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REGULAR ARTICLE

Kill or cure? The interaction between endophytic *Paenibacillus* and *Serratia* strains and the host plant is shaped by plant growth conditions

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Abstract
Aims Verticillium wilt is difficult to suppress, and causes severe yield losses in a broad range of crops. Five *Serratia* and five *Paenibacillus* endophytic isolates showing antagonistic properties against fungal pathogens were compared for their plant growth-promoting (PGP) potential under different plant growth conditions with the objective of evaluating the PGP of endophytic strains in different *ad planta* systems.
Methods Preselected isolates were applied to the surface-sterilized seeds of oilseed rape and cauliflower using bio-priming. The isolates' PGP effect and root colonization capacities were compared under gnotobiotic conditions. One strain from each genus was selected and tested for its PGP qualities in sterile and non-sterile soil.
Results *Serratia* treatment resulted in different levels of PGP, while *Paenibacillus* strains damaged roots under gnotobiotic conditions. *P. polymyxa* Sb3-1 did not have a significant effect on plant growth in non-sterile soil; however it did promote plant growth in the sterile soil. *S. plymuthica* 3RP8 and *P. polymyxa* Sb3-1 were selected for further testing of their biocontrol effect under field conditions.
Conclusions The choice of growth environments in the investigation of plant-bacterium interaction is crucial. Non-sterile soil is suggested as the ideal medium for use in studying the PGP effect.

Keywords Biocontrol · Bio-priming · Plant growth promotion · PGP · BIOCOMES · *Brassica* · *Serratia* · *Paenibacillus* · Verticillium wilt

Introduction

Verticillium spp. induce vascular wilting corresponding with high yield losses within a wide range of dicotyledonous plants, including economically important field crops such as oilseed rape (*Brassica napus* L.) and vegetables like cauliflower (*Brassica oleracea* L.) (Debode et al. 2005; Zhou et al. 2006; Dunker et al. 2008). Verticillium wilt caused by *Verticillium dahliae* Kleb. and *Verticillium longisporum* is a severe soil-borne plant disease with no fungicidal control available to date. Due to the genetically heterogeneous and polyphyletic character of *Verticillium* isolates as well as its ecological behavior, the fungus is one of the most challenging phytopathogens to control (Jiménez-Gasco et al. 2014). The Verticillium wilt disease incidents are predicted to increase in future mainly due to decrease in crop rotation time and global warming (Heale and Karapapa 1999; Siebold and Tiedemann 2012). The current trend in plant disease control goes towards

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sustainable and environmentally friendly agriculture (directive of the European parliament and of the council 2009/128/EC), so that biological control of Verticillium wilt is especially desirable. Beneficial bacteria have been intensively studied as biocontrol agents against soil-borne diseases including *Verticillium* (Handelsman and Stabb 1996; Weller 1988). They are known not only to promote plant growth and improve soil health, they also induce resistance in plants against pathogens and restrict them from reaching plant roots (Berg 2009). However, inconsistent effects under field conditions are a hurdle in commercialization of many interesting biocontrol systems (Berg et al. 2013). Although rarely reported in literature, biological treatments may also have the opposite effect to that desired, acting against its host, sometimes in combination with pathogens. Recently root endophytes were suggested as promising biocontrol agents against Verticillium wilt in olives (Prieto et al. 2009); they were able to induce resistance in the plant (Cabanás et al. 2014). In addition, fungal endophytes were identified as promising antagonists (Tyvaert et al. 2014). Because endophytes colonize the same microhabitat as *Verticillium*, they probably have substantial, currently under exploited potential to act as biocontrol agents (BCAs) against *Verticillium* spp. in different crops. Owing to their endophytic lifestyle they are better protected against adverse environmental conditions, which should allow more consistent beneficial effects in the field.

Strains from the genera *Serratia* and *Paenibacillus* are widely known for their plant growth promoting (PGP) and biocontrol qualities as well as for their endophytic lifestyle (Petersen and Tisa 2013; Rybakova et al. 2015). For example, *Serratia plymuthica* HRO-C48 has been successfully used for controlling Verticillium wilt and other soil-borne fungi as a soil amendment in strawberry fields (RhizoStar®) (Kurze et al. 2001). The application of *S. plymuthica* HRO-C48 to the seeds of the oilseed rape via bio-priming, pelleting or seed coating was shown to reduce the degree of Verticillium wilt in oilseed rape plants under greenhouse conditions (Müller and Berg 2008). While *Serratia* is a typical inhabitant of *Brassicaceae* (Kalbe et al. 1996), *Paenibacillus* strains have a broader host range and are cosmopolitans. *Paenibacillus* species are world-wide well known as commercially promising BCAs of plant diseases (Berg 2009; Lal and Tabacchioni 2009; Rybakova et al. 2015). One of the main advantages of *Paenibacillus* as a BCA is its ability to build endospores that increase survival of the species in extreme conditions. This provides advantages over the non-spore formers in product formulation and stable maintenance in soil (Emmert and Handelsman 1999). In addition, the broad spectrum of beneficial plant-microbe interaction support the selection of *Paenibacillus* as potential BCA as already shown in detail for *P. polymyxa* E681 (Timmusk and Wagner 1999; Timmusk et al. 2005) Although the biocontrol potential of *Serratia* as well as *Paenibacillus* was identified in scientific studies, *ad planta* systems are necessary to assess these effects.

