shows a variety of symptoms depending on the crop and stage concerned. Furthermore it can be seed-borne, air-borne, or soil-borne [5]. In addition, Styrian oil pumpkins can be affected by several bacteria from the class of Gammaproteobacteria, e.g. soft rot caused by *Pectobacterium carotovorum.* It was supposed that the bacterium is able to co-infect fruits synergistically with *D. bryoniae* [6]. Beside *P. carotovorum*, other bacteria such as *Pseudomonas viridiflava* and *Xanthomonas cucurbitae* can infest Styrian oil pumpkin plants as well [7]. Owing to the lack of effective fungicides and because a high proportion of oil pumpkin is cultivated under organic farming conditions, environmentally friendly and sustainable methods to protect pumpkins against microbial pathogens are desirable to control diseases.

Biocontrol using naturally occurring plant-associated microorganisms with beneficial effects on plant health provides promising perspectives for plant protection [8-10]. Especially endophytes of the genera *Bacillus* and *Pseudomonas* can efficiently support the host plants by growth promotion and/or antagonism towards phytopathogens [11-13]. For application of

antagonistic endophytes in biocontrol strategies, it is necessary to understand the microbial ecology of the host plant. Plant-associated microbial communities show a certain degree of plant specificity regarding species and cultivars [8], and different organs are often colonized by specific bacterial populations [14]. It is therefore essential to establish knowledge about pumpkin-specific endophytic microbial communities and their impact on plant growth and health.

The aim of this study was to analyze mainly endophytically living oil pumpkin-associated microbial communities with a special focus on the antagonistic potential towards the main oil pumpkin pathogens, the fungus *D. bryoniae*, and the bacteria *P. carotovorum*, *P. viridiflava* and *X. cucurbitae*. Samples were obtained from three different field-grown pumpkin cultivars 'Gleisdorfer Ölkürbis', 'Gleisdorfer Diamant' and 'Gleisdorfer Maximal', at three plant developmental stages (young, flowering, senescent), from four different microhabitats: from seeds (spermosphere), roots (endorhiza), flowers (anthosphere), and fruits (carposphere). We used a multiphasic approach combining DNA-based studies, molecular fingerprints performed by single-stranded conformation polymorphism (SSCP) of 16S rRNA genes for *Bacillus* and *Pseudomonas* and ITS genes for ascomycetous fungi, microscopic observations applying fluorescence *in situ* hybridization coupled with confocal laser scanning microscopy (FISH-CLSM) and a cultivation-dependent approach. On the basis of the latter the functional diversity was studied and broad-spectrum antagonists were selected. The effect of selected strains was characterized in more detail in a greenhouse experiment.

Methods

Experimental design and sampling

Plant samples were collected from fields located in Gleisdorf, Austria (47°05'23.96" N, 15°43'45.71" E, altitude 336 m). Samples were taken from three different Styrian oil pumpkin varieties: 'Gleisdorfer Ölkürbis', 'Gleisdorfer Diamant' and 'Gleisdorfer Maximal', (Saatzucht Gleisdorf, Gleisdorf, Austria) and from four different sites for each cultivar at three time points in 2009: 16th of June (young plants before flowering), 15th of July (flowering plants) and 26th of August (senescent plants with well-developed fruits). Four different microhabitats were investigated: seeds (spermosphere), roots (endorhiza), flowers (anthosphere), and fruits (carposphere). Seed-borne microorganisms were isolated from plants grown under gnotobiotic

conditions because in seeds microorganisms are mostly in a dormant stage and difficult to cultivate [15]. Roots were collected at all three time points, whereas female flowers were sampled only at the 2nd and fruits only at the 3rd time point. For each sampling time, cultivar and habitat, four independent composite samples were taken randomly from four different individual plants per site (number of root samples: 36, flower samples: 12, fruit samples: 12, seed samples: 5). These samples were used for DNA-dependent analysis as well as for cultivation.

Total community DNA isolation and analysis

Roots were washed with tap water until soil particles were completely removed prior to surface sterilization in 4% NaOCl for 5 min and subsequently washed three times with sterile water, sterility checks on culture media were performed according to Berg *et al.* [14]. Flowers were not surface sterilized to preserve endophytes occurring in thin petals. Fruit pulp was cut out of the inner fruit under aseptic conditions. The different plant tissues were homogenized in sterile 0.85% NaCl with mortar and pestle. Suspensions used for cultivation-dependent analysis were centrifuged for 20 min at 10,000 x g. DNA was extracted from the pellets using the FastDNA®Spin Kit for Soil (MP Biomedicals, Irvine, USA).

Bacterial fingerprints of *Pseudomonas* and *Bacillus* from roots, flowers and fruits were analyzed by single-strand conformation polymorphism (SSCP) analysis [16] using specific primers for *Pseudomonas*, *Bacillus* and mainly ascomycetous fungi [17-19]. Because only flowers contained a considerable number of culturable fungi (data not shown), we prepared fungal fingerprints only of the anthosphere community. All DNA fragments were separated with a TGGE Maxi apparatus (Biometra, Göttingen, Germany) at 400 V and 26°C. Silver staining of gels was applied for visualization of the bands [20]. Dominant bands of gels were cut out and the sequences determined [16]. Closest matches of obtained sequences were found with BLASTn [21] as implemented in the database of the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov).

Microscopic analysis of bacterial communities from the anthosphere

Manually cut fragments of petals and pistils from female flowers (Gleisdorfer Opal) were washed in several steps with PBS buffer (pH 7.4). Samples were stored in 1:1 PBS:96% ethanol at -20°C until further processing. Prior to microscopic analysis sections were placed on poly-L

lysine pre-coated microscopic slides (Thermo Fisher Scientific, Bremen, Germany). Samples were dried by using filter paper and a heating block (40°C) for 10 min. Fifty µl of 0.5 mg ml^{-1} lysozyme solution (Sigma-Aldrich, Steinheim, Germany) were added for 10 min before dehydration by ethanol series (50%, 80% and 96% ethanol, 3 min. incubation each one) and successive washing with PBS. FISH was performed as described by Lo Piccolo *et al.* [22] with modifications. Hybridization was carried out with 27 µl hybridization buffer [360 µl of 5 M NaCl, 40 µl of 1 M TRIS, pH of TRIS solution was set at pH 7.4, 300 µl of formamide (Sigma-Aldrich, Steinheim, Germany), 2 µl of 0.7 M SDS that was filled up to 2 ml with ddH_2O] and 3 µl of labeled probes (50 ng μl^{-1}) at 45°C for 2 h in a dark humid chamber. Probe EUB338MIX (Cy3 labelled) was used for overall bacterial communities, Gammaproteobacteria were probed with GAM42a (Cy5 labelled), Alphaproteobacteria with ALF968 (Cy5 labelled), Firmicutes were probed with LGC354MIX-FITC, and probe BET42a (ATTO488 labelled) was used for Betaproteobacteria [23-24]. All FISH probes were purchased from genXpress (Service & Vertrieb GmbH, Wiener Neudorf, Austria). The hybridization mix was removed by filter paper and slides were placed in preheated (47°C) washing buffer (3.18 ml of 5 M NaCl, 1 ml of 1M Tris at pH 7.4, filled up to 50 ml with ddH_2O) for 20 min. The slides with flower pieces were then dried before application of few drops of cold ddH_2O on the flower pieces prior to drying with compressed air. Mounted with ProLong Gold antifadent (Molecular probes, Eugene, USA) slides were kept dark prior to confocal laser-scanning microscopy (CLSM). Stained samples were analyzed with a Leica TCS SPE confocal laser-scanning microscope (Leica Microsystems, Mannheim, Germany) equipped with solid state and UV lasers. The software Imaris 7.0 (Bitplane, Zurich, Switzerland) was used for 3D rendering of confocal stacks and creation of isosurface-spot models.

Isolation of microorganisms from Styrian oil pumpkin plants

Microorganisms were isolated from suspensions obtained from plant materials on R2A medium for bacteria (Roth GmbH, Karlsruhe, Germany) and SNA medium for fungi (Spezieller Nährstoffarmer Agar) containing 1 g KH_2PO_4, 1 g KNO_3, 0.5 g $MgSO_4$ x 7 H_2O, 0.5 g KCl, 0.2 g glucose, 0.2 g sucrose, and 22 g agar per l (pH 6.5). After autoclaving for 20 min, the following antibiotics were added: 10 mg $liter^{-1}$ chlorotetracycline, 50 mg l^{-1} dihydrostreptomycine sulfate, 100 mg l^{-1} penicillin G. After five days incubation at 20°C, CFU

were determined and representative colonies were transferred onto LB (Luria Bertani) medium for bacteria and PDA (Potato Dextrose Agar) medium for fungi (all media from Roth).

In vitro assays to characterize antagonistic activity of isolated microorganisms

The following pathogenic strains were used in dual culture assays: *Didymella bryoniae* A-220-2b, *Pectobacterium carotovorum* subsp. *atrosepticum* 25-2, *Pseudomonas viridiflava* 2d1 and *Xanthomonas cucurbitae* 6h4. The fungal strain is from our own collection [6], whereas bacterial pathogenic strains were obtained from the Göttinger Sammlung Phytopathogener Bakterien: GSPB, University of Göttingen, Germany. Altogether, 1,748 bacterial isolates (152 from spermosphere, 930 from endorhiza, 336 from anthosphere and 330 from carposphere) were streaked out on nutrient agar (Sifin, Berlin, Germany) together with mycelium fragments of *D. bryoniae*. Mycelium fragments of fungal isolates (47 from spermosphere, 200 from endorhiza, 304 from anthosphere and 21 from carposphere) were placed on PDA plates together with *D. bryoniae*. Antagonistic activity was assessed after five days incubation at 20°C according to Berg *et al.* [14]. *D. bryoniae* antagonists were also tested for their broad-spectrum activity against the bacterial pathogens *P. carotovorum* subsp. *atrosepticum* 25-2, *P. viridiflava* 2d1, and *X. cucurbitae* 6h4. Bacterial suspensions grown overnight in TSB (Roth) at 30°C and were mixed with LB agar (*P. carotovorum* subsp. *atrosepticum* 25-2, *P. viridiflava* 2d1), nutrient agar (*X. cucurbitae* 6h4), and PDA (for fungal isolates) (always containing 1.2% agar). Subsequently, bacterial isolates were streaked out on these treated plates, whereas mycelium fragments were placed on the agar surface. After five days incubation at 20°C, presence or absence of clearing zones (due to growth inhibition of pathogens) surrounding the test strains was assessed. Isolates exhibiting antagonistic potential against *D. bryoniae* and at least two of the three tested bacterial phytopathogens were designated as broad-spectrum antagonists.

Genotypic characterization and identification of broad-spectrum antagonists

Bacterial DNA was isolated according to Berg *et al.* [14]. In a first step, strains were characterized taxonomically by Amplified Ribosomal DNA Restriction Analysis (ARDRA) using the restriction endonucleases *Hha*I (MP Biomedicals, Eschwege, Germany) and *Pst*I (New England Biolabs, Ipswich, UK) and by a more discriminative method based on whole genome-BOX-PCR fingerprints [14, 25]. Representative strains were selected according to their ARDRA

and BOX patterns and their partial 16S rRNA genes were sequenced and analyzed by BLASTn analysis [21].

Evaluation of mode of action of broad-spectrum antagonists and their effects *ad planta*

The capacity of antagonists to inhibit the growth of *D. bryoniae* A-220-2b via the production of bioactive volatile organic compounds (VOCs) was tested with dual culture assays comprising two-compartment petri dishes (side 1: 50 µl from overnight cultures in LB or TSB; side 2: a 0.3 cm mycelium fragment of *D. bryoniae* on PDA). After three days of incubation, diameters of *D. bryoniae* colonies were measured. Furthermore, the production of antimicrobial compounds against *D. bryoniae* was studied by analysis of supernatants of growth media in which antagonists were cultivated. Supernatants of 3-day-old liquid cultures of antagonists were filtered (0.45 µm pore size) and mixed with PDA (1.5 %) in a ratio 1:3. In the control treatments, sterile media were mixed with PDA. Mycelium fragments of *D. bryoniae* A-220-2b were placed on top of the supernatant-containing agar and diameters of colonies were measured after four days growth at 20°C. Both experiments were conducted in triplicate.

Effects of antagonists on disease severity (ds) caused by *D. bryoniae* on oil pumpkin was evaluated in a greenhouse experiment. Seeds of the cultivar 'Gleisdorfer Opal' were treated for 13 hours with suspensions at 6.9 x 10^9 CFU ml^{-1} of all six antagonists separately or with a mix of suspensions of strains *Lysobacter gummosus* L101, *Serratia plymuthica* S13, *Pseudomonas chlororaphis* P34, *Paenibacillus polymyxa* PB71 (one representative for each bacterial genus) in 0.85% NaCl. For the control treatment, seeds were only treated with 0.85% NaCl. Seeds were planted into peat moss substrate with clay (Gramoflor, Vechta, Germany). For each treatment always three 5 l pots with 5 plants per pot were studied. Plants were grown under artificial illumination (12 h per day) at 26 °C. 13 days after sowing, 50 µl of a conidial suspension (2.3 x 10^5 ml^{-1} of *D. bryoniae* SP2) were injected into petioles of first and second leaves [6]. Plants were covered with plastic bags to produce humid conditions, which support the infection. After 12 days, disease severity was observed on primary and secondary leaves and scored using the following numbers: 1 = no infestation on leaf, 2 = approx. 25% of leaf area infested, 3 = 50% of leaf area infested, 4 = 75 % of leaf are infested, and 5 = leaf was completely infested.

Statistical analysis

Banding patterns obtained from single isolates (ARDRA, BOX-PCR) as well as from community analyses (SSCP) were normalized and subjected to cluster analysis based on the unweighted pair group method using average linkages to the matrix of similarities obtained (UPGMA) using the Gel ComparII software (Version 5.1, Applied Maths, Kortrijk, Belgium). Similarity matrices were analyzed with R software version 2.12.1 (The R Foundation for Statistical Computing, ISBN 3-900051-07-0) to perform a permutation test ($P < 0.001$) in order to find significant differences between SSCP band patterns. Data from *Pseudomonas* and *Bacillus* fingerprints were additionally analyzed by Detrended Correspondence Analysis (DCA) with the Canoco software (Version 4.52, Biometris, Wageningen, The Netherlands). Analysis of Variance (ANOVA) in addition with Fisher's Least Significant Difference test (LSD; $P < 0.05$) was performed to compare mean diameters of *D. bryoniae* measured in the antibiosis assays (for soluble and volatile compounds). Duncan`s multiple range test ($P < 0.1$) was used to compare mean values for disease severity (ds) in *ad planta* tests. ANOVA and post-hoc tests were performed with Predictive Analysis Software (PASW, Version 18.0.0).

Nucleotide sequence accession numbers

Obtained sequences were deposited in GenBank under accession numbers HQ163897 to HQ163900, HQ163902 to HQ163914, HQ661145 to HQ661152, and JF796744 to JF796748.

Results

Abundances of oil pumpkin-associated microorganisms

Bacterial abundances from different field-grown Styrian oil pumpkin cultivars and microhabitats were in a range between 2.83 and 7.03 $\log_{10}$ CFU g^{-1} fresh weight of plant material (Table 1). The anthosphere exhibited the highest number of cultivable bacteria (6.99 – 7.03 $\log_{10}$ CFU g^{-1}) followed by the endorhiza (4.19 – 4.21 $\log_{10}$ CFU g^{-1}), carposphere (2.83 – 3.58 $\log_{10}$ CFU g^{-1}), and spermosphere (under the detection limit of < 2.0 $\log_{10}$ CFU g^{-1}). Fungal abundances above the detection limit were only found in the anthosphere (2.36 – 3.08 $\log_{10}$ CFU g^{-1}).

Table 1. Abundances in different microenvironments from the Styrian oil pumpkin cultivars 'Gleisdorfer Ölkürbis', 'Gleisdorfer Diamant' and 'Gleisdorfer Maximal'. Mean values of $\log_{10}$ transformed colony forming units (CFUs) of isolated microorganisms per g plant material are presented; values in brackets indicate standard deviations.

Micro-habitat	*Gleisdorfer Ölkürbis*		*Gleisdorfer Diamant*		*Gleisdorfer Maximal*	
	Bacteria	*Fungi*	*Bacteria*	*Fungi*	*Bacteria*	*Fungi*
Endo-rhiza	4.19 (± 0.44)	< 2.00	4.21 (± 0.39)	< 2.00	4.20 (± 0.37)	< 2.00
Anthos-phere	6.99 (± 0.22)	2.36 (±0.37)	7.01 (± 0.27)	3.08 (±0.34)	7.03 (±0.57)	2.94 (±0.39)
Carpos-phere	3.58 (±0.27)	< 2.00	3.47 (±0.32)	< 2.00	2.83 (±0.11)	< 2.00

Microbial fingerprints of oil pumpkin-associated communities

In order to characterize pumpkin-associated communities, SSCP fingerprints of *Pseudomonas* and *Bacillus* were analyzed; both genera represent dominant inhabitants and antagonists of plants [9, 10, 13]. For all microenvironments a high diversity for both genera was found. *Bacillus* and *Pseudomonas*-specific patterns were detected in the investigated microenvironments (Fig. S1). Statistically significant differences between different microenvironments were confirmed by permutation tests ($P < 0.001$). Furthermore, significant different *Pseudomonas* and *Bacillus* communities were found for the endorhiza at different plant developmental stages ($P < 0.001$). In contrast, only a minor, statistically insignificant influence of the cultivars on *Bacillus* and *Pseudomonas* species composition was found (data not shown). Differences between microbial communities from the microenvironments and at different plant stages are illustrated by detrended correspondence analyses (DCA) (Fig. 1, 2), which clearly show the dependence on biotic parameters.

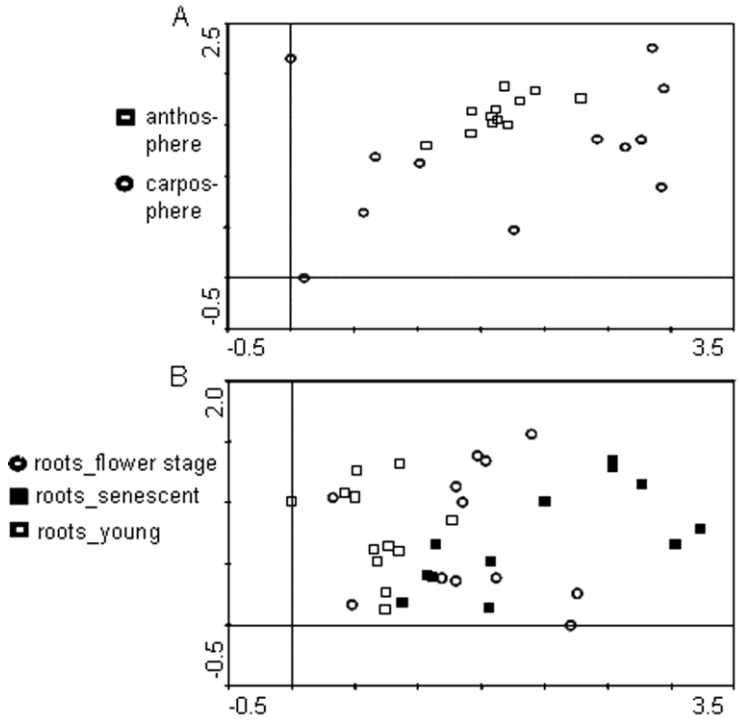

Fig.1 Detrended Correspondence Analysis based on *Pseudomonas*-specific patterns on SSCP gels originated from A) anthosphere and carposphere B) endorhizas of roots at different plant developmental stages from Styrian oil pumpkin.

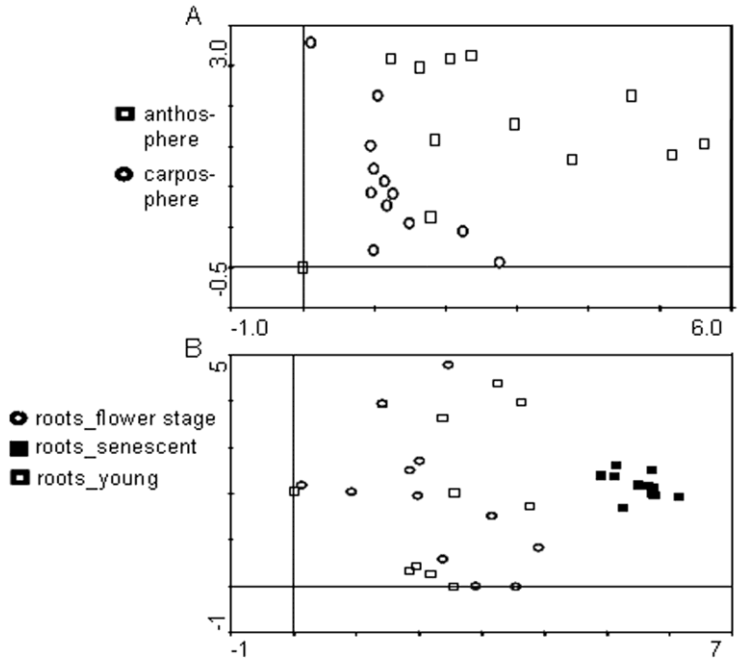

Fig. 2 Detrended Correspondence Analysis based on *Bacillus*-specific patterns on SSCP gels originated from A) anthosphere and carposphere B) endorhizas of roots at different plant developmental stages from Styrian oil pumpkin.