The objective of the project was to test the effects of the strains at different in vitro and *ad planta* conditions to simplify the selection process for an optimal candidate for protecting oilseed rape and Brassica vegetables against fungal pathogens using seed treatment with beneficial bacteria. In the search for an optimal candidate for the seed treatment of *Brassica* plants we selected five strains of *Paenibacillus* in addition to five strains of *Serratia* (Berg et al. 2002, 2005; Fürnkranz et al. 2012; Köberl et al. 2013; Müller and Berg 2008 and Zachow et al. 2013). Although all strains have different origins, they were mainly isolated from plants and selected according to their antagonistic potential against fungal plant pathogens (Table 1). The selected strains were compared for (1) their ability to inhibit the growth of *Verticillium* spp. in vitro, (2) the efficiency of the bacterial colonization in the oilseed rape and cauliflower and (3) their ability to induce plant growth promotion (PGP) in oilseed rape and cauliflower seedlings. In addition to these studies we compared the colonization patterns of the different BCAs on the roots using confocal laser scanning microscopy (CLSM) combined with fluorescent in situ hybridization and/or using microorganisms labelled with fluorescent markers. Although all *ad planta* systems presented different results, the comparison of the strains' properties and a comparative assessment allowed us to choose one strain of each genus for further testing. As the initial study was performed under soil-less gnotobiotic conditions which may not reflect the natural effects of the BCA on the plant, we additionally evaluated the PGP effects of the selected strains on the plants grown under different artificial *ad planta* conditions. The results observed allowed us to conclude that growth conditions in the investigation of plant-bacterium interaction are crucial and that the same bacteria applied to the seeds may even result in either death of the host plant or in growth promotion depending on plant growth conditions.

Table 1 Selected bacterial isolates and plasmids used in this study

Strains	Closest database match	Environmental source		Reference
Paenibacillus				
Sb3-1	*P. polymyxa*	Agricultural soil		Köberl et al. 2013
Mc2-9	*P. brasilensis*	Chamomile rhizosphere		Köberl et al. 2013
302P5BS	*P. polymyxa*	Lichen		Cernava et al. 2015
Pb71	*P. polymyxa*	Styrian oil pumkin spermosphere		Fürnkranz et al. 2012
GnDWu39	*P. peoriae*	Styrian oil pumkin rhizosphere and spermosphere		Fürnkranz et al. 2012; Liebminger et al. 2011
Serratia				
HRO-C48	*S. plymuthica*	Oilseed rape rhizosphere		Müller and Berg 2008
3Re4-18	*S. plymuthica*	Potato endosphere		Berg et al. 2005; Zachow et al. 2010
3RP8	*S. plymuthica*	Potato rhizosphere		Berg et al. 2002
SP1-3-1	*S. proteamaculans*	Sorgum (primed with primula microbial community) rhizosphere		Zachow et al. 2013
S13	*S. plymuthica*	Summer squash spermosphere		Fürnkranz et al. 2012
Plasmid	Strain	Label	Excitation/Emission optima (nm)	Antibiotic resistance
pSM1890	*S. plymuthica* HRO-C48	EGFP	488 /507	gentamycin
pIN69_mNep	*S. plymuthica* 3Re4-18	mNeptune	532/625	trimethoprim
pIN69_dsRed	*S. plymuthica* 3RP8	DsRed2	563/582	trimethoprim
pIN69_EGFP	*S. proteamaculans* SP1-3-1	EGFP	488/507	trimethoprim
pIN69_EGFP	*S. plymuthica* S13	EGFP	488/507	trimethoprim

Material and methods

Bacterial strains and growth conditions

The bacterial strains used in this study are listed in Table 1. The fungal pathogens used were *V. dahliae* Kleb. V-024 (fungal collection of the Agronomy Dpt. of University of Córdoba) and *V. longisporum* ELV25 Stark (Karapapa et al. 1997) (Messner et al. 1996; strain collection TU Graz, Environmental Biotechnology). *Paenibacillus* and *Serratia* strains were routinely grown on Standard I nutrient agar (NA, SIFIN, Berlin, Germany) at 30 °C. When required, gentamicin or trimethoprim were added at concentrations of 10 and 50 μg ml^{-1}, respectively.

Labelling of the *Serratia* strains with fluorescent proteins for CLSM

In order to study colonization of plant tissue by natural isolates of *Serratia* spp., four strains were transformed with one of the derivates of the rhizosphere-stable plasmid pIN69 (Gasser et al. 2011) carrying genes encoding for the fluorescent proteins DsRed2, EGFP or mNeptune, and a trimethoprim resistance cassette (Table 1). *S. plymuthica* HRO-C48 was labelled with the plasmid pSM1890 conferring a gentamicin resistance and EGFP fluorescence (Haagensen et al. 2002).

In vitro antagonistic assays

Bacterial isolates were screened for their activity towards *V. dahliae* and *V. longisporum* by a dual culture in vitro assay on Waksman agar according to Berg et al. (2002). All strains were tested in three independent replicates.

Seed treatment (bio-priming)

Paenibacillus und *Serratia* strains used for bio-priming were grown on NA. Four to eight plates were used for each priming experiment. Strains were grown for 72 h and harvested following the modified protocol described by D'aes et al. (2011). Cells were scraped from the plates and suspended in sterile 0.85 % (*w/v*) NaCl. Cell concentrations were estimated by measuring their optical density at 600 nm (OD_{600}). The cell concentration was adjusted with sterile 0.85 % (*w/v*) NaCl to an

optical density corresponding to OD_{600} of 10. The final cell concentration was estimated for each strain separately using direct cell counting in a Thoma chamber (depth 0.01 mm). Live cell concentration was measured by successive dilutions of bacterial suspension followed by plating. The winter oilseed rape *Brassica napus* l. partim, "Traviata H 605886" (KWS Saat Einbeck, Germany) or Hybrid cauliflower var. "freedom" (Seminis, Holland) seeds were surface-sterilized for 5 min using 2 % NaOCl. Bio-priming was performed in accordance with the modified protocol of Müller and Berg (2008). Seeds were immersed in the cell suspension for 4 h at 20 °C under agitation. Infiltrated seeds were dried for 1 h at 20 °C until they appeared dry. Seeds incubated with sterile 0.85 % (*w/v*) NaCl solution for 4 h served as a control. Twenty seeds were transferred into 2 ml sterile 0.85 % sodium chloride solution in order to determine cell counts. Each strain was tested in four replicates. Primed seeds were ground for 1 min using an autoclaved mortar and pestle. Suspensions were serially diluted and plated onto NA medium (two replicates per dilution). Plates were incubated for 24–48 h at 30 °C and colony forming units (CFUs) were counted in order to calculate the logarithmic means of CFUs ($\log_{10}$ CFU).

Germination assay

The germination assay was performed following the modified protocol described by Zachow et al. (2013). Germination of oilseed rape seeds primed with *P. polymyxa* Sb3-1 in two concentrations ($\log_{10}$ 5 and $\log_{10}$ 7) was tested on a folded wet filter paper in a (10× 15.5 cm) plastic container at 20 °C. Germination rate was evaluated after 10 days by comparison with the non-inoculated control. The germination rate of the seeds as well as the total weight of the seedlings was then estimated. Each treatment included four replications containing 25 seeds each.

Plant growth promotion and root colonization assays

Plant growth in germination pouches In order to determine the effect of the potential biocontrol strains on the growth of plants in germination pouches, seven bio-primed seeds from each strain and seven non-inoculated seeds (control) were aseptically placed into one germination pouch (Mega International, Minneapolis, USA) which had been filled with 10 ml of sterile water. The pouches were placed upright in sterilized plastic boxes for 14 days. After 14 days, the green parts were gently separated from the roots and the fresh weight of all plant parts was determined gravimetrically. The experiment was carried out in four replicates for each bacterial strain (14 seedlings per replicate). For each replicate, root material from 14 seedlings was sampled into sterile plastic bags and homogenized with mortar after adding 2 ml of NaCl solution (0.85 %). Suspensions were serially diluted and plated on NA as previously described. CFU were determined after 1–3 days of incubation at 30 °C and calculated to a CFU per gram root fresh weight. In experiments with the antibiotic resistant bacteria strains carrying fluorescent tags (Table 1), medium was supplemented with the respective antibiotic.

Plant growth in sterile soil For evaluation of the PGP effect of BCA in the sterile soil, the surface-sterilized oilseed rape seeds were bio-primed independently with $\log_{10}$ 9 for *S. plymuthica* 3RP8 and $\log_{10}$ 7 for *P. polymyxa* Sb3-1. The seeds were sown in autoclaved plastic containers with a volume of 5.6 l containing 1 l propagation compost (Einheitserdewerk, Uetersen, Germany) mixed with vermiculite (4:1, *v/v*). The potting soil mixture was autoclaved in plastic bags twice with a 48-h interval. When the experiments were finished, the plants were weighed for the purpose of analyzing the effects of each BCA on biomass production in comparison to an unprimed control. The experiment was performed in six replicates with 12 seeds planted into each plastic container and repeated at least two times.