Dominant bands from SSCP fingerprints were sequenced: the *Pseudomonas* species *P. oryzihabitans*, *P. putida*, *P. syringae*, *P. viridiflava*, and *P. fluorescens* were identified (Table 2). For *Bacillus*, *B. weihenstephanensis*, *B. flexus*, *B. psychrodurans*, *B. siralis*, *B. indicus*, *B. subtilis*, *B. gibsonii*, and *B. firmus* (only 96% 16S rRNA gene sequence match) were detected in different microhabitats of Styrian oil pumpkin. *B. flexus* was found in endorhizas and anthospheres, whereas *B. subtilis* was found in all three observed microhabitats. *B. weihenstephanensis*, *B. psychrodurans*, and *B. siralis* could only be detected in endorhizas, whereas *B. gibsonii*, *B. indicus*, and *B. firmus* were found only in anthospheres (Table 3).

Table 2. Taxonomic characterization of Styrian oil pumpkin associated *Pseudomonas* strains based on partial 16S rRNA gene sequences obtained from SSCP gels.

Microhabitat	*Clone*	*Taxonomic affiliation based on BLAST matches (accession numbers)*	*Similarity in %*
Endorhiza	PR1	*P. oryzihabitans* (EU977742.1)	100
	PR2	*P. putida* (CP002290.1)	100
	PR3	*P. syringae* (DQ294981.1)	97
Anthosphere	PB1	*P. viridiflava* (HM190229.1)	100
	PB2	*P. syringae* (HQ267378.1)	100
Carposphere	PF1	*P. putida* (HQ658765.1)	97
	PF2	*P. fluorescens* (HM016864.1)	100
	PF3	*P. syringae* (HQ267378.1)	100

Table 3. Taxonomic characterization of Styrian oil pumpkin associated *Bacillus* strains based on partial 16S rRNA gene sequences obtained from SSCP gels.

Microhabitat	*Clone*	*Taxonomic affiliation based on BLAST matches (accession numbers)*	*Similarity in %*
Endorhiza	R1	*B. weihenstephanensis* (AB021199.1)	100
	R2	*B. flexus* (AB021185.1)	100
	R3	*B. psychrodurans* (AJ277984.1)	99
	R4	*B. siralis* (NR_028709.1)	98
	R6	*B. subtilis* (AJ276351.1)	100
Anthosphere	B1	*B. indicus* (AJ583158.1)	97
	B2	*B. gibsonii* (X76446.1)	98
	B3	*B. flexus* (AB021185.1)	98
	B4	*B. firmus* (D16268.1)	96
	B5	*B. subtilis* (AJ276351.1)	99
Carposphere	F1	*B. subtilis* (AJ276351.1)	99

For fungal fingerprints, only the anthosphere was investigated. The other microenvironments were not considered because here fungi were under the detection limit in the cultivation approach. If the amount of fungal DNA is too low, it is not possible to perform reliable fingerprints. Again, an influence of the cultivar on structure was observed but this impact was not significant for all samples (data not shown). The following members of ascomycetes were identified: *Plectosphaerella cucumerina* (AJ246154.1), *Phoma herbarum* (FN868459.1), uncultured *Oidiodendron* (GQ338892.1), *Capnobotryella* sp. (AJ972854.1) and *Pleosporaceae* sp. (EF060611.1).

Microscopic analysis of bacterial communities from anthosphere

According to the high abundances and diverse fingerprints found in the anthosphere, bacterial colonies on petals and pistils were observed in more detail by FISH-CLSM. Female flowers were densely colonized by bacteria (Fig. 3A-D). Petals were colonized on their inner surface by diverse micro-colonies (Fig. 3A). On pistils, Beta- and Gammaproteobacteria and diverse unclassified bacteria were detected, all of them were found separately as micro-colonies (Fig.

3C). Furthermore, it was visualized that pollen grains on pistils were densely colonized predominantly by Gammaproteobacteria that formed dense patches (Fig. 3B, D).

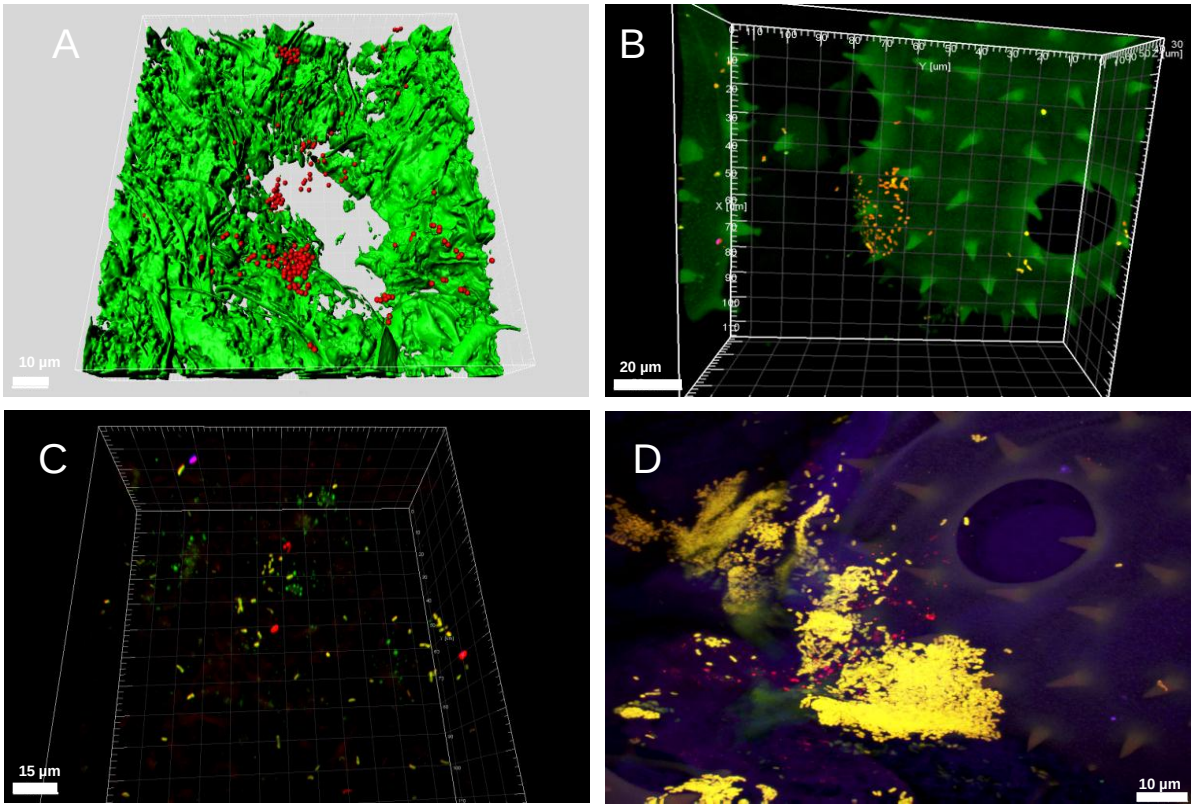

Fig. 3 Visualization of FISH-labelled bacteria inhabiting female oil pumpkin flower parts by CLSM. A) 3D rendered image (Imaris software) of overall bacterial communities (in red) on petals labelled with EUB338MIX-Cy3. B) Alphaproteobacteria (in yellow), not taxonomically classified bacteria (in red) and Firmicutes (in pinkish) labeled with ALF968-Cy5, EUB338MIX-Cy3 and LGC354MIX-FITC on pollen grains located on pistils. C) Gammaproteobacteria (in yellow), Betaproteobacteria (pinkish) and not taxonomically classified bacteria (in red) on pistils labeled with GAM42a-Cy5, BET42a-ATTO488 respectively with EUB338MIX-Cy3. D) Gammaproteobacteria (in yellow) and not taxonomically classified bacteria (in red) on pollen grains on pistils labeled with GAM42a-Cy5 and EUB338MIX-Cy3.

Cultivation-based screening for antagonists

Representative bacterial and fungal strains were selected from each microhabitat and cultivar, and screened in a first step for their *in vitro* activity towards *D. bryoniae*. Out of 2,230 tested bacterial and fungal isolates 128 (=7%) and 37 (=6.5%) strains, respectively, showed *in vitro* antagonism against *D. bryoniae* (Fig. S2). In general, the highest number of antagonists was found in the endorhiza, in contrast to low amounts of antagonists in anthosphere and

carposphere (Fig. 4). The highest proportion of bacterial antagonists was found in the spermosphere (16%) and for fungal antagonists in the endorhiza (16%).

The antifungal antagonists were screened against the bacterial pathogens of pumpkin *P. viridiflava* 2d1, *P. carotovorum* subsp. *atrosepticum* 25-2 and *X. cucurbitae* 6h4. Out of 128 bacterial and 31 fungal isolates, 49% and 32%, respectively, showed antagonistic activity against at least one of the bacterial pathogens; 34% of analyzed bacterial isolates exhibited growth inhibition of at least two of the observed strains, and 6% of the bacterial antagonists inhibited growth of all three pathogenic bacteria. None of the tested fungi demonstrated antagonism against more than one bacterial strain. A significant portion of bacteria and fungi (40% and 29%) were effective against *P. viridiflava*, followed by 30% and 3%, which suppressed growth of *X. cucurbitae* 6h4. Altogether, 19% of the bacterial strains could suppress *P. carotovorum*, whereas no fungal isolate showed inhibition of this pathogen. The 43 bacterial isolates exhibiting antagonistic potential against *D. bryoniae* and two bacterial pathogens were classified as broad-spectrum antagonists and further characterized. In contrast, no fungal broad-spectrum antagonist was selected due the missing activity of the fungal strains against bacterial pathogens.

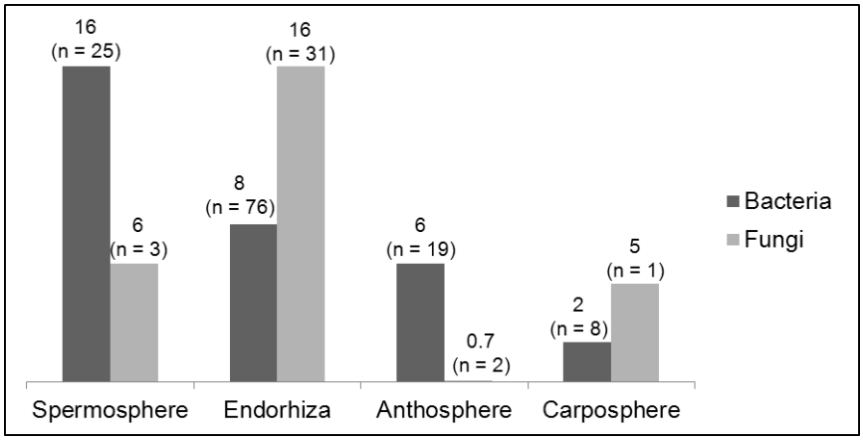

Fig. 4 *In vitro* antagonists against *Didymella bryoniae* isolated from different microhabitats of Styrian oil pumpkin presented as proportion in % of tested bacterial and fungal strains.

Genotypic characterization of broad-spectrum antagonists

To characterize the 43 antagonists at genotypic level and to select unique strains for the biocontrol strategy, two fingerprinting methods were applied. In a first step, ARDRA analysis was used to differentiate four groups (I - IV). In a second step, BOX-PCR was used to

characterize the strains within each ARDRA group at population level. The ARDRA groups could be sub-divided into one to five groups with similar patterns. For example, ARDRA group IV, which was the largest group with a high intraspecific diversity later identified as *Lysobacter*, clustered into five different BOX-PCR fingerprint patterns (Fig. 5). Finally, six broad-spectrum antagonists with unique BOX patterns were chosen for identification: always one representative of ARDRA groups I - III, and three of ARDRA group IV. Strains were identified as *Lysobacter gummosus*, *Lysobacter antibioticus, Paenibacillus polymyxa*, *Pseudomonas chlororaphis,* and *Serratia plymuthica* (Table 4).

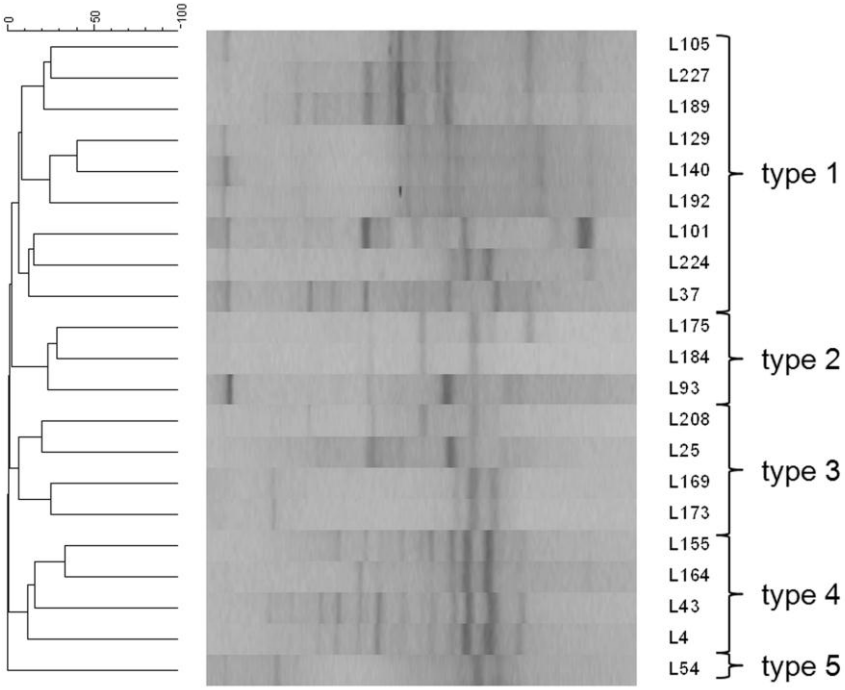

Fig. 5 Grouping of BOX-PCR band patterns obtained from broad-spectrum antagonists belonging to *Lysobacter* spp. (ARDRA group IV).

Table 4. Taxonomic affiliation of broad-spectrum antagonists including their abilities to produce bioactive soluble and volatile organic compounds and to decrease disease severity (ds) on Styrian oil pumpkin caused by *Didymella bryoniae***.**

Strain	*Origin*	*Identification*		*Synthesis of inhibitory substances against* D. bryoniae		*Interaction with plants*
		Taxonomic affiliation[a] *(assession numbers)*	*Similarity (%)*	*volatile*	*soluble*	*Decrease of ds*[b] *(%)*
L101	Endorhiza	*Lysobacter gummosus* (HQ163910.1)	98	+	+	8.6
L169	Endorhiza	*Lysobacter antibioticus* (HQ163912.1)	98	-	+	0.0
L175	Endorhiza	*Lysobacter antibioticus* (HQ163913.1)	99	-	-	9.7
S13	Anthosphere	*Serratia plymuthica* (HQ163914.1)	98	+	-	18.2
P34	Endorhiza	*Pseudomonas chlororaphis* (HQ163911.1)	97	+	+	20.4*
PB71	Spermosphere	*Paenibacillus polymyxa* (HQ163909.1)	99	+	+	11.8

[a] of partial 16S rRNA genes by BLAST analysis

[b] % reduction of disease severity compared to control treatment based on mean values of grade sums, stating degrees of infestation of infected leaves

+ signify significant lower diameters of *D. bryoniae* colonies compared to the control treatment (ANOVA; LSD, $P < 0.05$)

*significant different to control treatment (ANOVA; Duncan`s multiple range test, $P < 0.1$)

Mode of action and biocontrol effect

The majority of broad-spectrum antagonists produced soluble or/and volatile antibiotics *in vitro* (Table 4). The only exception was *L. antibioticus* strain L175. Secretion of soluble antimicrobial compounds was observed for *L. gummosus* L101, *L. antibioticus* L169, *P. chlororaphis* P34, and *P. polymyxa* PB71/1, whereas emission of antimicrobial volatiles was found for *L. gummosus* L101, *S. plymuthica* S13, *P. chlororaphis* P34 and *P. polymyxa* PB71.

To study the biocontrol activity of selected broad-spectrum antagonistic bacteria, oil pumpkin seeds were soaked in cell suspensions of the selected antagonists, grown in pots for 13 d and artificially infected with *D. bryoniae.* Reduction of disease severity was evaluated by comparison with a non-treated control (Table 4). Strain *L. antibioticus* L169 showed no antagonistic activity *ad planta* whereas the highest reduction in disease severity compared to the control treatment was observed for *P. chlororaphis* P34 with 20.4% (Table 4). Considerable reduction (19.4%) of disease severity compared to the control was exerted by a mixture of antagonists including *L. gummosus* L101, *P. polymyxa* PB71, *S. plymuthica* S13 and *P. chlororaphis* P34.

Discussion

Our study about the ecology of pumpkin-associated microorganisms revealed bacteria dominating the microbial communities. The community structure was influenced by plant stage and organ but not by the cultivar. The spermosphere and anthosphere were discovered as important new reservoirs for antagonistic bacteria. Six efficient bacterial antagonists against pathogens tested were selected and evaluated under greenhouse conditions. In contrast, fungi were less abundant and no broad-spectrum antagonist could be isolated from the fungal community.

Virtually, all plant species are colonized by microorganisms including bacteria and fungi, which are neutral or show positive plant-microbe interaction. For pumpkin we found bacteria dominated microbial communities, except for the anthosphere. In general, various biotic and abiotic parameters such as plant age, vegetation time or pH influence the plant-associated microbial communities [8]. We found an impact of plant stage and organ on the composition of *Bacillus* and *Pseudomonas* communities. Importance of both factors is also known from studies with other plants [8, 26]. Interestingly, analysis of flower-associated microbial communities in

Saponaria officinalis and *Lotus corniculatus* showed also a strongly different community structure for leaves and petals: Enterobacteriaceae dominated here the floral communities [27]. In contrast to other studies [8], we found no influence of the cultivar. Genetic differences between pumpkin cultivars are relatively low in comparison to other crops because a special breeding program for oil pumpkin exists in Styria only for 15 years. The plant microhabitat contributes to specific differences in the functional roles of the culturable fraction. The endorhiza is confirmed here as one of the main reservoir for antagonistic strains. In addition, we discovered the spermosphere and the anthosphere as potential habitats for antagonists. This merits further study in other plants, especially since both organs were till now regarded rather as reservoirs for pathogens, e.g. the anthosphere for *Erwinia amylovora* or *Botrytis cineria* and the spermosphere for diverse seed-borne pathogens. By cultivation-independent approach, we also identified not only potential beneficial but also strains known as pathogens. *Pseudomonas syringae* is well known as important plant-pathogen and *P. oryzihabitans*, present in the endorhiza, is an opportunistic pathogen of humans [28]. Although the identification of potential pathogens is only a hint, which needs further evidence and experiments, our findings support the view of the rhizosphere as a reservoir of opportunistic or emerging pathogens [29].

Plants acquire their microorganisms from the environment, primarily from the soil [8]. Recent investigations suggested also a vertical transmission of bacteria from the parent plant [30]. Also for mosses, the phylogenetically oldest land plants, a transmission of the core microbiome from the gametophyte to the sporophyte and *vice versa* was discovered. This moss-specific microbiome was found to be modified by abiotic factors (nutrient richness, pH) [31]. In the present study we found hints that bacteria are transferred with pollen, as suggested by microscopy and by shared occurrence of several strains in anthosphere and carposphere. This should be not only considered in the interpretation of floral traits, it is also important for pollination and microbial ecology [27]. Many reports have been published on the use of honey- and bumble-bees to disseminate biocontrol agents to flowers especially against *Botrytis cinerea.* To use specifically adapted bacteria from the anthosphere for this purpose is an interesting objective.

Although the proportion of fungal antagonists against *D. bryoniae* was similar to those of the bacteria in general, at last only bacterial strains were selected. The reason for this selection was the missing activity of the fungal strains against the bacterial pathogens. This agrees with

former studies, where a high antifungal but a low antibacterial potential within the indigenous plant-associated communities were found [32, 33]. All bacteria of our collection belong to well-known antagonistic species [34-36]. However, the extent of antagonistic activity is highly specific for each of these strains. This was found for the two *Lysobacter antibioticus* strains investigated: L175 showed no production of antibiotics *in vitro* but was able to reduce the disease index of *D. bryoniae*, while L169 produced antibiotics but showed no disease-suppressive activity. Members of the genus *Lysobacter* are well-known disease-suppressive bacteria with strain-specific effects [36]. Recent investigations of evolutionary genomics show that species are defined by the paleome (core genes, which allow basic metabolism), whereas strains are characterized by their cenome (genes, which allow cells to live in and explore a particular niche). The latter genomic content is made responsible for strain-specific properties [37]. This is important for the characterization of biocontrol strains: each strain of one species can have their specific antagonistic properties and can show different biocontrol effects in the field [13, 32]. Therefore, a selection strategy considering these strain-specific effects is necessary. Our strain-specific consortium is therefore optimally suited to suppress various pathogens which frequently co-occur in pumpkin disease. Diseases caused by cooperation of various pathogens are on the rise also due to climate change [6], for which biological control based on synergistic biocontrol agents needs to be further developed.