Plant growth in non-sterile soil In order to evaluate the PGP effects of BCAs on plants grown in the non-sterile soil oilseed rape seeds were primed independently with $\log_{10}$ 9 for *S. plymuthica* 3RP8 as well as with $\log_{10}$ 5 or $\log_{10}$ 7 for *P. polymyxa* Sb3-1. The seeds were sown in pots (three seeds per pot) with a volume of 250.0 ml containing propagation compost mixed with vermiculite (4:1, *v/v*). They were then grown and evaluated as described above. The seedlings were watered every second to forth day. The experiment was performed in four replicates with nine seedlings each for every bacterial strain and bio-priming concentration, and repeated at least three times.

For all three growth condition experiments the seedlings were kept in a phytochamber (Binder KBWF 720, Tuttlingen, Germany) at 22 °C, day–night regime of 12:12 h (7000 lux) for 14 days.

Isolation of endophytic bacteria from oilseed rape roots grown from seeds bio-primed with *P. polymyxa* Sb3-1

For endophyte enumeration, five 10-day old oilseed rape seedlings grown in germination pouches as described above were surface sterilized for 5 min in a surface sterilization solution (1 % sodium hypochlorite in 0.85 % NaCl solution). During the incubation period the plants were subjected to vortexing for 15 intervals of 10 s duration with 10 s of rest between intervals. This was followed by four washes with sterilized water. The wash solution from the final root rinse (1 ml) was cultured to determine the efficiency of sterilization. Seedlings were then macerated using a mortar, and the numbers of CFU were determined by plating preparations on NA.

Confocal laser scanning microscopy (CLSM) and fluorescent in situ hybridization (FISH)

CLSM was used to study colonization patterns of selected *Paenibacillus* and *Serratia* strains in oilseed rape and cauliflower seedlings grown in germination pouches for 14 days. Plant colonization by *Serratia* strains labelled with fluorescent markers was directly observed with a TCS SPE confocal microscope (Leica Microsystems, Germany) using laser lines/detection wavelengths as described in Table 1 for each fluorescent dye. The plant tissues (autofluorescence) were observed using a 405 nm laser line wavelength and detected at 425–490 nm. Confocal stacks were acquired with Z-step of 0.4–0.5 μm and sequential activation of laser lines/detection windows. Maximum projections of an appropriate number of optical slices were applied to visualize the root sections (confocal stacks).

The FISH technique was utilized in order to study the plant colonization ability of *Paenibacillus* strains. The roots and green parts of the 14 day old seedlings were fixed with 4 % paraformaldehyde/phosphate buffered saline (PBS) (3:1 *vol/vol*). The control group contained roots without bacterial treatment. The fixed samples were then stored in PBS/96 % ethanol (1:1) at −20 °C. The FISH probes for *Firmicutes* genera (LGC354A, LGC354B, LGC354C labelled with FITC; Meier et al. 1999) were used, and the in-tube FISH was performed as described by Cardinale et al. (2008). In this step, 45 % formamide was added to the samples which were then subsequently incubated in a water bath (41 °C) for 90 min. After hybridization, the samples were washed at 42 °C for 15 min. The second hybridization step directing eubacteria included an equimolar ratio of the FISH probes EUB338 (Amman et al. 1990), EUB338 II, and EUB338 III (Daims et al. 1999) labelled with Cy3 followed by another washing step. The unspecific binding of the probes to the plants or bacteria was analyzed by including a negative control sample. Additionally, the seedling roots and green parts were hybridized with NONEUB-FITC and NONEUB-Cy3 probes for the first and second hybridization steps, respectively, following the same protocol as for the positive samples. These samples served as a negative control to detect the unspecific probe hybridization. Microscopy and image capturing were performed as described above.

The evaluation of the colonization preferences of the *Serratia* strains in the oilseed rape and cauliflower seedlings was carried out using Universal Hood transilluminator (Bio-Rad, Austria) using excitation/detection wavelengths as described in Table 1 for each fluorescent dye.

Statistical analysis

The PGP effect of the microorganisms was statistically analyzed using the IBM SPSS program version 20.0 (IBM Corporation, Armonk, NY, USA). The selection of statistical test was done according to Gray and Kinnear (2012). For each of the treatments, at least three replicates were included in the analysis unless otherwise stated. The data was tested for normal distribution by using Q-Q plots and the Shapiro-Wilk test, and the homogeneity of variances was examined using the Levene's test (Bragina et al. 2013). The significance of the differences in plants' weights of the non-inoculated control versus each treatment (Tables 3 and 4) was (pairwise) calculated using a *t*-test with independent samples or by using the non-parametric Mann–Whitney *U* test, depending on the distribution of the variables (normal versus non-normal). The data was expressed as the geometric mean±standard deviation. For the dual culture assay, the data was analyzed for normal distribution and homogeneity of variances as described above. The significance of the differences between zones of inhibition of *Verticillium* growth by different bacterial strains (Table 2) was calculated using one-way ANOVA and Tukey's HSD tests. For both analyses, the P values<0.05 were considered to be significant.