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Supplementary material

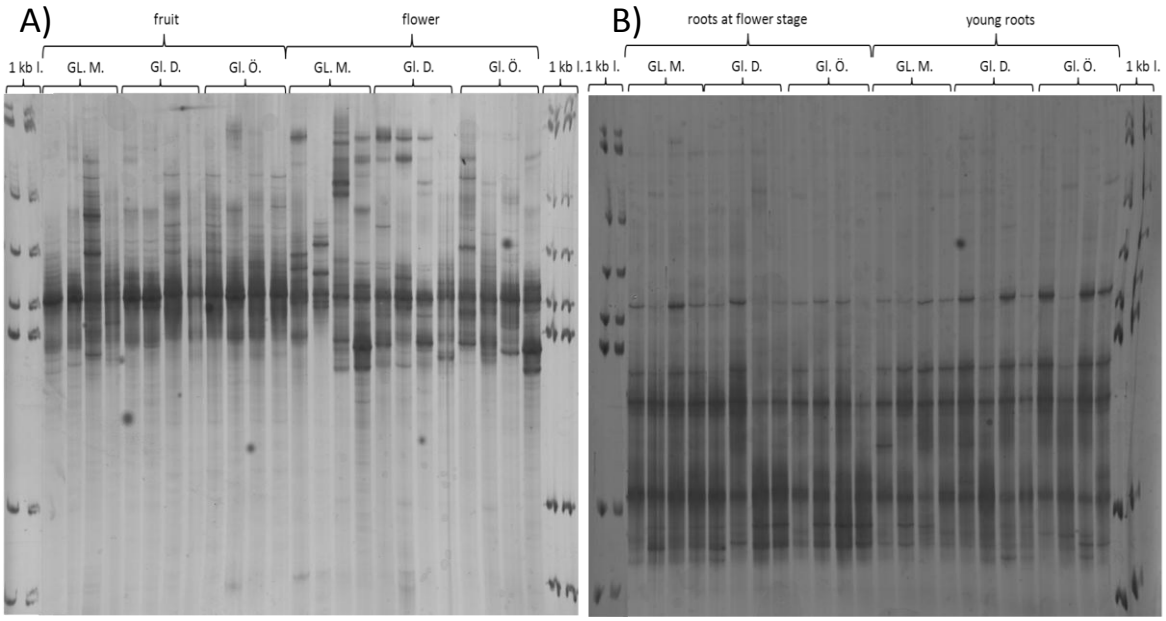

Fig. S1. *Bacillus*- (A) and *Pseudomonas*-specific (B) microbial communities obtained from different habitats (fruits, flowers, roots) of oil pumpkin cultivars ('Gleisdorfer Ölkürbis' = Gl. Ö., 'Gleisdorfer Diamant' = Gl. D., and 'Gleisdorfer Maximal' = Gl. M.) and visualized by SSCP gels. DNA size marker: 1kb ladder.

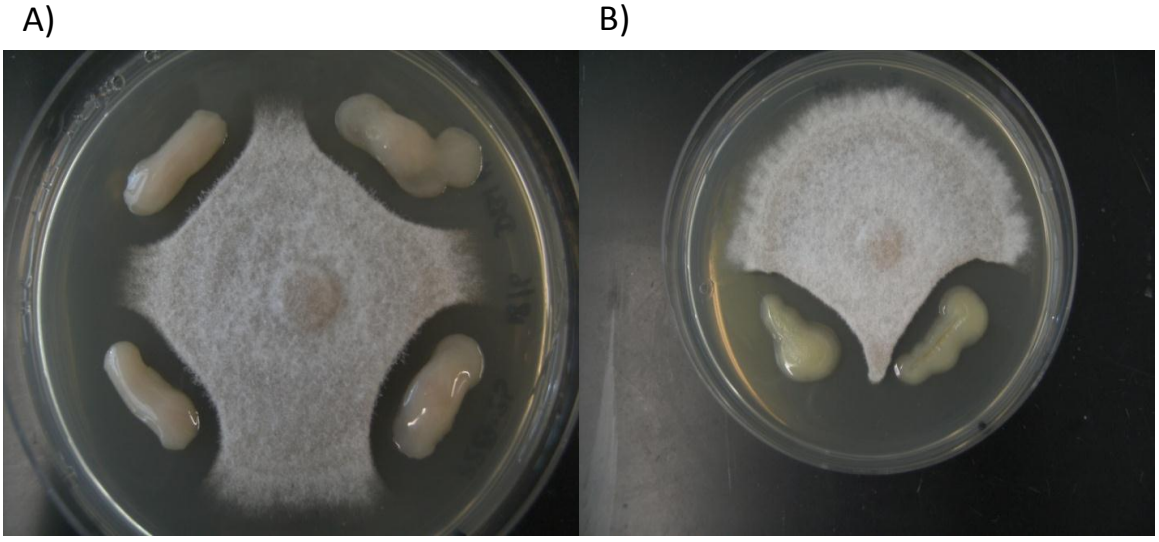

Fig. S2. Dual culture assay showing in vitro antagonisms between the pathogenic fungus *Didymella bryoniae* A-220-2b and the antagonistic strains *Serratia plymuthica* S13 (A) and *Lysobacter gummosus* L101 (B) on nutrient agar after five days growth at 20°C.

Publication III

Promotion of growth, health and stress tolerance of Styrian oil pumpkins by bacterial endophytes

Michael Fürnkranz, Eveline Adam, Henry Müller, Martin Grube, Herbert Huss, Johanna Winkler and Gabriele Berg. European Journal of Plant Pathology (2012), under revision

Personal contribution: analysis of bacteria for seed priming, seed priming and preparation of the primed seeds for the seed (e.g. drying, application of a chemical stripper), cooperation on: development of field designs and seed and field work, statistical analysis, and preparation of the manuscript

Abstract

Substantial yield losses of Styrian oil pumpkin caused by the fungus *Didymella bryoniae* and bacterial pathogens were recently reported. Here we applied bacterial endophytes with a broad antagonistic activity to pumpkin plants by seed priming. Effects of the bacterial inoculants with and without chemical seed treatments on plant growth and health were evaluated during three different field trials in two consecutive years (2010 and 2011). Biological seed treatments strongly supported the germination of pumpkin seeds. In 2010, the germination of the biologically treated seeds was comparable to the rate following a chemical treatment, while in 2011 effects of biological seed treatments were more obvious, including an increased emergence rate up to 109% by *Serratia plymuthica* S13. Furthermore, tolerance against desiccation stress was observed for *Serratia* as well as for *Lysobacter gummosus* L101 treatment. The biological treatment showed different effects against fungal diseases: no effect on fruit rot was observed, whereas powdery mildew could be significantly suppressed by *Paenibacillus polymyxa* PB71 and *L. gummosus* L101 in 2010. In addition, both strains led to reproducible increases in harvest yields. In this study, we found bacterial endophytes suitable as inoculants for plant growth promotion, biocontrol as well as enhancing stress tolerance of Styrian oil pumpkins.

Abbreviations: cw = calendar week

Introduction

During the last years, Styrian oil pumpkin (*Cucurbita pepo* L. subsp. *pepo* var. *styriaca* Greb.; Cucurbitaceae) has become an important oil crop world-wide. The cultivar originated from a natural mutation, which was first recognized in the 19th century in the Austrian region of Styria (Teppner 2000). Owing to the absence of woody outer hulls, seeds are usable for bakery industry and especially valued for the production of an unique, dark-green seed oil. Pumpkin seed oil is of traditional use locally but has become popular in gourmet cuisines world-wide. The oil is also known to prevent from atherosclerosis, lower urinary tract symptoms, benign prostatic hyperplasia, and to positively regulate the cholesterol level (Dreikorn 2002, Fruehwirth & Hermetter 2007). These effects have been attributed to the content of bioactive ingredients such as unsaturated fatty acids, vitamins, phytosterols, minerals and polyphenols.

For a long time, cultivation of oil pumpkins was only of regional importance in Styria but now there is an increasing production of this crop in several Southern European and African countries, China, Russia, and the USA. During the last years, substantial yield losses were reported in Styria due to fruit rot caused by the fungus *Didymella bryoniae* and the bacterial pathogen *Pectobacterium carotovorum* (Huss et al. 2007, Huss et al. 2009). Vegetative plant parts are affected by the Zucchini Yellow Mosaic Virus (ZYMV), *Pseudomonas* spp., *Xanthomonas cucurbitae* and *D. bryoniae* as well (Huss 2007, Huss & Mavridis 2007, Huss & Winkler 2009, Huss 2011). Powdery mildew can lead to significant damages on leaves especially when oil pumpkin plants are affected in an early developmental stage (personal communication H. Huss), and desiccation stress that affected crop plants negatively in Central Europe could play an even more important role in future due to the effects of climate change (Trnka et al. 2010). Conventional methods, e.g. use of partly resistant cultivars (e.g. cv. Gl. Diamant against black rot), pesticides like Metalaxyl-M or Fludioxonil, and crop rotation exist for diminishing the degree of pathogen infestation for oil pumpkin. In addition to the conventional agricultural practice, there is demand for environmentally friendly and efficient solutions to improve health of oil pumpkin in Austria. Furthermore, this request will be underlined by the proposed complete ban of copper-based fungicides in the European Union for organic farming.

Biological control agents (BCAs) and plant growth promoting bacteria (PGPB) offer the possibility to enhance plant growth and health. PGPB serve plants directly by supplying

nutrients, e.g. via the fixation of atmospheric nitrogen (N_2), phosphorous (P) solubilization, phytohormone synthesis (e.g. indole-3-acetic acid, IAA), and by lowering the hosts ethylene level due to ACC (1-aminocyclopropane-1-carboxylate) deaminase activity (Lugtenberg & Kamilova 2009). BCAs increase plant health by antagonizing pathogens via different modes of action: the production of antibiotics and extracellular enzymes, pathogen cell signal interference, predation and parasitism, and competition for nutrients (especially for iron) and niches. Furthermore, they can support the hosts` immune response by induced systemic resistance (Compant et al. 2005, Berg 2009, Lugtenberg & Kamilova 2009). Bacterial endophytes undergo an intimate and effective interaction with the host plant and should be considered as beneficial inoculants for plants (Sessitsch et al. 2005, Berg & Hallmann 2006). However, only a few products on endophytic strains such as *Pseudomonas trivialis* (Salavida©) are already on the market (Mei & Flinn 2010). In a previous study, we have analyzed endophytic microbial communities from seeds (spermosphere), roots (endorhiza), flowers (anthosphere), and fruits (carposphere), of three different pumpkin cultivars. In all microenvironments potential bacterial antagonists were present (Fürnkranz et al. 2012). Six broad-spectrum antagonists were selected according to their *in vitro* antagonism against the oil pumpkin pathogens *D. bryoniae*, *P. carotovorum*, *P. viridiflava* and *X. cucurbitae*, and their genotypic dissimilarity, whereas five of them were active under greenhouse conditions against gummy stem blight (Fürnkranz et al. 2012).

The objective of this study was to evaluate the most promising oil pumpkin derived biocontrol agents selected in our previous work, namely *Lysobacter gummosus* L101 (endorhiza), *Paenibacillus polymyxa* PB71 (spermosphere), *Pseudomonas chlororaphis* P34 (endorhiza), and *Serratia plymuthica* S13 (anthosphere), regarding their effect on plant growth, health and stress tolerance under field conditions. Oil pumpkin plants were inoculated with bacterial strains by seed priming with and without the addition of chemical seed treatment. The effect of the different treatments on germination rate, desiccation stress tolerance, harvest yield, and 100-corn weight was assessed during a field study in the vegetation period 2010 and two field trials in 2011. As fruit rot was a serious problem for the cultivation of oil pumpkin in Styria in recent years and a high degree of infestation by powdery mildew was noticed in 2010 and 2011, the effect of bacterial treatments against pumpkin rot and the leave disease was additionally monitored. The overall performance of tested bacteria *ad planta* was evaluated and finally three strains were selected and suggested as biological inoculants.

Materials and methods

Bacterial broad-spectrum antagonists

The bacterial broad-spectrum antagonists investigated in this study were: *Pseudomonas chlororaphis* P34 (HQ163911), *Serratia plymuthica* S13 (HQ163914), *Paenibacillus polymyxa* PB71 (HQ163909) and *Lysobacter gummosus* L101 (HQ163910). They were isolated from different microhabitats of Styrian oil pumpkin: *P. chlororaphis* P34 and *L. gummosus* L101 from the root endosphere, *S. plymuthica* S13 from the female flower and *P. polymyxa* PB71 was found seed-borne. These strains have the capacity to suppress growth of the oil pumpkin pathogens *Didymella bryoniae* A-220-2b, and at least two of the bacterial strains *Pectobacterium carotovorum* subsp. *atrosepticum* 25-2, *Pseudomonas viridiflava* 2 d1, and *Xanthomonas cucurbitae* 6 h4 *in vitro* (Fürnkranz et al. 2012). The strains are stored in the Culture Collection of Antagonistic Microorganisms (SCAM) at Graz University of Technology in LB medium containing 15% glycerol at -70°.

Seed priming

Cells were grown in LB medium at 30°C under agitation for 3-9 hours (until a favored cell density of each strain was reached), and centrifuged for 15 min at 8.850 x g before pellets were resuspended in 0.85% NaCl. For field trial I, the concentrations of cells were adjusted to 4 x 10^8 colony forming units (CFU) ml^{-1} and seeds were primed in these suspensions for 8 h under agitation. For the control treatment, seeds were primed with sterile 0.85% NaCl. One part of the seeds primed with the four bacterial suspensions was additionally treated with the chemical stripper Maxim® XL [2.4 % (w/v) Fludioxonil, 1.0% Metalaxyl-M]. Per 1 kg seeds 56 ml Sacrust® SK 76 including 2.2 ml Maxim® XL was used. Treated seeds were immediately sown. For field trials II and III, seeds were immersed for 9.5 h in bacterial suspensions containing 2.6 x 10^8 CFU ml^{-1}. The control treatment was performed as described above. In contrast to the procedure for field trial I, seeds were dried after priming in a climate chamber (Binder, KWBF 720) at 25°C until a residual moisture content of about 8% was reached. For field trials II and III, seeds treated with *L. gummosus* 101, *P. chlororaphis* P34, and *P. polymyxa* PB71 were sown only in addition with the chemical stripper (as described above) in contrast to *S. plymuthica* S13 that was applied without Maxim XL as well. This strategy was developed according to the results obtained from the field trial I in 2010: a high seedling emergence by *S. plymuthica* S13

was observed that could compensate the chemical treatment. An overview of the different treatments is given in Table 1.

Endophytic life-style of *S. plymuthica* S13

In contrast to the other tested broad-spectrum antagonists *S. plymuthica* S13 was not isolated from the endosphere originally. Therefore, it`s capability for an endophytic life-style was analyzed: Oil pumpkin seeds (GL Opal) were inoculated with the strain as described above for field trial I and resultant plants were grown until the two-leaf stage under greenhouse conditions (Fürnkranz et al. 2012). Subsequently, roots, shoots, and cotyledons were sampled. Surface-sterilized: roots were washed with tap water and placed in 5% NaOCl for two min with stirring (shoots and cotyledons were treated with NaOCl without washing with tap water). The plant materials were then washed three times with sterile water, dipped into 70% ethanol and flamed. Sterility checks of the superficial tissues were accomplished on agar plates with Luria-Bertani (LB) medium (Roth, Karlsruhe, Germany) that were incubated for 24 h at 30°C overnight. Subsequently, treated plant parts were homogenized with mortar and pestle in 0.85% NaCl. Out of resulting suspensions, dilution series were prepared and plated onto LB plates that were incubated at 30°C for 24 h. Molecular fingerprints of the colonies was compared with the inoculums by BOX-PCR (Rademaker & de Bruijn 1997) (Figure ESM4).

Field designs

Field trial I in Gleisdorf (2010)

In Gleisdorf (47°7´1´´ N, 15°42´2´´ E), the soil can be described as gleyed loose brown earth, loamy silt, and cover loams on quaternary terrace, which were neutral to slightly acidic and deficient in lime. According to the records, 116 kg ha^{-1} mineral fertilizer (DC Rot) including nitrogen (10%), phosphorus (8%), and potassium (20%) were applied on the test field in spring 2010 (before sowing). Seeds were seeded in three replicate plots per treatment (organized in a completely randomized plot design) in always two rows per plot. Eight seeds per row were seeded on 4th of May [calendar week (cw) 18] and the distance between two plants was 40 cm (240 seeds were sown per field trial I). In 1st rows chemically treated seeds were placed whereas in 2nd rows seeds without chemical stripper were seeded. A few hours after the sowing, the field

was treated with herbicides (Dual Gold, Centium, Flexidor). Plants were grown without artificial irrigation for 154 days.

Field trial II in Flöcking and field trial III in Gabersdorf (2011)

In Flöcking (47°5´3´´ N, 15°40´2´´ E), the soil was the same kind as described above for field trial I. In autumn of 2010 the field was treated with cow dung (40.000 kg ha^{-1}) and 116 kg ha^{-1} mineral fertilizer (as described for field trial I). The seed was on 6th of May (cw 18). In Gabersdorf (46°47´22´´ N, 15°35´37´´ E), the soil was profound loose sediment brown earth. Organic and mineral fertilizers were not applied here currently (neither in 2010 nor 2011). The sowing was on 27th of April (cw 17). Immediately and five days after the seed a herbicide treatment with the same substances used for field trial I was performed for field trials II and III respectively. Seeds were seeded in three replicate plots per treatment (organized in a completely randomized plot design). In each replicate plot seeds were seeded in 5 rows, and each row comprised 30 seeds (in a distance of 20 cm between seeds). Overall, 3.150 seeds were sown per field trial II and III respectively. After assessment of germination rates the number of plants was reduced to 15 per row (40 cm of distance between plants). Chemically treated and chemically untreated variants were sown in separate plots (in contrast to field trial I). Plants were grown without artificial irrigation for 125 days in Flöcking and 139 days in Gabersdorf.

Parameters assessed during field trials

The following criteria were evaluated for field trials I - III: germination rate (cw 20, 21, and 22 at field trials I, II, and III respectively), degree of fruit rot and dry weight of harvested seeds (cw 36, 37, and 40 at field trials II, III, and I respectively), and 100-corn dry weight of harvested seeds [always for each treatment, replicate plot and plant row (for field trial I)]. Due to heavy crow grub (Figure ESM2) at field trial III especially on the *P. chlororaphis* P34 variant (plus chemical stripper) and the non-chemical *S. plymuthica* S13 respectively control treatment paired with a low germination of these variants *per se*, they were excluded from observation in Gabersdorf. For evaluation of degrees of fruit rot and harvest yields for field trials II and III, only pumpkins from rows 2 – 4 (described above) were included. The number of rotten pumpkins was assessed and set in relation to the total number of pumpkins. For determination of harvest yields per treatment and replicate plot, seeds were mechanically extracted from pumpkins, dried and weighted. For field trials I and II desiccation stress resistance (in cw 27 at

both field sites) and degree of infestation of leaves by powdery mildew (*Sphaerotheca fuliginea*; cw 27 and 30 at field trials II and I respectively) was observed as well. Level of infestation by powdery mildew and extent of desiccation stress (percentage of leave area representing less turgor) was estimated always for each plant row for field trial I and for each replicate plot for field trial II (field designs are described above) by observing the respective leave areas from the top of a ladder. The degree of mildew infestation was described either as the percentage of leaf area covered by the pathogen (for field trial I) or by scoring the infestation severity by conferring the grades 0 (until 10% of leaf are infested), 1 (10 - 20% of leaf are infested), 2 (20-30% of leaf area infested), or 3 (> 30% of leaf area infested) (for field trial II). An overview of the different evaluated parameters for the different treatments is presented in Table 1.

Climate data

Data for precipitation and temperature (maximal day temperatures) were registered for each day during the oil pumpkin vegetation periods in 2010 and 2011 in Gleisdorf (47°7′1″ N, 15°42′23″ E) and provided by ZAMG (Zentralanstalt für Meteorologie und Geodynamik, Vienna; http://www.zamg.ac.at/). The obtained recordings are representative for the conducted field trials I and II.

Statistics

Analysis of Variance (ANOVA) in addition with Games-Howell and Duncan's post-hoc tests ($P < 0.1, 0.05, 0.01$) was performed with Statistical Product and Service Solutions for Windows, Rel. 11.5.1 (SPSS Inc.) to compare mean values of evaluated parameters indicating enhancement of plant growth and health.

Table 1. Overview of the evaluated parameters for different broad-spectrum antagonist treatments (including control treatments) in the course of oil pumpkin field trials at different field sites and vegetation periods.

Treatments	**Evaluated parameters tested at different field trials (site and vegetation period) with (+) and without (-) the addition of the chemical stripper Maxim XL**					
	Field trial I (Gleisdorf 2010)		Field trial II (Flöcking 2011)		Field trial III (Gabersdorf 2011)	
	+	-	+	-	+	-
Control	1 - 6	1 - 6	1 - 6	1 - 6	1 - 4	n.e.p.
S. plymuthica S13	1 - 6	1 - 6	1 - 6	1 - 6	1 - 4	n.e.p.
P. polymyxa PB71	1 - 6	1 - 6	1 - 6	n.e.p.	1 - 4	n.e.p.
L. gummosus L101	1 - 6	1 - 6	1 - 6	n.e.p.	1 - 4	n.e.p.
P. chlororaphis P34	1 - 6	1 - 6	1 - 6	n.e.p.	n.e.p.	n.e.p.

1: germination rate, 2: harvest yield, 3: 100-corn weight, 4: fruit rot, 5: desiccation stress tolerance, 6: mildew infestation, n.e.p: no evaluated parameter

Results

Endophytic life-style of *S. plymuthica* S13

BOX-PCR fingerprint patterns from bacteria, isolated from surface sterilized roots, shoots, and cotyledons that developed from oil pumpkin seeds that were primed with *S. plymuthica* S13, were identical to the band pattern of the inoculum (Figure ESM4). This demonstrates the capability of *S. plymuthica* S13 to live as an endophyte in oil pumpkin. The other test strains used in this study were originally isolated from the endosphere of oil pumpkin.

Parameters monitored during field trials

Germination rate

In order to assess plant growth promoting effects on Styrian oil pumpkin by selected broad-spectrum antagonists, germination rates of seeds treated with bacterial suspensions with and without addition of chemicals were monitored, compared to control treatments, and are summarized in Figure 1 (and Table ESM1). In field trial I, bacterial inoculation (without chemical treatment) increased germination rate by 27 to 47% compared to the non-inoculated control and could compensate the effect of the chemical stripper as it led to an equal or even better emergence of seedlings. Application of both, bacteria and fungicides, showed minor cumulative effects. The highest increase of germination rates were recognized for seeds inoculated with *S. plymuthica* S13 and *P. chlororaphis* P34 (always 20%). In comparison to field trial I, inoculation with *S. plymuthica S13* (without chemical treatment) led to a considerable increase in germination (109%) compared to the non-inoculated control in field trial II. Results from combined applications of bacteria and chemicals were similar to the results from the control, treated solely with the chemical stripper. In field trial III, the effect of *S. plymuthica* S13 applied without chemical stripper, as well as chemically treated seeds inoculated with *P. chlororaphis* P34, could not be monitored as crows fed on those seeds. In combination with chemical coatings, the highest germination rates were observed for *S. plymuthica* S13 (75.6%) followed by *L. gummosus* L101 (72.0%) and *P. polymyxa* PB71 (63.0%), and were comparable with the respective non-bacterized control.

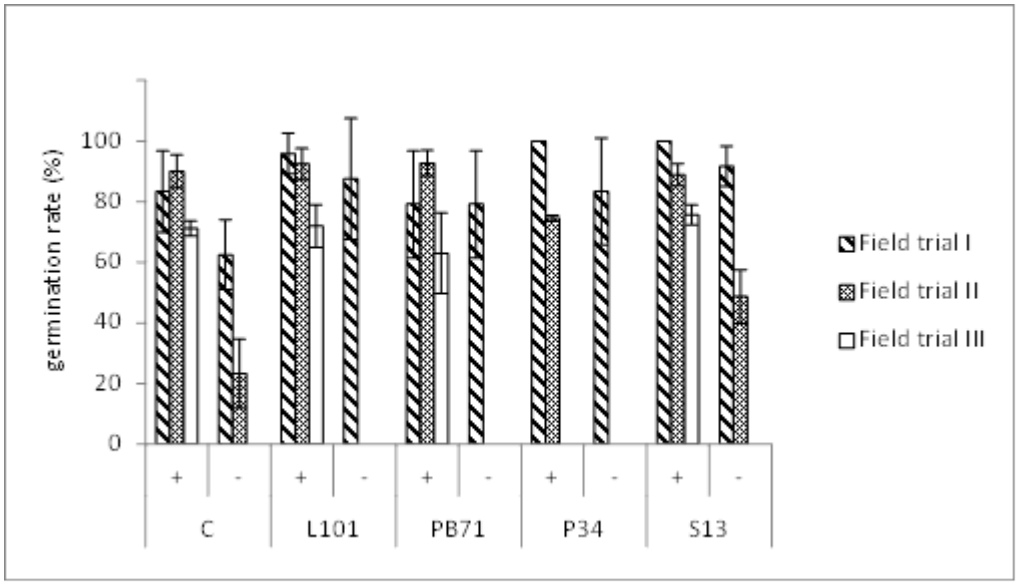

Figure 1. Mean values of germination rates (%) of Styrian oil pumpkin after seed priming with selected broad-spectrum antagonists *L. gummosus* L101, *P. chlororaphis* P34, *P. polymyxa* PB71, and *S. plymuthica* S13 (C = control treatments) with (+) and without (-) addition of a chemical stripper at different field sites. Error bars indicate 95% confidence intervals.

Dry weight of harvested seeds (harvest yield)

Harvest yield, the economically most important parameter, was monitored as follows. Mean values per treatment and replicate plot were evaluated (Fig. 2). Compared to the controls, combined treatments with bacteria and chemicals resulted in similar or higher harvest yields throughout the field trials. For plants inoculated with *L. gummosus* L101, increases of harvest yields by 26 and 20% (in comparison to the chemically treated controls) were observed in field trial II and III, respectively. The increment was statistically significant for field trial III (Duncan's multiple range test, $P < 0.1$). Inoculation with *P. polymyxa* PB71 resulted in higher yields in field trials I, II and III (+ 12%, 20%, and 15% respectively, compared to the chemically treated controls). In case of the chemically untreated variants, the greatest increase in harvest yield (in comparison to the respective control) was demonstrated for *S. plymuthica* S13 by 40.3% in field trial II; the effect was statistically significant ($P < 0.1$, Games-Howell test).

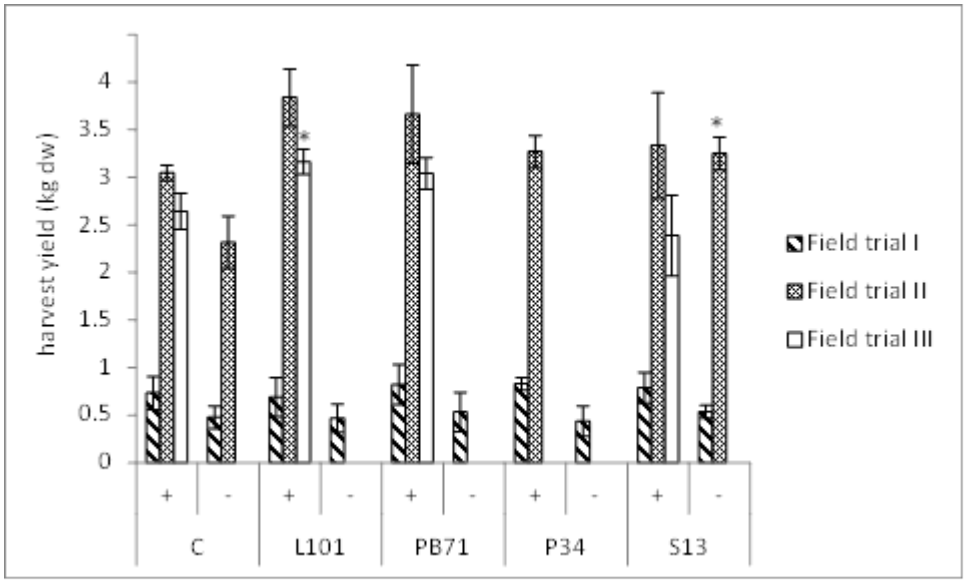

Figure 2. Mean values for harvest yields (kg dry weight) after seed treatments with selected broad-spectrum antagonists *L. gummosus* L101, *P. polymyxa* PB71, *S. plymuthica* S13, and *P. chlororaphis* P34 (C = control treatments) with (+) and without (-) the addition of a chemical stripper at different field sites. Error bars signify 95% confidence intervals. *significant differences compared to respective control treatments (Games-Howell and Duncan's post-hoc tests, $P < 0.1$).

100-corn weight

The 1000-corn weight is of particular importance for extraction of oil from pumpkin seeds. For our field trials, not 1000-corn weights but the weights of 100 seeds per treatment and replicate plot were compared. In field trial I, the chemically and non-chemically variants of the *P. polymyxa* PB71 treatment revealed highest increases by 8.7% and 5.0% respectively, compared to the corresponding controls. Beside this promising result, no considerable increases (> 5%) in 100-corn weight were observed for other inoculants at field trials I and II (Table 2) and field trial III (data not shown).

Biocontrol of fruit rot

The causal agents of the observed fruit rot were black rot (*D. bryoniae*), soft rot (*P. carotovorum*), and rotting due to *Sclerotinia sclerotiorum* especially in field trial II. Across the different field trials, no statistical effect of bacterial treatments on pumpkin fruit health could be investigated. In field trial I, the proportions of rotten pumpkins throughout the chemically treated variants were 5.0% for *S. plymuthica* S13, 5.4% for the control, 6.1% for *P. chlororaphis*

P34, 10.0% for *P. polymyxa* PB71, and 12.2% for *L. gummosus* L101. The degrees of rotten fruits within the chemically untreated alternatives in field trial I were: 2.7% for *S. plymuthica* S13, 3.0% for *P. chlororaphis* P34, 6.7% for *P. polymyxa* PB71, 12.9% for the control, and 15.2% for *L. gummosus* L101. In field trial II amounts of rotten pumpkins from chemically treated variants were 2.9% (*L. gummosus* L101), 4.8% (control), 7.8% (*P. chlororaphis* P34), 8.7% (*P. polymyxa* PB71), and 13.2% (*S. plymuthica* S13). The chemically untreated variants of *S. plymuthica* S13 and the control exhibited 3.3% and 4.4% respectively. For field trial III, the lowest degree of fruit rot after chemical seed treatment was found for the control (1.2%), followed by *P. polymyxa* PB71 (1.3%), *S. plymuthica* S13 (1.9%), and *L. gummosus* L101 (2.3%).

Biocontrol of powdery mildew

Leaf infestation with powdery mildew occurred in both vegetation periods. In 2010 (field trial I) it was even more pronounced due to a very intense rain event (511 mm) in cw 29 (Figure ESM1). In field trial I, the chemically treated variants exhibited a lower degree of mildew infestation. Lowest incidence of the pathogen on leaves was recorded for chemically treated variants of *P. polymyxa* PB71 (26.7% of infested leaf area) and *L. gummosus* L101 (53.3% of infested leaf area) that was statistically different to the respective control treatment (Duncan's multiple range test, $P < 0.01$ and 0.1 respectively) (Figure 3 and Figure ESM5). For field trial II, no effects on reduction of mildew by tested broad-spectrum antagonists were observed (Table 2 and Figure ESM6).

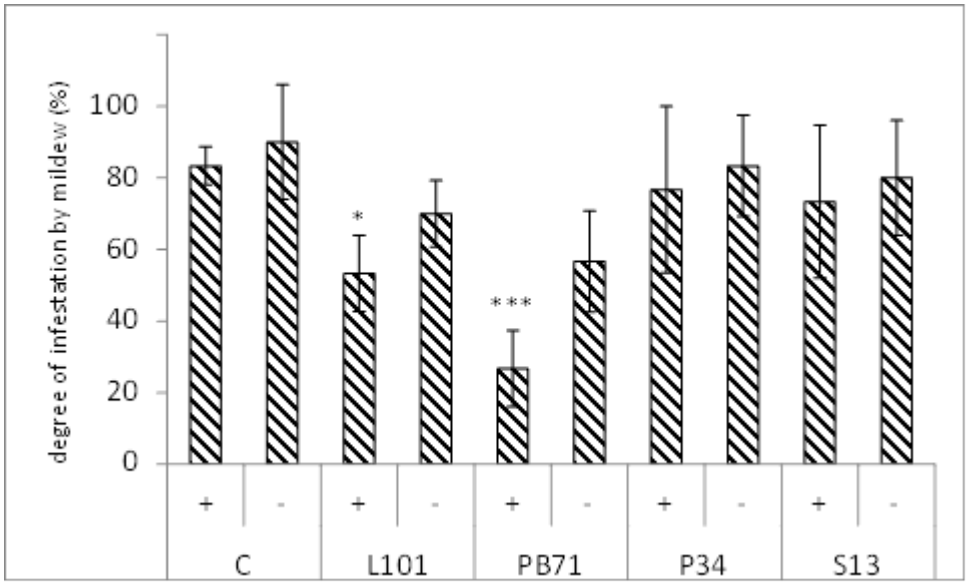

Figure 3. Mean values for leaf area of Styrian oil pumpkin infested with powdery mildew (in %) after seed priming with selected broad-spectrum antagonists *L. gummosus* L101, *P. chlororaphis* P34, *P. polymyxa* PB71, and *S. plymuthica* S13 (C = control treatments) with (+) and without (-) the addition of a chemical stripper at field trial I. Error bars indicate 95% confidence intervals. *, ***significant differences compared to the respective control treatment (Duncan's multiple range test, $P < 0.1$ and 0.01 respectively).

Desiccation stress tolerance

During both vegetation periods, the oil pumpkin plants showed signs for desiccation stress (Figure ESM3) due to relative dry periods (small amount of rainfall) in cw 26 and 27 in 2010 and 2011 (Figure ESM1), therefore the occurrence of desiccation stress symptoms on oil pumpkin plants was estimated (summarized in Table 2). Statistically significant effects were observed in field trial II by the chemical variant of the *L. gummosus* L101 treatment (Duncan's multiple range test, $P < 0.05$), leading to a 100% desiccation stress reduction compared to the respective control (0.0% in contrast to 6.7% of leaf area showed desiccation stress symptoms), and by the chemically untreated variant of *S. plymuthica* S13 (Duncan's multiple range test, $P < 0.1$), conferring a 75% reduction of desiccation stress in comparison to the respective control treatment by the representation of an affected leaf area of 1.7%. Treatments *S. plymuthica* S13 and *P. chlororaphis* P34 (both chemically treated) led to 3.3% of leaf area with less turgor, whereas 6.7% of leaf area lacking a healthy turgor was observed for the *P. polymyxa* PB71 treatment. In field trial I the lowest affected leaf area (23.3%) was observed for chemically stained *S. plymuthica* S13, followed chemically stained *P. chlororaphis* P34, *P. polymyxa* PB71, and control treatment (always 26.7%), followed by the chemically unstained *P. polymyxa* PB71

treatment (30.0%), followed by chemically unstained *S. plymuthica* S13, *P. chlororaphis* P34 and control treatment (always 33.3%). 36.7% and 40.0% of turgor-less leaf areas were found for the chemically unstained and stained *L. gummosus* L101 treatment respectively.

Overall performance of broad-spectrum antagonists *ad planta*

In order to select most promising antagonists for the development of a biological product for Styrian oil pumpkin, performances *ad planta* of the four test strains were compared. According to our evaluation scheme, *S. plymuthica* S13 exhibited the best overall performance (Table 2). *P. polymyxa* PB71 and *L. gummosus* L101 showed reproducible increases in harvest yields throughout different field trials II and III (Figure 2) and reached the 2nd positions in the evaluation scheme (see Table 2), whereas *P. chlororaphis* P34 had the least positive effect.

Table 2. Summary of the performance of selected broad-spectrum antagonists regarding promotion of growth and health of Styrian oil pumpkin after seed priming in the course of field trials I and II.

Field trial	**Bacterial strain**	**Increase in plant growth/health compared to the control treatment**					**Sum of weighted[a] plus**
		Germination rate	Harvest yield	100-corn weight	Reduction of infestation by mildew	Increase in desiccation stress tolerance	
I	*S. plymuthica* S13	+	+	-	+	+	15
		++	+	-	+	-	
II		-	+	-	-	++	
		+++	++*	-	-	+++*	
I	*P. chlororaphis* P34	+	+	-	-	-	5
		++	-	-	-	-	
II		-	+	-	-	++	
I	*P. polymyxa* PB71	-	+	+	++***	-	6
		+	+	+	++	+	
II		-	+	-	-	-	
I	*L. gummosus* L101	+	-	-	++*	-	6
		++	-	-	+	-	
II		-	+	-	-	+++**	

White and grey fields indicate treatments with and without addition of the chemical stripper Maxim XL respectively.
*, **, *** significant differences between respective mean values and means of corresponding control treatments (Duncan`s and Games-Howell post-hoc tests, $P < 0.1$, 0.05 and 0.01 respectively).
-increase in plant growth/health less than 5%; +, ++, +++increase in plant growth/health from: 5 – 30% (+), 30 – 60% (++) and > 60% (+++) respectively; [a]sum of plus from field trial I were halved due to the lower amount of plants in comparison to field trial II.

Observation of climate data

Amount of precipitation and temperature fluctuations were registered for field trials I and II in 2010 and 2011 (Figure ESM1) in order to explain differences in monitored parameters among the different vegetation periods. The maximum day temperatures in the vegetation period 2010 ranged from 11.5°C to 34.0°C with an average of 22.5°C, whereas in vegetation period 2011 the temperature range was from 15.5°C - 33.4°C (25.0°C in average). In 2010, an intense rain event (511 mm) correlated with a high infestation of oil pumpkin plants with powdery mildew (as described above). In 2011, the highest amount of precipitation was measured in cw 23 (460 mm). In cw 26 and 27, relative small amounts of rainfall were noticed in both vegetation periods, leading to the occurrence of desiccation stress symptoms on oil pumpkin plants (as described above).

Discussion

The selection of antagonists by *in vitro* assays is usually a straightforward procedure. To find BCAs that succeed under practical conditions in agriculture is actually much more complex and represents one of the main hurdle in the commercialization process (Berg 2009). Discrepancies between the antagonistic effects under *in vitro* conditions and the corresponding *in situ* efficacy are frequently reported (Weller & Cook 1983, Reddy et al. 1993). We revealed that three out of four bacteria, selected in a comprehensive, ecology-based study including greenhouse trials (Fürnkranz et al. 2012), were active under field conditions. Interestingly, we found out that the strains showed differential activities: while *Serratia plymuthica* S13 appeared as a PGPB, and was particularly suitable to promote the germination of pumpkin seeds, *Paenibacillus polymyxa* PB71 acted as BCA against powdery mildew infection. Furthermore, with *S. plymuthica* S13 and *Lysobacter gummosus* L101 rarely reported agents against abiotic stress were observed. However, considering all three performed field trials, differences in the efficacies of tested bacteria were found.

The highest variations were observed with respect to germination of pumpkin seeds. In field trial I, the effect of the chemical treatment on germination could be compensated by three of the four tested bacteria, whereas results from field trial II showed that the emergence of seedlings of the chemically non-treated variants was much lower compared to fungicide treated seeds. These discrepancies can be explained by the type of seed batch, which was used

for the priming procedure for field trials in 2011. The immersion bath negatively affected the cuticles due to the low seed quality, leading to a minor germination capacity. The different environmental conditions in vegetation periods 2010 and 2011 had probably a minor influence on observed germination rates. However, when the negative effect of priming on oil pumpkin seeds was subtracted from the beneficial effect on seedling emergence by the tested bacteria, oil pumpkin seed germination was increased up to 109% (by *S. plymuthica* S13 at field trial II). We therefore suggest the exploration of other application methods than seed priming for *S. plymuthica* S13 (Müller & Berg 2008) in order to develop a biological alternative for conventional fungicides as well as copper-based formulations, essential in organic farming. For *P. polymyxa* PB71 spore-based formulations can be developed; our preliminary results indicated a high shelf-life of encapsulated spores (no decrease of the inoculum after five months of storage at 4°C).

The effect of the selected bacterial strains against *D. bryoniae*, the causal agent of fruit rot, was actually of interest. Although all selected broad-spectrum antagonists showed *in vitro* inhibition of *D. bryoniae* (Fürnkranz et al. 2012), no biocontrol effect was observed in the field experiments. The degree of black rot depends mainly on the climatic conditions, the oil pumpkin cultivar, and field management (Huss et al. 2007, Babadoost & Zitter 2009, Huss 2011). In our study, the amount of rotten pumpkins was relatively low in comparison to other vegetation periods (Huss et al. 2007). The occurrence of affected fruits on the fields was irregular, indicating a high influence of soil characteristics, especially drainage, on black rot. These circumstances did not allow a representative evaluation of selected bacteria on the suppression of oil pumpkin rot. However, a preventive biocontrol strategy to diminish the risk for black rot and gummy stem blight, which is a world-wide problem for other crops belonging to Cucurbitaceae (Lee et al. 1984, Sitterly & Keinath 1996, Keinath 2010), is urgently needed. In contrast to the low degree of fruit rot, powdery mildew on leaves was significant. The symptoms ranged from partly affected to fully covered leaves by the fungus. In field trial I, a statistically significant reduction of disease symptoms was observed for treatments with *L. gummosus* L101 and especially *P. polymyxa* PB71. In field trial II, the degree of mildew infestation was low due to climatic conditions, and therefore disease suppression by the bacteria was hardly assessable.

Positive effects also on the physiological status of field grown oil pumpkin plants were observed for tested bacteria. Especially *L. gummosus* L101 and *S. plymuthica* S13 were

able to suppress symptoms of desiccation of pumpkin plants. Although several mechanisms of biocontrol and plant growth promotion have already been reported, e.g. for *S. plymuthica* (Koo & Cho 2009, Müller et al. 2009), nothing is known about the relation of plant-associated bacteria and desiccation stress of plants. Due to challenges of climate change, bacterial inoculants can serve here as a promising solution. The better physiological constitution of oil pumpkin plants after bacterial inoculation was accompanied with increased harvest yields. Consistently enhanced crop yields among different field sites were obtained by *L. gummosus* L101 and *P. polymyxa* PB71 in 2011. Another parameter, which is correlated with harvest yield, is the 100-corn weight. Especially for oil extraction from pumpkin seeds, a high seed size is desirable. Here, also strain *P. polymyxa* PB71 offers potential but this effect was not observed in all field trails and should be investigated further.

In this study we found bacterial strains suitable as inoculants for plant growth promotion, biocontrol as well as stress tolerance for Styrian oil pumpkins. Only one of the four tested strains, *P. chlororaphis* P34, showed no clear effects under field conditions. Further investigations are necessary to develop a formulation with a high shelf-life for the three promising bacteria. Although all strains belong to risk groups 1 (without risk for humans and environments), further risk assessment is important (Berg et al. 2009).