Table 2 Antagonistic activity of preselected *Paenibacillus* and *Serratia* strains towards *V. dahliae* Kleb. and *V. longisporum* Stark ELV25

Strains	*V. longisporum* Stark ELV25 The means of the zones of inhibition (mm)*	*V. dahliae* Kleb. The means of the zones of inhibition (mm)*
P. polymyxa Sb3-1	4.3[a]	5.7 [ab]
P. peoriae GnDWu39	4.0[ab]	4.7 [bc]
P. brasilensis Mc2-9	4.0[ab]	8.7 [a]
P. polymyxa Pb71	3.9 [ab]	2.0 [cde]
P. polymyxa 302P5B5	2.9 [ab]	5.0 [bc]
S. plymuthica 3Re4-18	3.2 [ab]	3.7 [bcd]
S. plymuthica HRO-C48	2.3 [ab]	2.0 [cde]
S. plymuthica 3Rp8	1.2 [ab]	1.7 [cde]
S. plymuthica S13	0.8 [ab]	0.5 [de]
S. proteamaculans SP1-3-1	0 [b]	0 [e]

* The bacteria and *Verticillium* strains were grown on Waksman agar. Zones of inhibition were measured and statistically analysed after 6 days at 20 °C. According to the Tukey's HSD Test at $P=0.05$ the means from three independent replicates that are followed by a common letter for each isolate do not differ significantly

Results

Characterization and antagonistic effects of preselected *Serratia* and *Paenibacillus* strains towards *Verticillium*

Five selected strains of *Serratia* and *Paenibacillus* (Table 1) were screened for their in vitro activity against two pathogenic *Verticillium* strains: *V. dahliae* Kleb. V-024 and *V. longisporum* Stark ELV25. All strains with the exception of *S. proteamaculans* SP1-3-1 showed inhibition effects on both *Verticillium* strains in vitro (Table 2) while *P. polymyxa* Sb3-1 and *P. brasilensis* Mc2-9 exhibited the highest antagonistic potential for both *Verticillium* strains among tested strains. When *Serratia* strains were compared with each other, *S. plymuthica* 3Re4-18 exhibited the highest antagonistic effect towards both *Verticillium* strains, followed by *S. plymuthica* HRO-C48 and *S. plymuthica* 3Rp8. *S. proteamaculans* SP1-3-1 showed no antagonistic effect to both *Verticillium* strains.

Alteration of the bacterial abundances on the bio-primed seeds and the roots of the seedlings grown in germination pouches

The inoculation of the seeds with the preselected *Paenibacillus* strains with inoculum concentrations spanning from $\log_{10}$ 5.6 to $\log_{10}$ 7.3 CFU ml^{-1} resulted in the attachment of $\log_{10}$ 2.6 to $\log_{10}$ 4.1 CFUs per seed (Table 3). Although we attempted to keep the inoculation concentration constant by using both OD_{600} measurements and a Thoma cell counting chamber to adjust the inoculation concentration, the concentration of the live *Paenibacillus* cells varied. Of interest, the *P. polymyxa* Pb71 that was used for bio-priming at its highest concentration ($\log_{10}$ 7.3 CFU ml^{-1}) had the least abundance of live cells on the seeds after priming ($\log_{10}$ 2.9±0.2 CFU seed^{-1} for oilseed rape and $\log_{10}$ 2.6±0.2 CFU seed^{-1} for cauliflower seeds). We also noted that this strain did not sporulate under conditions used for bio-priming as detected using light microscope (data not shown). On the other hand, *P. polymyxa* GNDwu39 that was inoculated with the lowest concentration among tested *Paenibacillus* strains ($\log_{10}$ 5.6 CFU ml^{-1}) demonstrated the highest abundance of living cells on the seeds after priming ($\log_{10}$ 4.4± 0.1 CFU seed^{-1} for oilseed rape and $\log_{10}$ 4.2± 0 CFU seed^{-1} for cauliflower seeds). The selected *Serratia* strains were applied to the seeds in concentrations ranging from $\log_{10}$ 8.4 to 9.7 CFU ml^{-1}. This resulted in higher abundance of the bacteria on the oilseed rape seeds after priming than observed for *Paenibacillus* strains, ranging from $\log_{10}$ 5.8 CFU seed^{-1} for HRO-C48 to $\log_{10}$ 6.9 CFU seed^{-1} for SP1-3-1 (Table 3). A similar tendency was observed for the bacterial abundancies on the cauliflower seeds (Table 3). The amount of *Serratia* spp. that attached to the seeds during bio-priming was approximately 300 times higher on average when compared to that of the *Paenibacillus* spp.

Table 3 Effect of the 10 selected *Paenibacillus* and *Serratia* strains on the 2 weeks old oilseed rape and cauliflower seedlings grown in germination pouches

		Oilseed rape					Cauliflower				
	Priming concentration (log_{10} CFU ml^{-1})	Abundance on the seed (log_{10} CFU $seed^{-1}$)	Abundance on the root (log_{10} CFU (g) $roots^{-1}$)	Root weight (10 $plants^{-1}$ (mg)	Weight of the green parts (10 $plants^{-1}$ (mg)	Germination (%)	Abundance on the seed (log_{10} CFU $seed^{-1}$)	Abundance on the root (log_{10} CFU (g) $roots^{-1}$)	Root weight (mg 10 $plants^{-1}$)	weight of the green parts (10 $plants^{-1}$ (mg)	Germination (%)
Control	0	0.0±0	0.0±0	243±30	409±9.1	100	0.0±0	6.6±0.9[a]	157±23	351±77	98
Serratia											
HRO-C48	8.8	5.8±0.3	7.7±0.2	276±31	464±38	96	6.3±0.3	7.9±0.1	170±27	408±22	86
S13	9.5	6.4±0.1	8.7±0.2	253±20	429±21	95	6.4±0.1	8.8±0.1	112±26	375±11	91
3RP8	9.5	6.5±0.2	9.0±0.1	325±39*	448±44	93	6.4±0.2	8.9±0.2	201±26	408±58	86
RE4-18	8.4	6.0±0.3	8.4±0.1	337±48*	436±37	98	6.1±0.2	8.8±0.1	149±10	378±12	93
SP1-3-1	9.7	6.9±0.1	8.9±0.2	271±38	451±44	100	6.7±0.2	8.9±0.1	200±32	439±39	89
Paeni bacillus											
Pb71[b]	7.3	2.9±0.2	8.1±0.3	98±58*	154±134*	79	2.6±0.2	0.0±0	0±0	0±0	0*
GnDW-u39	5.6	4.4±0.1	8.4±0.2	44±15*	197±31*	59*	4.2±0	8.5±0.4	34±7	27±25	20*
Sb3-1	7.1	3.9±0.2	8.0±0.1	68±28*	260±30*	75	3.8±0	8.2±0.3	42±15	100±48	27*
Mc2-9	6.1	3.6±0	7.7±0.3	64±5*	271±29*	86	3.6±0.1	7.8±0.6	30±19	57±57	29*
302P5BS	6.5	4.1±0.2	8.2±0.7	46±43*	199±29*	61	4.0±0.1	8.2±0.7	6±11	26±45	11*

[a] Bacteria found on the roots of the seedlings used for negative control were not the strains used for priming

[b] Cauliflower seeds primed with *P. polymyxa* Pb71 did not germinate; For the data evaluating PGP effect (root weight, weight of the green parts and germination columns) the asterisk (*) denotes values that varied significantly from non-primed control group values ($P<0.05$). For the weights of the roots and green parts the fresh weight was determined

After 14 days, the roots of the seedlings were analyzed to determine cell densities. Although the abundances of the bacteria on the roots of the plants fluctuated within each genus, they appeared quite similar when both genera were compared to each other (Table 3). On average, $\log_{10}$ 8.0 *Paenibacillus* and $\log_{10}$ 8.5 *Serratia* CFUs were isolated from 1 g of oilseed rape roots. Cauliflower seedlings contained on average $\log_{10}$ 8.1 and $\log_{10}$ 8.7 CFUs (g) roots $^{-1}$ of *Paenibacillus* and *Serratia* strains, respectively.

Effect of bio-priming with *Serratia* and *Paenibacillus* strains on oilseed rape and cauliflower seedlings grown in germination pouches

The evaluation of the PGP effect of the selected BCAs (Table 1) showed that while *Serratia* treatment resulted in different levels of PGP, the opposite effect was found after *Paenibacillus* evaluation. The treatments with all *Paenibacillus* strains resulted in significant reduction of the growth of the seedling with Sb3-1 being the least damaging for the plant among *Paenibacillus* strains (Table 3). Priming of the oilseed rape and cauliflower seeds with the *S. plymuthica* 3RP8 and 3Re4-18 strains had a significant PGP effect on the root weights of the oilseed rape seedlings, while other *Serratia* strains showed no significant effects on plant growth. In combination with the results of the in vitro dual culture assay, *S. plymuthica* 3RP8 was chosen for further testing.