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Supplementary material

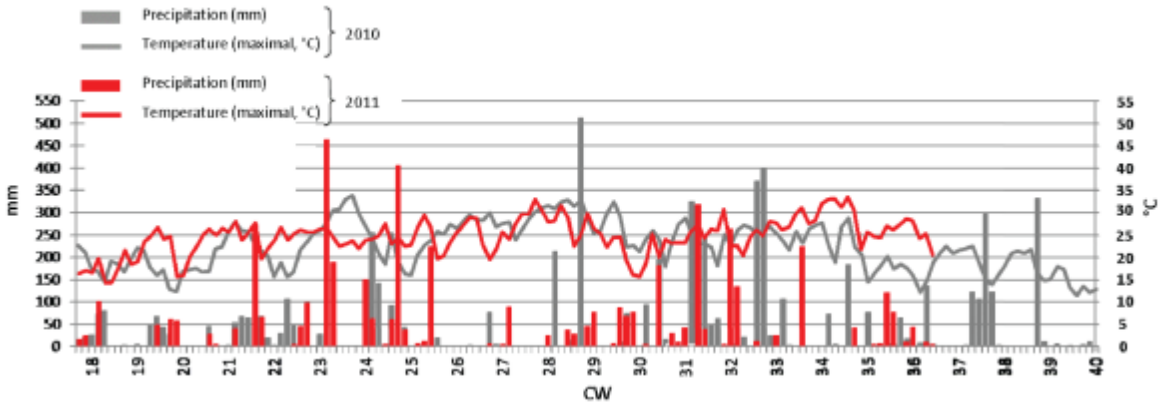

Fig. 1 Climate data (precipitation and maximal temperature) during the vegetation periods of oil pumpkin (from seed until harvest) in 2010 and 2011, indicated for different calendar weeks (cw). Data were obtained by a climate observatory station in Gleisdorf and represent exactly the climate conditions for field trials I (in Gleisdorf, 2010) and II (in Flöcking, 2011).

Fig. 2 Illustration of the crow grub, affecting especially chemically unstained variants and the *P. chlororaphis* P34 treatment in Gabersdorf (field trial III).

Fig. 3 Illustration of desiccation stress symptoms of oil pumpkin. The upper photo shows patches with plants representing leaves with less turgor. The lower photo shows oil pumpkin plants that are not suffering from desiccation stress, but exhibiting a healthy turgor.

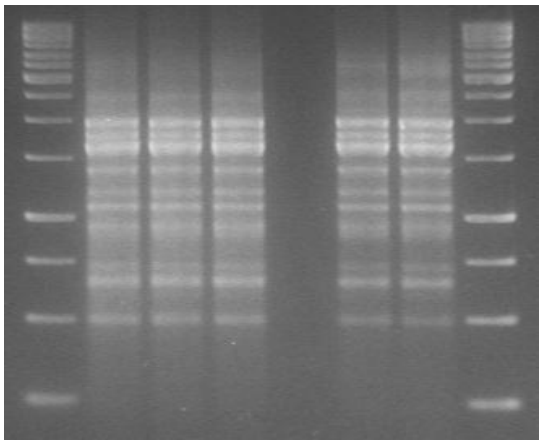

Fig. 4 Box-PCR fingerprint patterns from bacteria that were isolated from surface-sterilized roots, shoots, and leaves of oil pumpkin plants at the two-leaf stage that developed from seeds that were inoculated with *S. plymuthica* S13 by priming (lanes 1 – 5). Lane C represents the Box-PCR fingerprint patterns of the inoculum (for comparison); 1kbl. = 1 kb ladder as size marker.

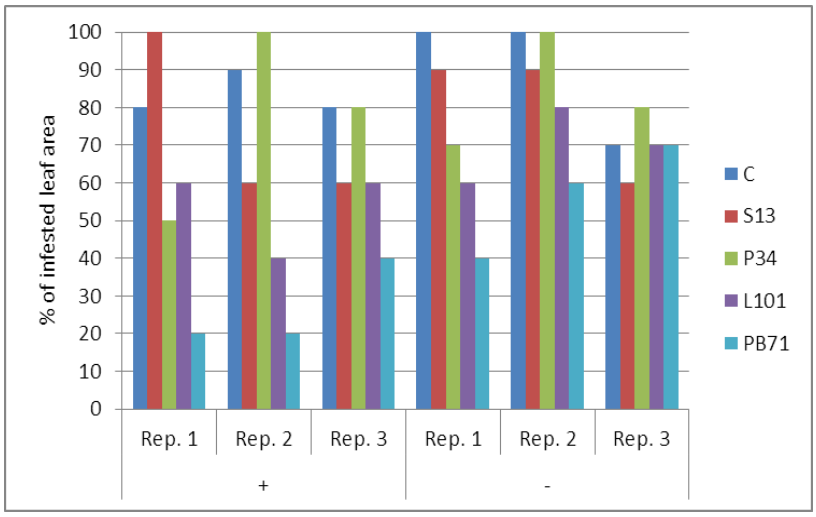

Fig. 5 Degree of mildew infestation in % for different treatments and replicate plots with (+) and without (-) the addition of the chemical stripper at field trial I.

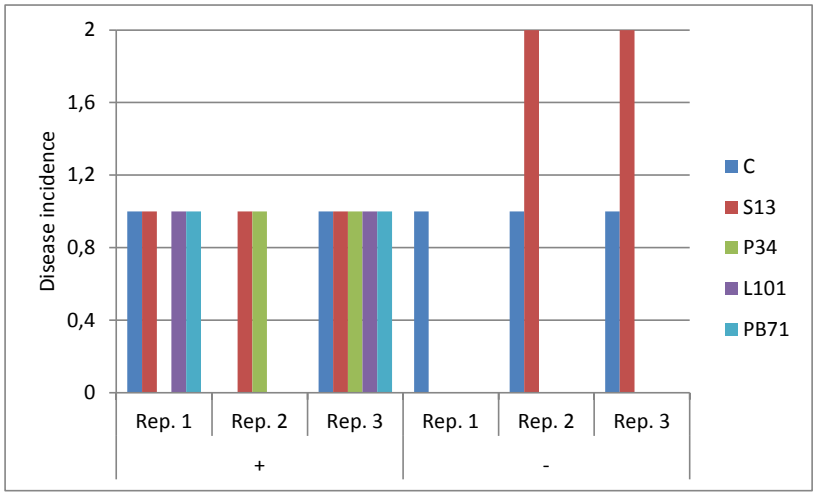

Fig. 6 Degree of mildew infestation described as disease incidence (the lower the value the less leaf are is affected) for different treatments and replicate plots with (+) and without (-) the addition of the chemical stripper at field trial II.

Table 1. Seedling emergence (mean values) after seed primings with bacterial broad-spectrum antagonists (including control treatment) with and without the addition of a chemical stripper in case of three different field trials.

Treat-ment	**Chemical stripper**	**Field trial I**			**Field trial II**			**Field trial III**		
		sown	germinated	%±CI[a]	sown	germinated	%±CI[a]	sown[b]	germinated	%±CI[a]
C	+	8	6.67	83.38±13.34	150	135.00	90.00±5.48	136	96.86	71.22±2.39
	-	8	5.00	62.50±11.55	150	35.00	23.33±11.31	79	n.e.	n.e.
S13	+	8	8.00	100.00±0.00	150	133.35	88.90±	143	108.18	75.65±3.25
	-	8	7.33	91.63±6.67	150	73.01	48.67±	112	n.e.	n.e.
PB71	+	8	6.33	79.13±17.64	150	139.01	92.67± 4.31	130	81.93	63.02±13.30
	-	8	6.33	79.13±17.64	150		n.e.	n.e.	n.e.	n.e.
L101	+	8	7.67	95.88±6.67	150	138.66	92.44±5.16	137	98.60	71.97±7.00
	-	8	7.00	87.50±20.00	150		n.e.	n.e.	n.e.	n.e.
P34	+	8	8.00	100.00±0.00	150	111.66	74.44± 0.94	120	n.e.	n.e.
	-	8	6.67	83.38±17.64	150		n.e.	n.e.	n.e.	n.e.

[a]confidence interval (95%)

[b]minus crow grub

Additional publication I

Characterization of plant growth promoting bacteria from crops in Bolivia

Michael Fürnkranz, Henry Müller and Gabriele Berg. Journal of Plant Diseases and Protection (2009) 116: 149-155, ISSN 1861-3829

Personal contribution: accomplishment of the underlying practical work at the field and in the laboratory, and comprehensive preparation of the manuscript

Abstract

The use of plant growth promoting bacteria (PGPB) is an economically and environmental friendly alternative to the application of chemical fertilizers resp. pesticides in agriculture. To obtain novel bacterial strains that could be used for plant growth promotion (PGP) in the agriculture of Bolivia, plant associated bacteria derived from horseradish tree (*Moringa oleifera*), sorghum (*Sorghum vulgare*), sunflower (*Helianthus annuus*), and safflower (*Carthamus tinctorius*) were screened for direct and indirect *in vitro* PGP traits. Subsequently, most promising strains were selected for *ad planta* studies. According to *in vitro* experiments, out of 59 tested isolates, 19% were declared as diazotrophs, 41% as solubilizers of phosphorus, 10% were able to reduce the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC), and 17% exhibited phytohormone (IAA) synthesis. Only a small proportion of rhizobacterial strains (7% out of 276) showed *in vitro* antagonism against plant pathogenic *Colletotrichum* sp., whereas none of isolated stem and leaf endophytes inhibited growth of *Verticillium chlamydosporum* or *Bipolaris maydis* in dual culture. Based on results of *in vitro* screens, the rhizospheric strains *Pectobacterium cypripedii* M56, *Pantoea agglomerans* M72, and *P. agglomerans* M81 were selected for *ad planta* applications. Results revealed significant increases in number of beans per black bean plant resp. diameters of flower heads of sunflower plants compared to controls by *P. agglomerans* M72. We conclude that the assessment and selection of plant associated bacteria based on traits conferring theoretically PGP can provide the basis for the development of new microbial inoculants for agricultural purposes.

Zusammenfassung

Die Anwendung pflanzenwachstumsfördernder Bakterien in der Landwirtschaft kann eine profitable und umweltschonende Alternative zum Einsatz von chemischen Düngemitteln und Pestiziden darstellen. Um neue, bakterielle Pflanzenwachstumsförderer für die bolivianische Landwirtschaft zu gewinnen, wurden pflanzenassoziierte Bakterien von Meerrettichbaum (*Moringa oleifera*), Sorghumhirse (*Sorghum vulgare*), Sonnenblume (*Helianthus annuus*) und Färberdistel (*Carthamus tinctorius*) isoliert, und *in vitro* auf direkte und indirekte Mechanismen der Pflanzenwachstumsförderung untersucht. Ausgehend von 59 unter Laborbedingungen getesteten Isolaten, waren 19 % diazotroph, 41 % wurden als Phosphor-Solubilisierer deklariert, 17 % zeigten Phytohormonsynthese (IAA) und 10 % wurden als Verwerter der Ethylen-Vorläufer-Substanz 1-Aminocyclopropan-1-Carbonsäure (ACC) erklärt. Nur ein geringer Anteil rhizobakterieller Stämme (7 % von 276) zeigte antagonistische Aktivität *in vitro* gegen das Pflanzenpathogen *Colletotrichum sp*., während keiner der von Stängeln oder Blättern isolierten Endophyten das Wachstum von *Verticillium chlamydosporum* oder *Bipolaris maydis* in Dualkultur inhibieren konnte. Basierend auf den Resultaten der *in vitro* Tests, wurden die Rhizosphären-Stämme *Pectobacterium cypripedii* M56, *Pantoea agglomerans* M72 und *P. agglomerans* M81 für die Applikation in Freilandexperimenten selektiert, wobei *P. agglomerans* M72 zu signifikant höheren Bohnenzahlen bei schwarzer Bohne bzw. Blütenkorbdurchmessern bei Sonnenblume im Vergleich zu Kontroll-Behandlungen geführt hat. Die Bewertung und Selektion pflanzenassoziierter Bakterien, basierend auf deren pflanzenwachstumsfördernden Eigenschaften *in vitro*, stellt die Grundlage für die Entwicklung neuer biologischer Produkte für landwirtschaftliche Zwecke dar.

Introduction

Plant growth promoting bacteria (PGPB) and biological control agents (BCAs) (BASHAN and HOLGUIN 1998; CHERNIN and CHET 2002) perform beneficial effects on plants via direct resp. indirect mechanisms. PGPB directly serve the plants by supplying nutrients [e.g. via the fixation of atmospheric nitrogen (N_2), phosphorous (P) solubilization, segregation of iron (Fe) by siderophores], by means of phytohormone synthesis (e.g. indole-3-acetic acid, IAA), and by lowering the hosts ethylene level due to ACC deaminase activity (DART 1986; BAR-NESS et al. 1991; BLOEMBERG and LUGTENBERG 2001; GULL et al. 2004;

GLICK et al. 2005). BCAs support plant health via the suppression of plant pathogens, e.g. due to competition for nutrients and space, the synthesis of antimicrobial compounds, parasitism, or the induction of systemic resistance in host plants (O`SULLIVAN and O`GARA 1992; VAN LOON 2007; RAAIJMAKERS et al. 2008). Whereas it is sometimes difficult to screen biocontrol agents under *in vitro* conditions due to discrepancies between the antagonistic effects under *in vitro* conditions and the corresponding *in situ* efficacy (WELLER and COOK 1983; REDDY et al. 1994), PGP traits can be assessed under laboratory conditions and allow the selection of strains that could lead to increased plant growth (CATTELAN et al.1999).

The inoculation of legumes with *Rhizobium* spp. is a prominent example for an effective, cheap, and environmental friendly method to ensure supply of nitrogen to agriculturally used areas (KADAM et al. 1977; DOBBELARE et al. 2003). Also non-symbiotic bacterial strains have notable potentials to promote plant growth and health, and were even subjected to the development of ecosensitive products for the agricultural market (KURZE et al. 2001; WHIPPS 2001). However, plant associated microhabitats harbour a large number of bacterial strains with yet unknown abilities for direct and/or indirect PGP. The rhizosphere, as the soil compartment directly encompassing the roots, constitutes an attractive niche for bacterial colonization (FAURE et al. 2008). Furthermore the interior of plant tissues termed as endosphere represents a niche of close plant vicinity in that PGPB and BCAs may be selectively enriched (BERG and HALLMANN 2006; BERG et al. 2008).

The aim of this study was to isolate and characterize new strains of plant associated bacteria that could serve in terms of PGP for a farm in Bolivia. This farm, which is named ´San Rafael` and owned by the company DESA (Desarrollos Agrícolas), is located in the Bolivian department Santa Cruz, cultivated an area of 12.300 ha. On the farm different crop plants including soybean, sunflower and cotton are cultivated and, additionally, citrus fruits in plantations are grown. Interestingly, resident farmers produce its own biocontrol agents such as *Trichoderma harzianum* and *Beauveria bassiana*. To find PGPB with a broad host range for self-production, bacteria derived from agricultural plants grown at the Bolivian farm were isolated and screened *in vitro* for N_2 fixation, IAA synthesis, ACC deaminase activity, P solubilization, and antagonistic activity against fungal plant pathogens. A screening scheme for the selection of most promising strains was designed and three chosen isolates were

applied *ad planta.*

Materials and Methods

Sampling and isolation of plant associated bacteria

For isolation of plant associated bacteria, horseradish tree (*Moringa oleifera* Lam.) and safflower (*Carthamus tinctorius* L.) plants were collected from fields of the farm San Rafael/DESA, whereas sorghum (*Sorghum vulgare* PERS.) and sunflower (*Helianthus annuus* L.) plants were collected from fields adjacent to this farm, located in the Bolivian department Santa Cruz. Samples of roots with adhering soils, stalks, and leaves were taken from six safflower- and four sorghum individuals from always four different sites of corresponding fields. From sunflower, samples of roots and shoots were taken from two individual plants from always four different field sites whereas in case of horseradish tree, roots from three individuals grown at different plantation sites were collected.

For preparation of bacterial rhizosphere suspensions, four to seven grams of roots with adhering soils were amended with 40 ml of sterile 0.85% NaCl in 50 ml tubes and shaken thoroughly by hand for 1.5 min. Suspensions of endophytic bacteria were obtained as follows: tap water washed roots as well as unwashed stalks and leaves were surface sterilized in 4% sodium hypochlorite (NaOCl) for five min, except for sorghum leaf samples, that were treated only for three min with NaOCl, and then washed three times with sterile water. Afterwards plant materials were imprinted on R2A medium as a sterility check. Only colonies that derived from samples showing negative sterility test results resp. exhibited different colony morphologies compared to bacteria grown on corresponding sterility test plates were used for subsequent analysis. Two to six grams of surface sterilized roots, stalks, and leaves were amended with five resp. 10 ml sterile 0.85% NaCl in a mortar and homogenized with a pestle. Resulting rhizosphere and endosphere bacterial suspensions were serially diluted with sterile 0.85% NaCl and plated onto R2A medium. Agar plates were incubated for five days at 24°C and afterwards colony forming units (CFUs) were counted and their means were assessed per g of sample fresh weight. Bacterial isolates selected from PGP screenings (as described below) were purified and stored in sterile nutrient broth containing 50% glycerol at –70°C.

In vitro screenings for direct PGP abilities

Overall, 54 bacterial isolates derived from the horseradish tree rhizosphere as well as selected bacterial *in vitro* antagonists, isolated from rhizospheres of sorghum (two), sunflower (two), and safflower (one), that exhibited growth inhibition of plant pathogenic *Colletotrichum* sp. that exceeded five mm in a dual culture assay (as described below), were subjected to the following screenings:

N_2 fixation and ACC deaminase activity

Bacteria were transferred to Brown & Dilworth (BD) minimal medium (BROWN and DILWORTH 1975) with and without the addition of 150 µl 5% ACC solution that was spread on BD medium as the unique nitrogen source for ACC deaminase active isolates. Bacteria were grown for five days at room temperature (RT) and their extent of growth was assessed after five days. Bacteria that exhibited heavily growth on BD plates were declared as diazotrophs and isolates showing more pronounced growth on BD + ACC solution in comparison to ACC free medium were declared as ACC utilizing strains.

IAA synthesis

Isolated strains were grown in five ml minimal growth medium (5 g glucose, 0.025 g yeast extract, 0.204 g L-tryptophan) in culture tubes in absence of light for 72 hours at 20°C under agitation (120 rpm). Afterwards 1 ml of bacterial suspensions were transferred to 1.5 ml Eppendorf tubes and centrifuged at 16.750 × g for 10 min. 90 µl of resulting supernatants were added to 60 µl Salkowski reagent (0.5 M $FeCl_3$ and 35% perchloric acid in a mixture 1:49) in the cavities of a 96 well plate and incubated for 30 min in the dark. Afterwards absorbance at 530 nm was measured with a microplate spectrophotometer (Infinite M200, Tecan). Absorbance arising from tested isolates was compared with that one of the IAA producing strain *Serratia plymuthica* HRO-C48. Strains whose corresponding absorbance values exceeded that of *S. plymuthica* HRO-C48 were declared as IAA synthesizers.

P solubilization

Bacterial isolates were transferred to National Botanical Research Institute`s phosphate growth agar (NBRIP) containing per liter: 10 g glucose, 5 g $Ca_3(PO_4)_2$, 5 g $MgCl_2 \times 6\ H_2O$,

0.25 g $MgSO_4 \times 7\ H_2O$, 0.2 g KCl, 0.1 g $(NH_4)_2SO_4$, and 15 g agar to perform screening for P solubilization on plates. Isolates were grown for 5 days at RT and afterwards presence (P solubilizing positive) or absence (P solubilizing negative) of visible halo zones at the isolates growth sites on the plates was noted.

In vitro screenings for indirect PGP qualities by detecting antagonistic activity against isolated fungal plant pathogens

Root associated bacteria isolated from sorghum (50), sunflower (80), and safflower (81) as well as six selected rhizosphere strains derived from horseradish tree, positively tested in at least two of the four aforementioned *in vitro* screening criteria for direct PGP, were screened for their antagonistic capacity against the soilborne pathogen *Colletotrichum* sp. Moreover, bacterial endophytes from sorghum (11), sunflower (12), and safflower (15), isolated from stalks, were tested against stem-infesting *Verticillium chlamydosporum* whereas bacterial leaf endophytes harboured by sorghum (11) and safflower (10) were tested against the foliar pathogen *Bipolaris maydis*. Bacterial strains and respective pathogens were transferred on Waksman agar (per liter: 5 g bactopeptone, 10 g glucose, 5 g NaCl, 3 g meat extract, 20 g agar) and incubated at RT. When moulds were grown close enough to bacterial isolates, sizes of possible growth inhibition of the pathogens, outgoing from bacterial strains, were assessed.

Colletotrichum sp. was isolated from dry field stubbles of soybean. *V. chlamydosporum* was isolated from symptomatic sunflower stalks, and *B. maydis* was isolated from corn leaves exhibiting symptoms of Southern Corn Leaf Blight. Pathogenic fungi were isolated from plant material of the farm San Rafael/DESA, Santa Cruz, Bolivia, and identified by morphological observations. Identity of *Colletotrichum* sp. and *B. maydis* was confirmed by sequence analysis of the ribosomal internal transcribed spacer region as described below.

Selection of screened isolates for *ad planta* experiments

Based on direct PGP criteria a point scheme was generated to assess *in vitro* performance of tested strains and to select most promising candidates for *ad planta* studies. The point scheme was designed by the assignment of one point for each of the aforementioned positive fulfilled criteria for direct PGP (max. four points) to tested strains, points were summed up subsequently and strains to that highest sums of points were ascribed, were selected for *ad*

planta experiments.

Inoculation of black bean (*Phaseolus vulgaris*) and sunflower (*Helianthus annuus*)

Two weeks old black bean plantlets and three weeks old sunflower plantlets (both at the four leaf stage) were inoculated with horseradish tree derived rhizobacterial strains *Pectobacterium cypripedii* M56, *Pantoea agglomerans* M72 or *Pantoea agglomerans* M81, selected by *in vitro* experiments (as described above). Chosen strains were grown in nutrient broth (NB: 5 g l^{-1} tryptone, 3 g l^{-1} meat extract) for 48 h at RT under agitation and plants were inoculated by 15 min root dipping in respective bacterial suspensions that contained 10^9 cells ml^{-1} for black bean inoculation and 2×10^9 cells ml^{-1} for sunflower inoculation. Plant roots dipped in sterile NB served as control. Inoculated and control plants were put in sterile water until they were transplanted. Black bean plants were transferred to pots containing loamy soil obtained from the superficial layer of a field of the farm San Rafael/DESA, Bolivia, mixed with sand in a proportion of 3:1. Sunflower plants were transplanted to a field with loamy soil located at farm San Rafael/DESA, Bolivia. 27 resp. 24 days after inoculation/transplantation 15 ml of 10^9 resp. 7×10^8 cells ml^{-1} containing suspensions prepared out of aforementioned strains, grown in NB for 44 hours at RT, were applied to each stem base (a control treatment, by applying 15 ml sterile NB, was performed as well) of black bean plants resp. sunflower plants. It is noteworthy that the used soil for the black bean experiment was taken from a boarder site of a field where no agricultural plants were grown and that was heavily exposed to solar radiation and may reflected approximated gnotobiotic conditions. Each treatment was performed with 40 black bean plants (8 plants per pot and 5 repetitions) and 84 sunflower plants (14 plants per plot with 6 repetitions) and both experiments were organized in a randomized complete block design (RCBD). Plants were irrigated by watering can and were grown without fertilization. Cultivation time of black bean plants was from May to August and from June to September 2008 for sunflower. As indicators for plant growth and harvest yields, numbers of beans per adult plant resp. diameters of flower heads of sunflowers were assessed.

Isolation of DNA from bacterial strains possessing pronounced *in vitro* PGP qualities

Bacterial cell material were transferred to 1.5 ml tubes, containing 300 µl extraction buffer [0.2 M Tris-HCl (pH 8), 0.25 M NaCl, 0.5% sodium dodecyl sulphate], and mixed well. Cell

suspensions were transferred to 2 ml tubes containing 200 µl glass beads and were agitated two times for 30 s at level five with a Fast Prep™ machine (Qbiogene BIO 101® systems, Carlsbad, CA, USA). Then 150 µl of 3 M sodium acetate were added, tubes were vortexed and stored for 30 min at –20°C. Afterwards tubes were centrifuged for 10 min at 16,750 × g and supernatants were transferred to 1.5 ml tubes, DNA was purified by the phenol-chloroform extraction method and precipitated by the addition of isopropanol. DNA was dissolved in 50 µl of PCR grade water and stored at –20°C.

Isolation of DNA from fungal plant pathogens

100 – 250 mg of mycelium of *Colletotrichum* sp. and *B. maydis*, grown on potato dextrose agar (PDA), were transferred to 2 ml tubes containing 400 µl salt homogenizing buffer (0.4 M NaCl, 10 mM Tris-HCl pH 8.0, 2 mM EDTA pH 8.0) and agitated with a Fast Prep™ machine (Qbiogene BIO 101® systems, Karlsbad) for 15 s at level 6.5. Then, 40 µl of 20% sodium dodecyl sulphate were added and tubes were vortexed. Afterwards tubes were incubated at 60°C for one hour followed by the addition of 300 µl of 6 M NaCl. Then samples were centrifuged for 30 min at 10,000 × g, supernatants were transferred to fresh tubes and an equal volume of isopropanol was added before samples were incubated at –20°C for one hour. After tubes were centrifuged for 20 min at 16.750 × g, DNA was washed with 70% ethanol, dried, suspended in 50 µl PCR grade water, and stored at –20°C.

Partial 16S rRNA gene sequence analysis of selected bacterial strains exhibiting pronounced *in vitro* PGP capabilities

To get information of the identity of bacterial strains, exhibiting pronounced direct and/or indirect *in vitro* PGP activities, partial 16S rRNA genes from respective isolates were sequenced. For that, in a primary step, 16S rDNA fragments were amplified in a 60 µl master mix, containing 12 µl of 5 × Taq &GO ™ PCR Mastermix (Qbiogene), always 0.5 µM of primers Eubac1-forward (5‘-GAG TTT GAT CCT GGC TCA G-3‘) and 1492r (5‘-TAC GGY TAC CTT CGT TAC GCA CTT-3‘) as well as 3 µl of template DNA. PCR conditions were as follows: initial denaturation at 95°C for 5 min followed by nine amplification cycles consisting of 95°C for 30 s, 52°C for 30 s, and 72°C for 100 s followed by 19 amplification cycles consisting of 95°C for 30 s, 52°C for 30 s, and 72°C for 30 s (+ 10 s/cycle), and a final elongation step at 72°C for 5 min. PCR products were purified using the peqGOLD

MicroSpin Cycle Pure Kit (PEQLAB, Erlangen, Germany). Purified PCR products were sequenced using the genetic analyzer AB3730 (Applied Biosystems) and the ABI PRISM BigDye Terminator v3.1 Ready Reaction Cycle Sequencing Kit (Applied Biosystems). Obtained data was subjected to BLAST analysis (ALTSCHUL et al. 1997) with the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov).

Internal transcribed spacer rDNA sequence analysis of fungal plant pathogens

For confirmation of morphological identification of isolated fungal plant pathogens *Colletotrichum* sp. and *B. maydis*, their ribosomal internal transcribed spacer (ITS) rDNA regions were sequenced. The PCR master mix for amplification of fungal ITS genes for subsequent sequencing consisted of 8 µl of 5 × Phusion GC reaction buffer (Finnzymes), 0.2 mM dNTPs mix, 2 µl of 100% dimethyl sulfoxide, 2 units Phusion^{TM} DNA Polymerase (Finnzymes), always 1 µM of primers ITS 1f (5‘-TCC GTA GGT GAA CCT GCG G-3‘) and ITS 4r (5‘-TCC TCC GCT TAT TGA TAT GC-3‘), 1.5 mM $MgCl_2$, and 2 µl template DNA in a final reaction volume of 40 µl. Touchdown PCR was performed as follows: initial denaturation at 95°C for 5 min followed by 37 amplification cycles consisting of 95°C for 30 s, 62°C (–0.32°C/cycle) for 35 s, 72°C for 40 s, and a final elongation step at 72°C for 10 min. PCR products were purified using the peqGOLD MicroSpin Cycle Pure Kit (PEQLAB Erlangen, Germany) for the *B. maydis* derived PCR product whereas for *Colletotrichum sp.* the respective PCR product was cut out from an agarose gel and purified using the GENECLEAN®Turbo Kit (Qbiogene). Purified PCR products were sequenced and subjected to phylogenetic analysis as described above.

Statistical analysis

Via Statistical Product and Service Solutions for Windows, Rel. 11.5.1 (SPSS Inc.) mean numbers of beans per black bean plant (DMS, $P<0.05$) resp. diameters of flower heads of sunflowers, (Duncan`s multiple range test, $P<0.15$) evaluated in *ad planta* studies, were compared.

Nucleotide sequence accession numbers

Obtained sequences were deposited in GenBank under accession numbers FJ669155 to

FJ669165 (16S rRNA gene sequences) and FJ669166 to FJ669167 (ITS sequences).

Results

Isolation of plant associated bacteria

Bacterial counts (expressed as $\log_{10}$ CFU g^{-1} fresh weight of plant material) derived from microhabitats of horseradish tree (*M. oleifera*), sunflower (*H. annuus*), sorghum (*S. vulgare*), and safflower (*C. tinctorius*) were the highest for rhizosphere of *M. oleifera* (5.39×10^{10}, SD $\pm$ 4.97 x 10^{10}) and lowest for endosphere of shoot of *H. annuus* (5.48×10^{3}, SD $\pm$ 5.81 x 10^{3}) as declared in Table 1.

Table 1: Mean values of bacterial cell abundances in CFUs (± SD) per g fresh weight of plant material from different host plants and microhabitats

Plant species	Habitat	CFU g^{-1} plant material
Horseradish tree (*Moringa oleifera*)	Rhizosphere	5.39×10^{10} SD ± 4.97
Sunflower (*Helianthus annuus*)	Rhizosphere	1.66×10^{8} SD ± 1.37
	Endorhiza	1.16×10^{5} SD ± 0.79
	Shoot endosphere	5.48×10^{3} SD ± 5.81
Sorghum (*Sorghum vulgare*)	Rhizosphere	1.14×10^{10} SD ± 1.38
	Endorhiza	1.92×10^{7} SD ± 1.82
	Shoot endosphere	1.57×10^{4} SD ± 0.99
	Leaf endosphere	2.22×10^{5} SD ± 1.18
Safflower (*Carthamus tinctorius*)	Rhizosphere	4.98×10^{8} SD ± 4.93
	Endorhiza	8.32×10^{5} SD ± 7.00
	Shoot endosphere	2.35×10^{5} SD ± 1.39
	Leaf endosphere	3.81×10^{5} SD ± 2.88

In vitro screenings for direct PGP abilities

To select strains that promote plant growth directly, 54 bacterial strains originated from the rhizosphere of horseradish tree as well as selected isolates from rhizospheres of sorghum (two), sunflower (two), and safflower (one) that exhibited pronounced *in vitro* antagonism against fungal plant pathogens (as shown below) were screened for direct PGP abilities. Among all 59 tested strains, 19% were declared as N_2 fixers, 41% were assigned to be P solubilizers, 17% synthesized IAA, and 10% were able to degrade ACC (data not shown). Altogether, 66% of tested strains were declared positive for at least one PGP criterion, 14% for at least two criteria and three strains (5%) for three criteria, whereas no ´allround strain`

was observed that fulfilled all four direct PGP criteria (data not shown). Organisms, declared positive for at least two screening criteria are listed in Table 2.

Table 2: Selected rhizobacterial strains exhibiting pronounced *in vitro* PGP traits by fulfilling at least two of the four tested direct PGP criteria and/or by representing halos of growth inhibition of *Colletotrichum* sp. ≥ 5 mm in a dual culture assay. Shaded lines indicate strains that were selected for *ad planta* experiments

Isolate	Host plant species	Closest NCBI match for partial 16S rRNA gene sequences/% similarity	IAA synthesis[a]	P solubilization[b]	ACC deaminase activity[c]	Growth on nitrogen-free medium[d]	Sum of points for direct PGP criteria[e]	*In vitro* growth inhibition of *Colletotrichum* sp.[f]
M41	*Moringa oleifera*	*Agrobacterium tumefaciens*, *Rhizobium* sp./100	+	–	–	+	2	–
M56	*Moringa oleifera*	*Pectobacterium cypripedii*/99	+	+	–	+	3	–
M71	*Moringa oleifera*	*Ochrobactrum* sp./100	+	+	–	–	2	–
M72	*Moringa oleifera*	*Pantoea agglomerans*/99	+	+	–	+	3	–
M80	*Moringa oleifera*	*Bacillus* sp./100	+	–	–	+	2	–
M81	*Moringa oleifera*	*Pantoea agglomerans*/100	+	+	–	+	3	–
S18	*Sorghum vulgare*	*Burkholderia* sp./100	–	+	–	+	2	+++
S36	*Sorghum vulgare*	*Burkholderia vietnamiensis*/99	–	+	–	–	1	++
H9	*Helianthus annuus*	*Streptomyces* sp./100	–	+	–	–	1	++
H112	*Helianthus annuus*	*Bacillus subtilis*/100	–	–	+	–	1	+++
C54	*Carthamus tinctorius*	*Burkholderia vietnamiensis*/99	–	+	+	–	2	+++

a IAA synthesis: – represents no IAA synthesis, + represents IAA synthesis.
b P solubilization: – represents no P solubilization activity, + represents P solubilization activity.
c ACC deaminase activity: – represents no ACC deaminase activity, + represents ACC deaminase activity.
d Growth on nitrogen-free medium: – represents no pronounced growth on nitrogen-free medium, + represents pronounced growth on nitrogen-free medium.
e Sum of points for direct PGP criteria: for each fulfilled criterion for direct PGP (as indicated by +), one point was assigned to respective isolates and points were summed up subsequently.
f Zones of growth inhibition of *Colletotrichum* sp. in a dual culture assay: – no growth inhibition (GI), ++ 5 – 10 mm GI, +++ > 10 mm GI.

In vitro screenings for indirect PGP abilities

For selection of strains that potentially function as BCAs, bacterial strains isolated from plant derived microhabitats from sorghum (72), sunflower (92), and safflower (106) as well as six selected rhizosphere strains, derived from the horseradish tree rhizosphere, positively tested in at least two of the four aforementioned *in vitro* screening criteria for direct PGP, were analyzed for their capacity to inhibit growth of fungal pathogens *Colletotrichum* sp., *V. chlamydosporum* and *B. maydis*. The interaction of pathogens and their potential antagonists was analyzed according to their microhabitat origin (as described above). Conformable to that

screen, highest amount of rhizobacterial antagonists that showed at least minimal antagonistic activity were harboured in the rhizosphere of sorghum (12%), followed by 9% and 8% of antagonists in rhizospheres of sunflower and safflower (Table 3). While 4% of sorghum associated bacteria from the endorhiza exhibited growth inhibition of *Colletotrichum sp*., in the corresponding microhabitat of sunflower and safflower none strain was tested positively (Table 3). Activity towards the soil-borne pathogen was found for 2% of all tested rhizobacterial strains (listed in Table 2). Conspicuously, none of the bacterial isolates obtained from endospheres of stems and leaves were tested positively for antagonistic activity against *V. chlamydosporum* resp. *B. maydis* (Table 3).

Table 3: Amount of bacteria associated with different microhabitats and host plants showing at least minimal antagonistic activity *in vitro* against plant pathogenic *Colletotrichum* sp., *Verticillium chlamydosporum* or *Bipolaris maydis*

	Rhizosphere		Endorhiza		Stem endosphere		Leaf endosphere	
	∑ strains tested	% pos.	∑ strains tested	% pos.	∑ strains tested	% pos.	∑ strains tested	% pos
Sorghum vulgare								
Colletotrichum sp.	26	12	24	4				
Verticillium chlamydosporum					11	0		
Bipolaris maydis							11	0
Helianthus annuus								
Colletotrichum sp.	57	9	23	0				
Verticillium chlamydosporum					12	0		
Carthamus tinctorius								
Colletotrichum sp.	65	8	16	0				
Verticillium chlamydosporum					15	0		
Bipolaris maydis							10	0

Selection of most promising strains for *ad planta* studies

To select most promising strains for *ad planta* experiments, a point scheme based on direct PGP criteria was generated by the assignment of one point for each of the aforementioned positive fulfilled PGP criteria (max. four points) (by even *in vitro* antagonists). Thus a selection of isolated bacteria that putatively serve for plant growth and health was achieved (Table 2).

The isolates *Pectobacterium cypripedii* M56, *Pantoea agglomerans* M72, and *Pantoea agglomerans* M81, declared in Table 2 to that most points (three) in point scheme were ascribed, were selected for *ad planta* studies.

***Ad planta* experiments**

Effects of treatments with selected bacteria on number of beans per adult plant as an indicator for PGP and harvest yield were assessed and compared with the control treatment. Application of strain *P. agglomerans* M72 resulted in a maximum mean number of beans per plant (8), that was significantly (DMS, P <0.05) more than in case of fewest beans per plant (6) harbouring control plants (Fig. 1).

As an indicator for PGP and harvest yield for sunflower plants, diameters of flower heads were measured and compared with control plants. Application of strain *P. agglomerans* M72 resulted in the highest mean for flower head diameters (10.13 cm) that was significantly different (Duncan`s multiple range test, P<0.15) to control plants (9.72 cm) (Fig. 2). Lowest mean diameter of flower heads resulted from application of strain *P. cypripedii* M56 (9.66 cm) (Fig. 2).

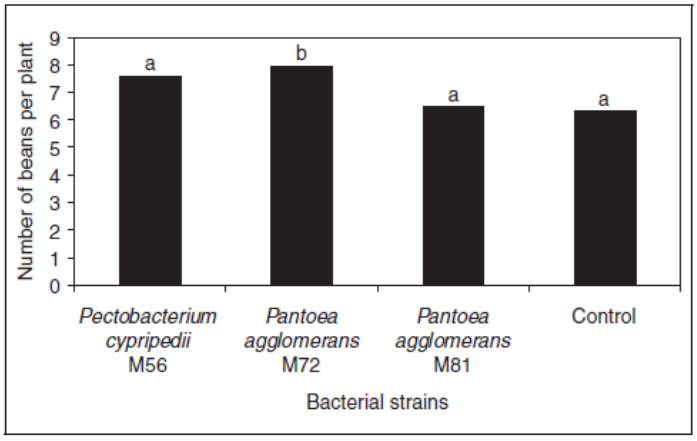

Fig. 1: Mean number of beans per black bean plant after treatment with bacterial strains selected from *in vitro* studies. Different letters signify significant differences between mean values (DMS, P<0.05).

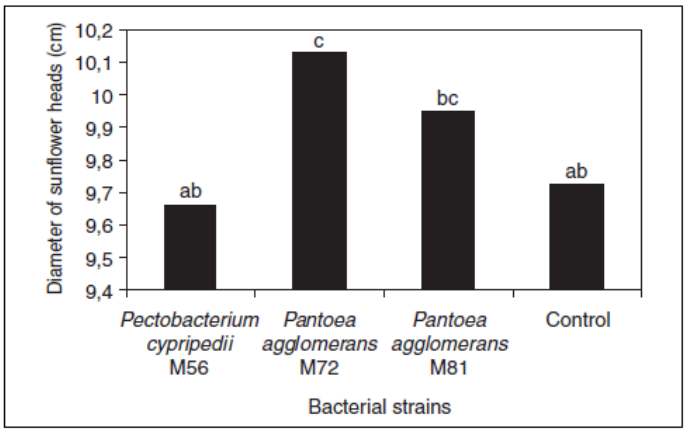

Fig. 2: Mean diameters of flower heads of sunflower plants treated with bacterial strains selected from *in vitro* studies. Different letters signify significant differences between mean values (Duncan`s multiple range test, P<0.15).

Partial 16S rRNA gene sequence analysis of selected bacterial strains exhibiting pronounced *in vitro* PGP capabilities

Potential PGPB were subjected to 16S rRNA gene sequence and BLAST analysis to obtain information about their taxonomic affiliation. The 11 strains could be assigned to eight different bacterial genera: *Agrobacterium*/*Rhizobium*, *Pectobacterium*, *Ochrobactrum*, *Pantoea*, *Bacillus*, *Burkholderia*, and *Streptomyces*. The genera *Bacillus* and *Pantoea* could always be assigned to two isolates, whereas the genus *Burkholderia* could be affiliated to three isolates. Partial 16S rDNA sequences showed at least 99% similarities to database entries.

ITS rDNA sequence analysis of fungal plant pathogens

To confirm identification of plant pathogenic *Colletotrichum* sp. and *B. maydis* based on morphological observations, their respective ITS rDNA sequences were subjected to BLAST analysis that revealed 100% similarities to *Colletotrichum* sp. resp. *Cochliobolus heterostrophus* (sexual stage of *B. maydis*).

Discussion

As microbial ecosystem, each plant harbours microorganisms which confer plant growth and health to their hosts (BERG et al. 2005; COMPANT et al. 2005; RAAJMAKERS et al. 2008). Results obtained by this study suggest that 66% of tested bacterial isolates, obtained from

rhizospheres of horseradish tree, sorghum, sunflower, and safflower in Bolivia, possess at least one of four tested properties (N_2 fixation, ACC deaminase activity, P solubilization, IAA synthesis) that are involved in PGP. This is in agreement with a comparative study investigating bacteria derived from the rhizosphere of soy bean and bulk soil, which revealed that 68% of screened isolates exhibited positive results for IAA synthesis, ACC deaminase activity or P solubilization (CATTELAN et al. 1999). Based on the fact that especially in the rhizosphere of the investigated plants extraordinary high abundances up to 10^{10} g^{-1} were found, a high indigenous plant growth promoting potential of microbial populations was determined. In comparison to the antagonistic potential in the rhizosphere, which is approximately 1/3 of the microbial populations (BERG et al. 2006), the potential to promote directly growth of observed host plants was higher.

By the use of a scheme to assess PGP traits *in vitro* of tested strains, a selection of promising candidates to support plant growth and health was obtained. Taxonomic analysis revealed that the majority of selected strains belong to *Pantoea, Bacillus, Burkholderia, Streptomyces*; bacterial genera that are well known as PGPB resp. BCAs (EL-SHANSHOURY 1989; KREBS et al. 1998; SESSITSCH et al. 2005; SERGEEVA et al. 2007). In contrast, *Pectobacterium cypripedii* is a novel discovered PGPB. It is the only non-phytopathogenic and non-pectinolytic species in the genus *Pectobacterium*. However, isolate M41 is phylogenetically highly related to *Agrobacterium tumefaciens*, which is a notorious phytopathogenic organism. This underlines the importance of an early taxonomic analysis of presumable BCAs or PGPB.