We found that the root system of the *Paenibacillus* primed oilseed rape and cauliflower seedlings under the described conditions was stunted and appeared damaged (Fig. 1b). The macroscopic appearance of the roots was similar for seedlings primed with bacteria of the same genera (data not shown). The influence of each of the tested strains showed similar tendencies when comparing oilseed rape and cauliflower with each other. Cauliflower seedlings, however, appeared to be more negatively affected by priming with *Paenibacillus* strains than oilseed rape seedlings.

The germination rate of cauliflower seedlings in germination pouches was negatively affected by the bio-priming with *Paenibacillus* strains resulting in a 60–80 % seed germination rate, while no reduction in germination rate was observed when seeds were primed with *Serratia* strains. With respect to the results, the *S. plymuthica* 3RP8 and *P. polymyxa* Sb3-1 were chosen for further experiments.

Effect of bio-priming with *S. plymuthica* 3RP8 and *P. polymyxa* Sb3-1 on oilseed rape seedlings grown in sterile and non-sterile soil

Because priming with *Serratia and Paenibacillus* spp. had a controversial effect on the seedlings grown in artificial gnotobiotic conditions, we decided to evaluate their effect on plants grown in soil. Therefore the oilseed rape seeds primed with *S. plymuthica* 3RP8 and *P. polymyxa* Sb3-1 were sown in either sterile or non-sterile soil. We found that the average fresh weight of the plants primed with *S. plymuthica* 3RP8 did not differ significantly from that of the unprimed seedlings grown in non-sterile and sterile soil (Fig. 1a). Plants primed with *P. polymyxa* Sb3-1 and grown in non-sterile soil were also not significantly different from the unprimed control. Interestingly, the evaluation of the fresh weights of oilseed rape seedlings grown in sterile soil showed an opposite correlation to the germination pouch experiment: seedlings primed with *P. polymyxa* Sb3-1 had significantly higher average weight when compared to the non-primed control (Fig. 1a).

Effect of the priming concentration of the P. polymyxa Sb3-1 on the seed germination and weight of the oilseed rape seedlings

In order to investigate whether the deleterious effect of *Paenibacillus* spp. observed when seedlings were grown in germination pouches correlates with the concentration of the inoculate, we applied $\log_{10}$ 5 and $\log_{10}$ 7 CFU ml^{-1} of *P. polymyxa* Sb3-1 to the surface-sterilized oilseed rape seeds in two independent experiment sets. The seedlings were grown under semi-sterile conditions on the folded filter paper for 10 days in one experiment set (germination assay) and in the non-sterile soil for 14 days in another experiment set. The weights of the seedlings grown on the filter paper was significantly reduced in comparison to the unprimed control in which seeds were primed with *P. polymyxa* Sb3-1 (Table 4). However, no significant differences in the weights of the plants grown in either non-sterile soil or on filter paper was observed when seedlings primed with different bacterial concentrations were compared to one another.