According to the results obtained *in vitro*, *Pectobacterium cypripedii* M56, *Pantoea agglomerans* M72, and *Pantoea agglomerans* M81, isolated from the rhizosphere of horseradish tree, were selected for *ad planta* studies. As the unique isolates they fulfilled three out of four assessed criteria for direct PGP. Most positive responses regarding number of beans per black bean plant and flower head diameters of sunflower plants, parameters that indicate plant growth and harvest yield as well, were noted from the application of strain *P. agglomerans* M72, as mean values for aforementioned parameters determined from plants inoculated with respective strain were always highest. The effect of *P. agglomerans* M72 on plant growth and harvest yield was more pronounced for the black bean experiment (DMS, $P<0.05$) than for the sunflower inoculation study (Duncan`s multiple range test, $P<0.15$) when compared to respective control treatments. Although the effect on PGP resp. harvest yield by

P. agglomerans M72 was less intensive in case of the sunflower field trial, *P. agglomerans* M72 may has the capability to serve as a PGPB for different agricultural plants by exploiting its full potential that could be assessed by dose-response assays (BONATERRA et al. 2003). The PGP effect of *P. agglomerans* M72 is not surprising as *P. agglomerans* strains are known for serving as PGPB (SERGEEVA et al. 2007).

By our knowledge this is the first report that deals with the analysis of bacteria for PGP that were associated with *M. oleifera*. The Moringa tree (that originates from India) belongs to the plant family of Moringaceae and is mainly used for oil production (technical purposes and cosmetic industry) and as vegetable. The synthesis of antimicrobial compounds like glucosinolates and isothiocyanates by horseradish tree is remarkable (FAHEY et al. 2001; KJAER et al. 1979). In other plant species with this defence system such as canola, a significant impact on its associated microbial community was shown (RUMBERGER and MARSCHNER 2003).

Due to the phenomenon of plant-driven selection of plant associated microbes (HARTMANN et al. 2009) it is not self-evident that a horseradish tree derived bacterial strain is able to establish itself in rhizospheres shaped by other host plants like black bean or sunflower. Although the rhizosphere competence, as a prerequisite for plant growth promoting rhizobacteria (BLOEMBERG and LUGTENBERG 2001), was not assessed for *P. agglomerans* M72, results obtained by this study suggest at least the effective colonization of the black bean rhizosphere by *P. agglomerans* M72 due to its significant reaction on that host plant. The soil used for the black bean inoculation survey, that was heavily exposed to solar radiation and might corresponded to approximated gnotobiotic conditions (as described above), may also be a reason for the stronger beneficial effect on black bean plants in comparison to sunflower plants by *P. agglomerans* M72.

Results obtained by *in vitro* screenings for the assessment of bacterial PGP cannot hundred percent reflect the reality e.g. at the field or in the greenhouse. Not all bacterial traits that confer plant growth can be determined *in vitro* as e.g. for induced systemic resistance. However, *in vitro* screenings for bacterial PGP can provide a tool to select strains out of the vast amount of bacteria living in plant associated habitats that ´fulfill *in situ* what they promise *in vitro*`.

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Additional publication II

Using ecological knowledge and molecular tools to develop effective and safe biocontrol strategies – Strategy to control a multi-species disease in the Styrian oil pumpkin

Michael Fürnkranz, Birgit Lukesch, Martin Grube and Gabriele Berg. Pesticides in the Modern World – Pests Control and Pesticides Exposure and Toxicity Assessment (2011) pp. 15-19. ISBN 978-953-307-457-3

Personal contribution: accomplishment of the underlying practical work at the field and in the laboratory, except FISH-CLSM analysis, and comprehensive preparation of the manuscript

Styrian oil pumpkin (*Cucurbita pepo* L. subsp. *pepo* var. *styriaca* Greb.) is a pumpkin variety that bears its name according to its origin of cultivation that is the Austrian district Styria. The specialty of this cultivar is the absence of a wooden seed shell that facilitates the production of pumpkin seed oil. Beside the culinary aspect of this dark green oil it is famous as a very healthy nutritional supplement containing high levels of polyunsaturated fatty acids, antioxidants, vitamins A, B1, B2, B6, C, D, E and counteracts diseases of bladder and prostate.

In recent years, dramatic yield losses of Styrian oil pumpkin were reported in Styria due to black rot of pumpkins caused by *Didymella bryoniae* (Auersw.) Rehm, anamorph *Phoma cucurbitacearum* (Fr.) (Huss et al., 2007). The ascomycete has a broad host range within the Cucurbitaceae and causes symptoms on vegetative plant parts known as gummy stem blight (Keinath et al., 1995). It spreads from temperate to tropical regions of the world (Sitterly & Keinath, 1996). Fruits, leaves and flower scars are invaded by the pathogen and it can also be seed-borne (Lee et al., 1984; Ling et al., 2010; de Neergaard, 1989; Sitterly & Keinath, 1996). By cultivation-independent SSCP fingerprinting of the fungal ribosomal internal transcribed spacer (ITS) region in combination with DNA sequencing and BLAST analysis (Altschul et al., 1997), it was detected as well in roots of oil pumpkin (data not

shown). This underlines the potential establishment of the pathogen even in soils (Bruton, 1998). The analysis of the phenotypic and genotypic variability of the pathogen across different oil pumpkin fields in Styria resulted in a remarkable high morphological versatility in contrast to a low genetic diversity (Zitzenbacher, pers. communication). Styrian oil pumpkins are also affected by bacterial pathogens *Pectobacterium carotovorum* subsp. *carotovorum* and subsp. *atrosepticum*, *Pseudomonas* spp. and *Xanthomonas cucurbitae* causing soft rot of pumpkins and leaf diseases (Huss, 2011). The transport of these bacterial phytopathogens by the fungus was observed *in vitro* (Zitzenbacher, pers. communication) suggesting synergistic interactions between them in the course of co-infections.

In order to manage microbial diseases of Styrian oil pumpkin based on autochthonous bacterial and fungal antagonists, initial studies to discover the microbial diversity associated with this host plant were conducted. Roots, female flowers and fruit pulp from three different oil pumpkin cultivars ("Gleisdorfer Ölkürbis", "Gleisdorfer Diamant" and "GL Maximal") at a field site in Styria were collected. Root samples were taken at three time points (before flowering, time of flowering, fruits well developed). Bacterial genera *Pseudomonas* and *Bacillus* that are known for their plant beneficial interactions (Haas & Défago, 2005) were analysed by SSCP analysis. Data revealed a greater impact of the microhabitat on community structure for *Pseudomonas*, whereas the plant stage had a stronger impact for *Bacillus* populations. Female flowers as possible gates for bacterial and fungal infections were analysed in more detail. For *Bacillus* and *Pseudomonas* and ascomycete communities, no effect of the plant cultivar on population structure was observed. However, in the flower, the communities are well-structured. FISH-CLSM studies revealed a dense bacterial colonisation of pollen grains that act as propagation vehicles between pistils especially for Alphaproteobacteria (Fig. 6) and shaped in this way the bacterial community structure of the oil pumpkin anthosphere.

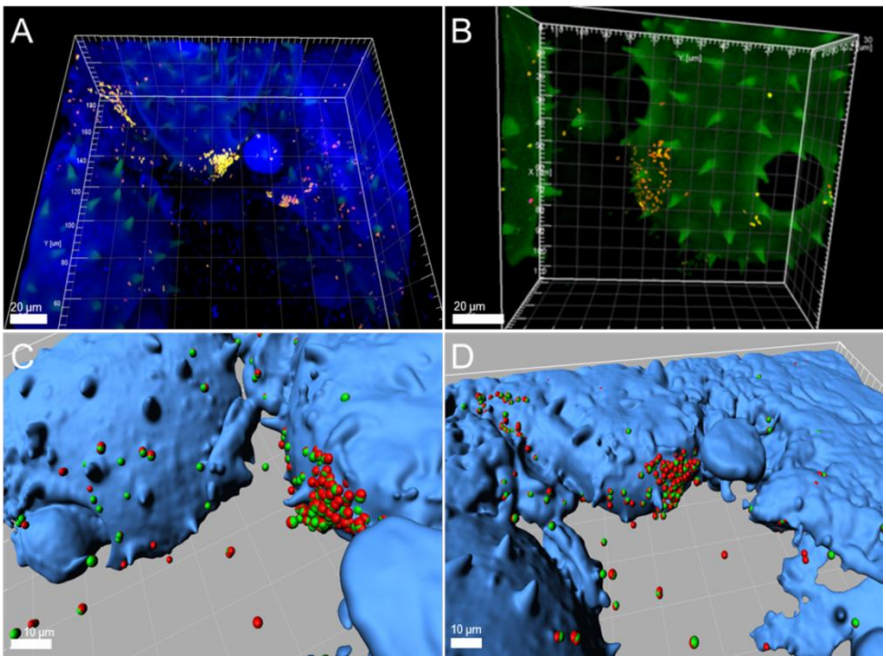

Fig. 6. FISH-stained bacteria colonising pollen grains located on pistils of oil pumpkin (GL Opal) visualized by CLSM. A) Alphaproteobacteria (in yellow) and not taxonomically classified bacteria (in red) labelled with ALF968-Cy5 and EUB338MIX-Cy3. B) Alphaproteobacteria labelled with ALF968-Cy5 (yellow), Firmicutes labelled with LGC354MIX-FITC (pink) and taxonomically undefined bacteria (in red) labelled with EUB338Mix-Cy3. C,D) 3D rendered image (Imaris software) of overall bacterial communities (in red) labelled with EUB338MIX-Cy3 and Alphaproteobacteria (red and green) labelled with ALF968-Cy5.

To obtain oil pumpkin-associated microorganisms for testing their antagonistic properties against *D. bryoniae* and bacterial pathogens, bacterial and fungal strains were isolated from oil pumpkin cultivars and microhabitats as described above. Endophytes were cultivated from roots and fruit pulp. In addition, seed borne microbial strains were obtained from aforementioned varieties by the isolation from roots, stems and leaves from plants that seeds were surface sterilized and grown under gnotobiotic conditions. Finally 2320 isolates (1748 bacteria and 572 fungi) were subjected to dual culture assays against *D. bryoniae* A-220-2b to test their antagonistic potential against this pathogen. Of tested bacteria, 7.3% inhibited growth, whereas 12.4% of observed fungi showed either growth inhibition or overgrowth of *D. bryoniae* (Fig. 7).

Potential antagonists (128 bacteria and 71 fungi) were subsequently screened *in vitro* for effects on growth inhibition of *Pectobacterium carotovorum* subsp. *atrosepticum* 25-2, *Pseudomonas viridiflava* 2d1 and *Xanthomonas cucurbitae* 6h4 to find broad-spectrum

antagonists. Altogether, 32% of fungal as well as 49% of bacterial *D. bryoniae* antagonists were positively tested against at least one, 34% of tested prokaryotes against at least two and 6% of investigated bacterial strains against all three bacterial phytopathogens, whereas no fungal *D. bryoniae* antagonist was effective against more than one bacterial pathogen (Fig. 8).

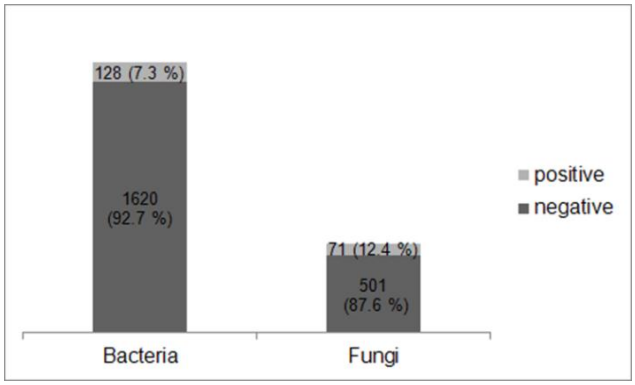

Fig. 7. Amount of oil pumpkin-associated bacterial and fungal isolates positively or negatively tested for *in vitro* antagonism against *D. bryoniae* A-220-2b.

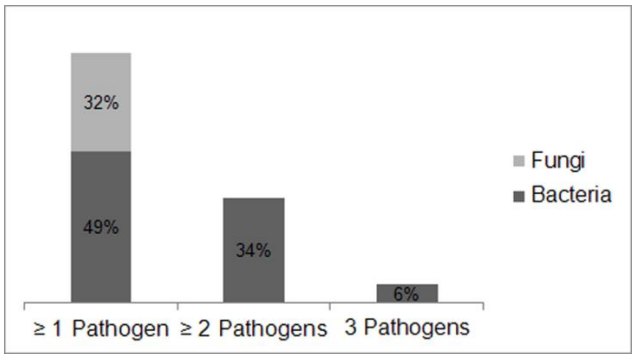

Fig. 8. Percentage of fungal and bacterial *D. bryoniae* antagonists positively tested against at least one, two or all three bacterial pathogens *Pectobacterium carotovorum* subsp. *atrosepticum* 25-2, *Pseudomonas viridiflava* 2d1 *and Xanthomonas cucurbitae* 6h4.

Broad-spectrum antagonists that have the potential to suppress *D. bryoniae* as well as at least two bacterial phytopathogens of oil pumpkin were characterized genotypically by ARDRA. This resulted in a grouping of 43 bacterial isolates into four different genera: *Pseudomonas*, *Paenibacillus*, *Serratia* and *Lysobacter*. As a relative high number of isolates belong to *Paenibacillus* and *Lysobacter* they were further analysed by BOX PCR (Rademaker & de Bruijn, 1997) to get insight into the intra-genera diversities. Within the group of *Paenibacillus* a negligible variability between BOX patterns was observed in contrast to strains of *Lysobacter* that were divided into five groups. Finally five potential broad-spectrum

antagonists were chosen for further analysis: one representative for *Pseudomonas*, *Paenibacillus* and *Serratia* and two representatives from the *Lysobacter* cluster. Partial sequencing of 16S rRNA genes with subsequent BLAST analysis (Altschul et al., 1997) was performed for their identification and the following species could be affiliated to respective strains: *Pseudomonas chlororaphis* P34, *Paenibacillus polymyxa* PB71, *Serratia plymuthica* S13, *Lysobacter antibioticus* L175 and *L. gummosus* L101.

To learn more about the mode of antagonism of chosen broad-spectrum antagonists against *D. bryoniae*, dual culture assays in which growth inhibition of *D. bryoniae* A-220-2b by either soluble or volatile antimicrobial compounds secreted by the five test strains was assessed were performed. Results suggest a high capability of broad-spectrum antagonists to synthesize bioactive compounds: sterile culture supernatants from *P. chlororaphis* P34, *L. gummosus* L101 and *P. polymyxa* PB71 as well as volatile organic compounds (VOCs) excreted from these bacteria and *S. plymuthica* S13 as well suppressed growth of the fungus significantly compared to control treatments (ANOVA; LSD, $p < 0.05$; data not shown).

Performances of broad-spectrum antagonists in terms of promoting plant growth and health will facilitate the selection of bacterial strains that will be analysed for the production of a biological strengthener for Styrian oil pumpkin. Studies with the model organism *C. elegans* (Zachow et al., 2009) will give insight into the potential pathogenicity of remaining test strains. The manufacture of the final product will further depend on the finding of an appropriate formulation procedure that guarantees a high stability of the ultimate BCAs/PGPR.

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Additional publication III

Multi-pathogen disease caused by *Didymella bryoniae* and bacteria on Styrian oil pumpkin: microbial ecology and biocontrol

Michael Fürnkranz, Eveline Adam, Birgit Lukesch, Henry Müller, Martin Grube and Gabriele Berg. Conference proceeding: 2nd Asian PGPR Conference, Beijing, 21st – 23rd of August 2011

Personal contribution: accomplishment of the underlying practical work in the laboratory, except FISH-CLSM analysis, and at the field, assistance during the field trial, and comprehensive preparation of the manuscript

Abstract

Styrian oil pumpkin, *Cucurbita pepo* L. subsp. *pepo* var. *styriaca* Greb., is a crop of cultural, medical and commercial importance in Austria but also in other regions of the world, e.g. in China. In the recent years, fruit rot and black rot caused by the ascomycete *Didymella bryoniae* (Fuckel) Rehm led to dramatic yield losses in Styria. In the field, the fungal disease was usually associated with characteristic symptoms of bacteriosis. Bacterial pathogens include *Pectobacterium carotovorum*, *Pseudomonas viridiflava*, *Pseudomonas syringae* and *Xanthomonas cucurbitae*. The high coincidence of fungal and bacterial disease suggests mutualistic effects in pathogenesis. In this study, Styrian oil pumpkin-associated microbial communities with focus on bacterial endophytes were analyzed by microbial fingerprints performed by PCR-Single Strand Conformation Polymorphism analysis (SSCP) and Fluorescence *in situ* hybridisation (FISH) in combination with confocal laser scanning microscopy. Computer-assisted comparisons of community profiles revealed microhabitat-dependent community structures for *Pseudomonas*, whereas *Bacillus* communities were more influenced by the plant development stage. By cultivation dependent methods, the fraction of Styrian oil pumpkin inhabiting in vitro antagonists against *D. bryoniae* and bacterial pathogens was determined: 9% (= 199 strains) of bacterial and fungal isolates showed an antagonistic potential against the fungus. From these in vitro *Didymella* antagonists, 43 bacterial strains inhibited growth of at least two of the three tested bacterial pathogens. Based

on genotypic characterization of these isolates, five potential broad-spectrum antagonists were identified: strains of *Lysobacter* spp., *Pseudomonas chlororaphis*, *Paenibacillus polymyxa* and *Serratia plymuthica*. They were successfully evaluated in field trials. On their basis, a biological product to protect the Styrian oil pumpkin against multi-pathogen disease is currently under development.

Introduction

Styrian oil pumpkin (*Cucurbita pepo* L. subsp. *pepo* var. *styriaca* Greb.) is a pumpkin variety that bears its name according to its origin of cultivation that is the Austrian district Styria. The specialty of this cultivar is the absence of a wooden seed shell that facilitates the production of pumpkin seed oil. Beside the culinary aspect of this dark green oil it is famous as a very healthy nutritional supplement containing high levels of polyunsaturated fatty acids, antioxidants, vitamins A, B1, B2, B6, C, D, E and counteracts diseases of bladder and prostate. Nowadays, Styrian oil pumpkin is mainly cultivated in China and Russia; however it is also economically and culturally important for Styria and Austria.

In recent years, dramatic yield losses of Styrian oil pumpkin were reported in Styria due to black rot of pumpkins caused by *Didymella bryoniae* (Auersw.) Rehm, [anamorph *Phoma cucurbitacearum* (Fr.)] (Huss et al., 2007). The ascomycete has a broad host range within the Cucurbitaceae and causes symptoms on vegetative plant parts as well that are known as gummy stem blight (Keinath et al., 1995). Styrian oil pumpkins are also affected by bacterial pathogens *Pectobacterium carotovorum*, *Pseudomonas* spp. and *Xanthomonas cucurbitae* causing soft rot of pumpkins and leaf diseases (Huss, 2011).

To understand the structure and function of pumpkin-associated microorganisms, endophytic microbial communities from roots-, fruits-, and flower-associated microfloras from three different pumpkin cultivars were analyzed by a multiphasic approach based on microbial fingerprints of 16S rRNA genes, FISH-CLSM studies and cultivation-dependent methods to obtain oil pumpkin associated microbial antagonists against *D. bryoniae* and bacterial pathogens. Selected antagonists were tested for their efficacy under practical conditions at the field and are subjected to the development of a biological product for Styrian oil pumpkin.

Materials & Methods

Experimental design and sampling

Plant samples of roots, flowers and fruit pulp from three different oil pumpkin cultivars, cv. Gleisdorfer Ölkürbis, Gleisdorfer Diamant and GL Maximal, from always 4 different individual plants from always 4 different sites at a field located in Gleisdorf, Austria, were collected. Roots were washed with tap water and then surface sterilized in 0.54 M NaOCl for 5 min. and subsequently washed three times with sterile water. The different plant materials were homogenized in sterile 0.15 M NaCl with mortar and pestle. For microscopic analysis of the female oil pumpkin flower, samples were taken from petals and pistils from the oil pumpkin cultivar GL Opal.

Total community DNA isolation and fingerprint analysis

Suspensions of homogenized plant parts (as described above) were centrifuged for 20 min. at 10.000 × g. From corresponding pellets DNA was extracted using the FastDNA®Spin Kit for Soil. Bacterial fingerprints of *Pseudomonas* and *Bacillus* from roots, flowers and fruits were analyzed by single-strand conformation polymorphism (SSCP) analysis (Schwieger and Tebbe 1998). 16S rRNA genes from *Pseudomonas* and *Bacillus* were amplified using Taq-&GOTM PCR Mastermix (Qbiogen BIO 101 Systems, Carlsbad, USA) by a nested PCR design: for the first amplification of *Pseudomonas* we used 0.4 µM of primers F311 Ps (5‘-CTG GTC TGA GAG GAT GAT CAG T-3‘) and 1459 rPs (5‘-AAT CAC TCC GTG GTA ACC GT-3‘), $MgCl_2$ (1.5 µM) and 1 µl of DNA in a 20 µl reaction mix. PCR conditions were: initial denaturation at 95℃ for 5 min., followed by 26 cycles consisting of 95℃ for 20 s, 63℃ for 30 s and 72℃ for 60 s, and a final elongation step at 72℃ for 10 min. Obtained PCR products were applied in a nested PCR reaction. For that we used Unibac-II-515f/phosphorylated Unibac-II-927r as primers (Lieber et al., 2002). For *Bacillus*-specific PCR we used 0.5 µM of primers Bspez 3f (5‘-AGA CTG GGA TAA CTC CG-3‘) and BACr833 (5‘-CTA ACA CTT AGC ACT CAT-3‘) and 1 µl of DNA in a 20 µl reaction mix. For the semi-nested PCR we used 0.5 µM of primers Bspez 6f (5‘-CGA CCT GAG AGG GT-3‘) and phosphorylated BACr833. Conditions for both PCRs were: 95℃ for 5 min. initial denaturation, 30 cycles of 95℃ for 45 s, 54℃ for 30 s, 72℃ for 45 s and final elongation at

72℃for 10 min. PCR products were purfied with the peqGOLD MicroSpin Cycle Pure Kit and DNA fragments were separated with a TGGE Maxi apparatus at 400 V and 26℃. Silver staining of gels was applied for visualization of the bands (Bassam et al., 1991).

Microscopic analysis of bacterial communities on the female flower

Samples of flowers parts were fixed in paraformaldehyde and then used for fluorescence in situ hybridization by applying the following probes: EUB338MIX (Cy3 labelled) for detection of the overall bacterial community, GAM42a (Cy5 labelled) for sensing Gammaproteobacteria, ALF968 (Cy5 labelled) for visualization of, Alphaproteobacteria, BET42a (6-FAM labelled) for detection of Betaproteobacteria and LGC354MIX (FITC labelled) for visualization of Firmicutes. Stained samples were analysed with a Leica TCS SP confocal laser-scanning microscope (Leica Microsystems, Wetzlar, Germany) equipped with argon and helium/neon lasers.

Isolation of oil pumpkin associated microorganisms

Dilution series with obtained suspensions from plant samples (as described above) were prepared and 0.1 mls of the dilutions were plated out onto R2A (for bacteria) and SNA (for fungi) medium. Agar plates were incubated at 20℃ until colonies were countable that were then transferred onto LB (for bacteria) and PDA (for fungi) plates after assessment of CFUs. In addition, seed borne microbial strains were obtained from aforementioned varieties by the isolation from roots, stems and leaves from plants that seeds were surface sterilized and grown under gnotobiotic conditions.

Selection, characterization and identification of antagonists

The pathogens *Didymella bryoniae* A-220-2b, *Pectobacterium carotovorum* subsp. *atrosepticum* 25-2, *Pseudomonas viridiflava* 2d1 and *Xanthomonas cucurbitae* 6h4 were provided by Herbert Huss (Bundesanstalt für alpenländische Landwirtschaft, Gumpenstein, Austria) and Athanassios Mavridis (University of Göttingen, Germany).

Oil pumpkin derived microbial isolates (1,748 bacteria and 572 fungi) were characterized in vitro in dual culture assays. Broad-spectrum antagonists that had the potential to suppress *D. bryoniae* as well as at least 2 bacterial phytopathogens of oil pumpkin were

characterized genotypically by Amplified Ribosomal DNA Restriction Analysis (ARDRA) and BOX PCR (Rademaker & de Bruijn, 1997). Selected isolates were identified by partial sequencing of 16S rRNA genes and BLAST analysis (Altschul et al., 1997).

Evaluation of broad-spectrum antagonists in a field trial

Seeds of the variety GL Opal were primed with suspensions of 5 selected broad-spectrum antagonists separately. Additionally a control treatment was performed with 0.15 M NaCl. 16 seeds per treatment were sown on always three replicate plots that were organized in a complete randomized plot design. Health statuses of plants were monitored and furthermore harvest yields and 100-corn weights were evaluated.

Statistical analysis

Band patterns from ARDRA and BOX-PCR gels were normalized and subjected to cluster analysis based on the unweighted pair group method using average linkages to the matrix of similarities obtained (UPGMA) using the Gel ComparII software (Version 5.1, Applied Maths). Analysis of Variance (ANOVA) in addition with Duncan‘s multiple range test ($P<0.1$) was performed with Predictive Analysis Software (PASW, Version 18.0.0) for evaluating data obtained from the field experiment.

Results and Discussion

Oil pumpkin associated community structures of *Pseudomonas* and *Bacillus*

To observe community structures of *Pseudomonas* and *Bacillus* from oil pumpkin, SSCP profiles were generated from specific plant organs (roots, flowers, fruit pulp). Investigated communities were strongly influenced by the microhabitat and by the plant stage. No significant effect on microbial communities by the oil pumpkin cultivar was noticed.

FISH-CLSM analysis

The female oil pumpkin flower as an underexplored and a spatially very heterogeneous microhabitat offering various niches for bacterial colonization was analysed in more detail by FISH-CLSM studies. Beta- and Gammaproteobacteria were observed on petals and pistils.

Furthermore, pollen grains were mainly colonized by Gammaproteobacteria. This result suggest pollen acts as a dissemination vehicle for bacteria between plants and shape by this means the flower- and pumpkin-associated bacterial community of oil pumpkin.

Selection, characterization and identification of broad-spectrum antagonists

Broad-spectrum antagonists, which have the potential to suppress *D. bryoniae* as well as at least two bacterial phytopathogens, were characterized genotypically by ARDRA. This resulted in a grouping of 43 bacterial isolates into four different genera: *Pseudomonas*, *Paenibacillus*, *Serratia* and *Lysobacter*. As a relative high number of isolates belonged to *Paenibacillus* and *Lysobacter* they were further analysed by BOX PCR to get insight into the intraspecific diversities. Within the group of *Paenibacillus*, a negligible variability between BOX patterns was observed in contrast to strains of *Lysobacter* which were divided into five groups. Finally, five potential broad-spectrum antagonists were chosen for further analysis: one representative for *Pseudomonas*, *Paenibacillus* and *Serratia*, and representatives for two *Lysobacter* clusters. Partial sequencing of 16S rRNA genes with subsequent BLAST analysis was performed for their identification, and the following species could be affiliated to respective strains: *Pseudomonas chlororaphis* P34, *Paenibacillus polymyxa* PB71, *Serratia plymuthica* S13, *Lysobacter antibioticus* L175 and *L. gummosus* L101.

Evaluation of broad-spectrum antagonists *ad planta*

Selected broad-spectrum antagonists were tested in a field study for their antagonistic efficacy against *D. bryoniae* and their impact on plant health and growth of the Styrian oil pumpkin cultivar GL Opal. Germination rate was highest for *S. plymuthica* S13 (95.8%), and was even higher than seed emergence by a chemical stripper. Germination rates observed for treatments with *L. gummosus* L101 and *P. chlororaphis* P34 (91.7%) were significantly higher compared to the control treatment as well. As an important parameter for the production of pumpkin seed oil, harvest yields obtained from 100 seeds were compared that revealed significant highest values after treatment with *P. polymyxa* PB71. Due to an extensive infestation by mildew, degree of leaf area covered with the pathogen was assessed and compared between treatments: *P. polymyxa* PB71 and *L. gummosus* L101 suppressed this pathogenic pressure significantly.

In further field studies, the selection of bacterial strains will be evaluated as single or combined applications for the development of a biological strengthener for Styrian oil pumpkin.

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Additional publication IV

Analysis of the antagonistic potential of microorganisms derived from the Styrian oil pumpkin against its pathogens

Michael Fürnkranz, Sabine Zitzenbacher, Martina Köberl, Henry Müller, Martin Grube and Gabriele Berg. IOBC-Bulletin 2010

Personal contribution: accomplishment of the underlying practical work at the field and in the laboratory, and comprehensive preparation of the manuscript

Abstract

Black rot and gummy stem blight, caused by *Didymella bryoniae* (Fuckel) Rehm, led to high yield losses of Styrian oil pumpkins (*Cucurbita pepo* var. *styriaca*) in recent years. Beside infections with this ascomycete, pumpkin plants are frequently co-infected by the bacterial pathogens *Pectobacterium carotovorum*, *Pseudomonas viridiflava* and *Xanthomonas cucurbitae*. To obtain insight into the antagonistic potential of oil pumpkin associated microorganisms, bacteria and fungi from different oil pumpkin varieties and microenvironments (with focus on endophytes and including seed-borne strains) were isolated and subjected to hierarchical dual culture screenings against the addressed oil pumpkin pathogens. Altogether, 200 (9 %) of 2,321 isolated oil pumpkin associated microorganisms showed an antagonistic potential against *D. bryoniae in vitro*. Out of 160 selected *in vitro* antagonists of *D. bryoniae* 46%, 28% resp.5% exhibited growth inhibition against at least one, two, resp. all three mentioned bacterial oil pumpkin pathogens. Potential broad spectrum antagonists were genotypically characterized and will be assessed in *ad planta* experiments on Styrian oil pumpkin.

Introduction

Cultivation of Styrian oil pumpkin (*Cucurbita pepo* var. *styriaca*) is an important economical factor for Styria (Austria). Further, "Styrian pumpkin oil" is an approved designation of origin in Europe and of high cultural value for the region.

Since 2004 increased crop yield losses of oil pumpkins have been recorded in Styria (Huss, 2007). These losses were primarily attributed to black rot which is caused by the ascomyceteous fungus *Didymella bryoniae* (Fuckel) Rehm. Additionally, oil pumpkins in Styria can be co-infected by bacteria, which were identified as *Pectobacterium carotovorum*, causing bacterial soft rot (Huss, 2009). Beside fruit rotting plant pathogens can cause leaf diseases on Styrian oil pumpkin plants: *Pseudomonas viridiflava*, *Xanthomonas cucurbitae* and *D. bryoniae* as well can cause typical symptoms on leafs and leaf stalks (Huss, 2007). As pumpkin rot represents a multipathogen syndrome, sustainable pest management is a particular challenge.

As no permitted pesticides are available in Austria and a high proportion is cultivated organically, the objective of our work is to develop a biological plant strengthener/protection product for the Styrian oil pumpkin that is based on autochthonous endophytic microorganisms with antagonistic properties. Therefore, hierarchical *in vitro* screenings for the assessment of the antagonistic potential of oil pumpkin associated microbial isolates against oil pumpkin pathogens were conducted.

Materials and methods

Oil pumpkin pathogens

Didymella bryoniae A-220-2b, *Pectobacterium carotovorum* ssp. *atrosepticum* 25-2, *Pseudomonas viridiflava* 2d1 and *Xanthomonas cucurbitae* 6h4 were provided by Dr. Herbert Huss (Bundesanstalt für alpenländische Landwirtschaft, Gumpenstein, Austria) and Dr. Athanassios Mavridis (University of Göttingen, Germany).

Isolation of microorganisms from field plants

Plant samples were collected from a field located in Gleisdorf, Austria. Samples were taken from three different oil pumpkin cultivars, cv. Gleisdorfer Ölkürbis, Gleisdorfer Diamant and Gleisdorfer Maximal, and from 4 different sites for each variety. Furthermore, samples were taken at three time points in 2009: 16th of June (before flowering), 15th of July (time of flowering) and 26th of August (fruits well developed). Roots were collected at all three time points, female flowers at the 2nd time point and pulp at the 3rd time point whereas plant material of always 4 different individual plants per site was combined for one sample (except

for root sampling at the 3rd time point: one sample was composed from material of one plant per site).

Roots were washed with tap water and then surface sterilized in 4 % NaOCl for 5 min. and subsequently washed three times with sterile water. Roots were dried on filter paper and weighted. Flowers were not surface sterilized (weighted directly) and pulp was cut out of the inner fruit under aseptic conditions and weighted. The different plant materials were homogenized in sterile 0.85 % NaCl with mortar and pestle. Dilution series with obtained suspensions were prepared and 0.1 mls of the dilutions were plated out onto R2A (for bacteria) resp. SNA (for fungi) medium. Agar plates were incubated at 20°C until colonies were countable and transferred onto LB (for bacteria) resp. PDA (for fungi) plates after assessment of CFUs.

Isolation of microorganisms from plants grown under gnotobiotic conditions

Plant agar based approach: Seeds of the three cultivars were washed under tap water, treated with 3.5 % NaOCl for 3 min. and washed three times with sterile water. Afterwards seed coats were peeled of and seeds were pre-germinated in humid chambers. When the root tips had developed the seedlings were transferred into sterile Gamborg B5 medium (3.05 g/l) supplemented with plant agar (5 g/l). From plants that were grown under sterile conditions until the two-leaf-stage roots, stems and leaves were used to isolate microorganisms.

Perlite based approach: Seed coats of seeds from the three different pumpkin varieties were removed (as described above) and placed into sterile perlite that was drenched with sterile Gamborg B5 medium. From plants that were grown under sterile conditions until the 4-leaf-stage roots, stems and leaves were used to isolate microorganisms (as described above, without surface sterilization).

Dual culture assays with isolated microorganisms and *D. bryoniae*

Bacterial isolates were streaked out on nutrient agar (5 g trypton, 3 g meat extract, 15 g agar per l) and pieces of *D. bryoniae* were placed on these plates as well. Pieces of fungal isolates were put on PDA plates together with *D. bryoniae*. Plates were incubated at room temperature and monitored until presence or absence of growth inhibition zones (≥ 1cm in size for

bacteria) of *D. bryoniae* was assessable. Growth of fungal isolates over *D. bryoniae* was scored as antagonistic property as well.

Dual culture assays with potential antagonists against *D. bryoniae* and bacterial oil pumpkin pathogens

Fungi tested positively against *D. bryoniae* were divided into morphotypes and one isolate per morphotype and corresponding oil pumpkin variety resp. per plant organ (root, stem, leaf) in case of isolates grown under gnotobiotic conditions was tested against the bacterial pathogens. *P. carotovorum* ssp. *atrosepticum* 25-2, *P. viridiflava* 2d1 and *X. cucurbitae* 6h4 were grown O/N in TSB (at 30°C, 120 rpm). Bacterial suspensions were mixed with 1.2 % LB agar (for *P. carotovorum* ssp. *atrosepticum* 25-2, *P. viridiflava* 2d1), nutrient agar (containing 1.2 % agar, for *X. cucurbitae* 6h4) resp. 1.2 % PDA (for testing fungal antagonists) in a ratio 1:3. Subsequently bacterial antagonists were streaked out on that plates whereas pieces of antagonistic fungi were placed on them and then plates were incubated for three days at room temperature (for bacterial antagonists) resp. 5 days at 20 °C (for fungal antagonists). After incubation time presence or absence of clear zones (due growth inhibition of pathogens) surrounding the antagonists was assessed.

Results and discussion

Antagonistic potential of oil pumpkin associated microbes against *D. bryoniae*

The amount of fungal antagonists against *D. bryoniae* (13 %) showing growth inhibition of the pathogen due to the formation of inhibition zones or the overgrowth of the fungus was higher than the amount of bacterial antagonists (7 %) leading to inhibition zone formation (Fig. 1). Nevertheless the proportion of fungal isolates leading only to a growth inhibition zone of *D. bryoniae* was lower (6.6 %) in comparison to bacteria (7.3 %) (data not shown).

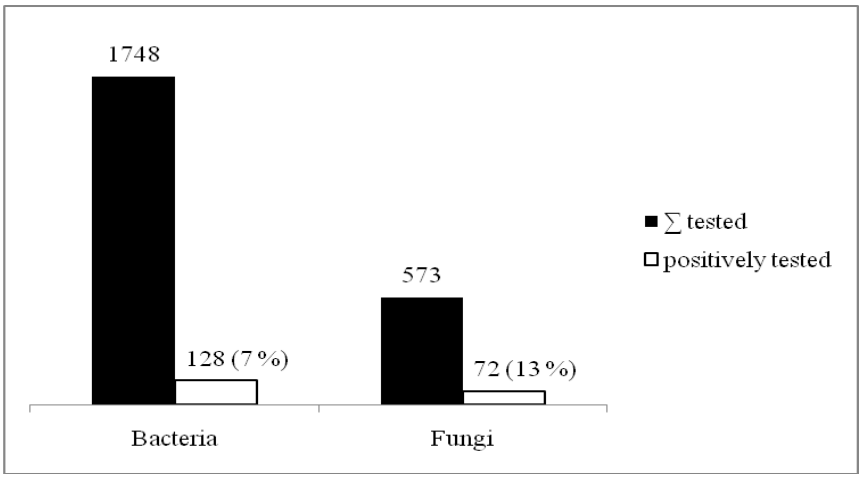

Figure 1. Number of positively tested bacteria and fungi against *D. bryoniae* in dual culture

Antagonistic capacity of potential *D. bryonia* antagonists against *P. viridiflava* 2d1, *P. carotovorum* subsp. *atrosepticum* 25-2 and *X. cucurbitae* 6h4

Altogether, 49 % resp. 34 % of tested bacteria resp. fungi showed antagonistic activity against at least one of the bacterial pathogens tested. 34 % resp. 3 % of analyzed prokaryotic resp. eukaryotic isolates exhibited growth inhibition of at least two of the observed strains. 6 % of obtained bacterial *D. bryoniae* antagonists inhibited growth of all 3 pathogenic isolates whereas none of the tested fungi demonstrated this broad spectrum antagonistic capacity (Fig. 2).

38 % of potential *D. bryoniae* biocontrol strains inhibited growth of *P. viridiflava* 2d1 followed by 26 % that showed antagonism against *X. cucurbitae* 6h4 and 15 % of tested bacteria and fungi had an effect on growth of *P. carotovorum* subsp. *atrosepticum* 25-2 (data not shown).

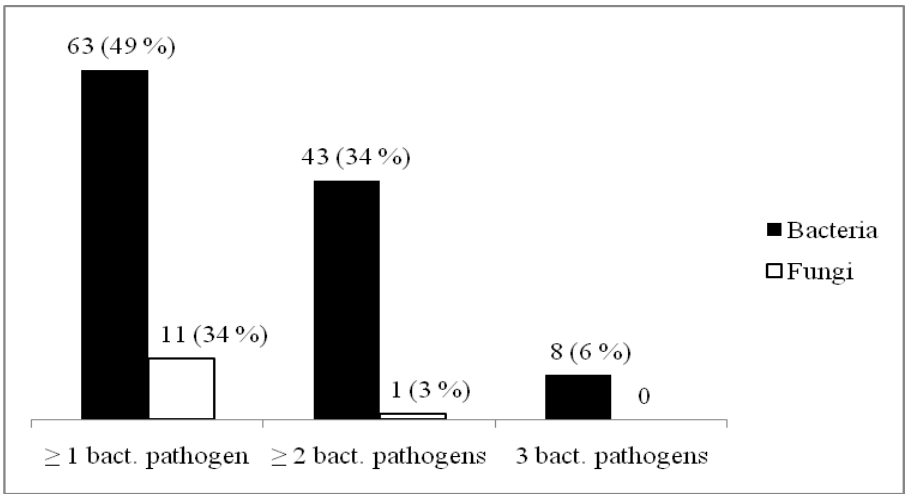

Figure 2. Number of positively tested bacteria and fungi against at least one, two or all 3 tested bacterial oil pumpkin pathogens (*P. viridiflava* 2d1, *P. carotovorum* subsp. *atrosepticum* 25-2 and *X. cucurbitae* 6h4)

The amount of plant associated antagonistic microbes discovered in this study is in an expectable range (Berg et al., 2006; Fürnkranz et al., 2009). Our results indicate that microbial oil pumpkin microhabitats are a source for promising antagonists against *D. bryoniae* and phytopathogenic bacteria. Selected isolates will be identified and tested for their activity as inoculums for the Styrian oil pumpkin.

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Publication list

Book chapter

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Oral presentations

Untersuchungen zur Bekämpfung der Fruchtfäule und Bakteriosen des Steirischen Ölkürbis. Fürnkranz et al. Meeting of the project partners, Graz, 1st of October 2009, 14th of October 2010, 14th of November 2011.

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Investigations for suppression of fruit rot and bacterioses in Styrian oil pumpkin. Fürnkranz et al. Seminar, Graz, 1st of October 2010.

Endophytes of oil pumpkin. Fürnkranz & Lukesch. Seminar, Graz, 21st of June 2011.

Multi-pathogen disease caused by *Didymella bryoniae* and bacteria on Styrian oil pumpkin: microbial ecology and biocontrol. Fürnkranz et al. 6th DocDay, Graz, 15th of July 2011.

Multi-pathogen disease caused by *Didymella bryoniae* and bacteria on Styrian oil pumpkin: microbial ecology and biocontrol. Fürnkranz et al. 2nd Asian PGPR Conference, Beijing, 21st – 23rd of August 2011.

Multi-pathogen disease caused by *Didymella bryoniae* and bacteria on Styrian oil pumpkin: microbial ecology and biocontrol. Fürnkranz et al. 14th International Alpe-Adria Biosymposium, Maribor, 15th – 19th of November 2011.

Poster presentations

Didymella bryoniae: characterization of a new pathogen and its interactions on the Styrian oil pumpkin. Fürnkranz et al. DocDay, Graz, 9th of July 2010. IMC9-Conference, Edinburgh, 1st - 6th of August 2010.

Statutory declaration

I declare that I have authored this thesis independently, that I have not used other than the declared sources/resources, and that I have explicitly marked all material which has been quoted either literally or by content from the used sources.

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