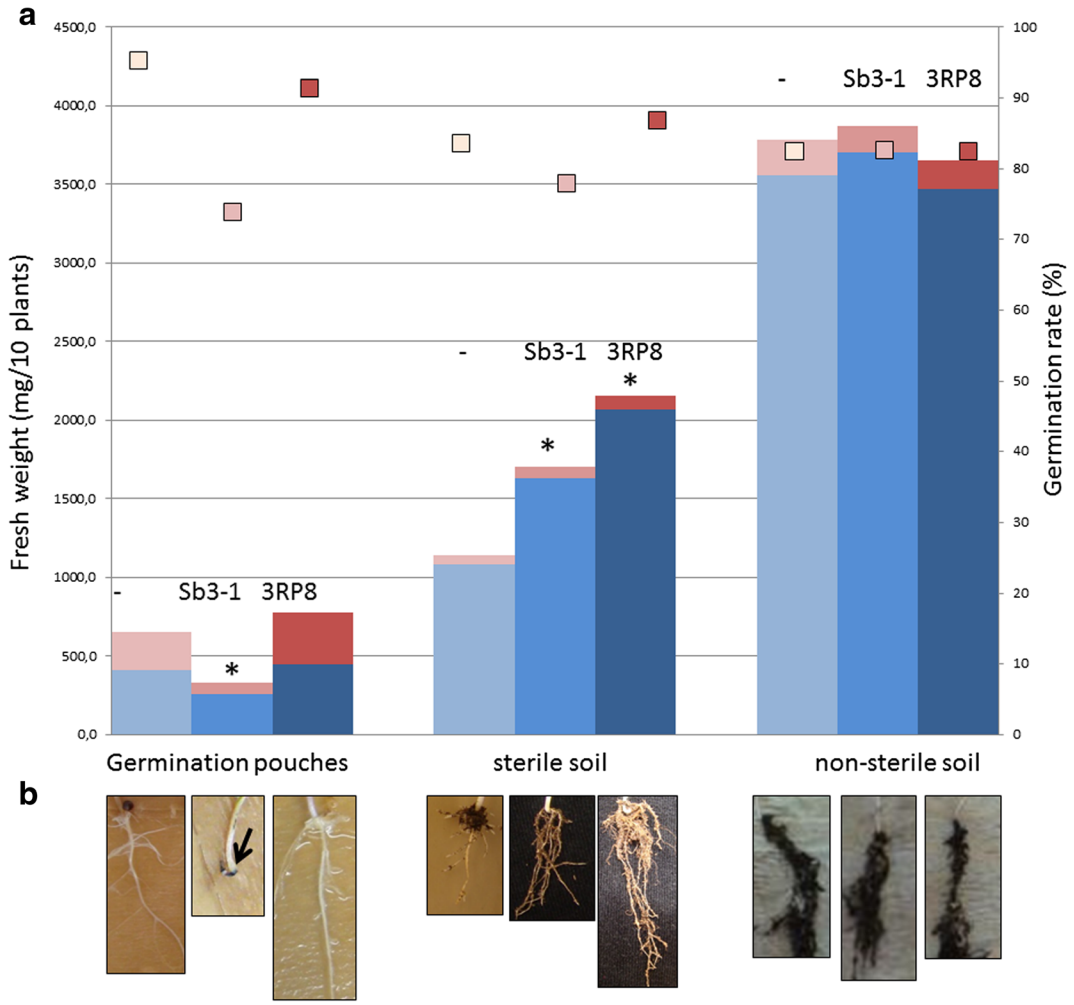


Fig. 1 **a** Comparison of the green part weights of oilseed rape seedlings after 2 weeks of growth in different conditions (sterile germination pouches, sterile soil and non-sterile soil). The seeds were primed with either NaCl solution (negative control, labelled "-") or *P. polymyxa* Sb3-1 (labelled "Sb3-1") or *S. plymuthica* 3RP8 (labelled "3RP8"). The *blue columns* denote mean weights of *green* parts (mg per 10 plants), *red columns* signify mean weights of roots (mg per 10 plants). The *squares* symbolize means of germination rate for each strain and experimental design in % (second axis). For details please refer to Tables 3 and 4. **b** Representative images of root bases of the oilseed rape seedlings corresponding to each bar from (**a**). *Arrow* denotes a stunned root system that was typical for seedlings primed with *Paenibacillus* spp. grown in germination pouches. The *asterisk* (*) denotes values that were significantly different from the non-primed control group values ($P<0.05$)

Table 4 Germination rate and total weight of the oilseed rape seedlings primed with different concentrations of *P. polymyxa* Sb3-1 grown in the non-sterilized soil (14 days) and under semi-sterile conditions on filter paper (10 days)

Inoculum concentration	Non-sterile soil		Semi-sterile filter paper	
	total weight / 10 plants^{-1} (mg)	Germination (%)	total weight / 10 plants^{-1} (mg)	Germination (%)
Non-innoculated control (0)	3536.9±511	83	375.4±32	97
$\log_{10}5$	3514.7±492	90	285.6±6*	90
$\log_{10}7$	3552.0±429	85	288.2±9*	96

The asterisk (*) denotes values significantly different from non-primed control group ($P<0.05$)

Colonization patterns of BCAs in the plant tissue of the seedlings grown in germination pouches

The ability of the biocontrol strains to colonize plants and their interactions were additionally assessed using the CLSM. *Serratia* isolates were tagged with either DsRed2, GFP or mNeptune enabling direct visualization of the bacterial colonies in the plant tissue (Table 1). *Paenibacillus* isolates were visualized using FISH with genera specific probes. The evaluation of the colonization preferences of the *Serratia* strains in the oilseed rape seedlings showed that fluorescent cells were mostly observed on the upper parts of the roots (Fig. 2a). This was confirmed by the screening of different parts of the root using CLSM. Within the root system, *Serratia* cells were found in either the upper parts of the root, or in the middle part of the root in fewer quantities, but not in the root tips (data not shown). The *Serratia* cells were either found as clouds around the roots, or they formed large micro-colonies in the root tissue (Fig. 2b). The cells were mostly observed in either the rhizosphere or in the intercellular space inside of the root tissue. Similarly shaped colonies, in a reduced abundance, were also found in the leaf tissue of the oilseed rape (Fig. 2c). The *Paenibacillus* colonies were often detected in the areas surrounding damaged root and leaf tissues (Fig. 2d) or in cavities (Fig. 2e and f) where they formed large micro-colonies. Similarly to the *Serratia cells*, the majority of the *Paenibacillus* cells were observed in the upper parts of the root. Bacterial colonization patterns within the same genus appeared to be similar. Furthermore, no notable differences between oilseed rape and cauliflower colonization patterns were observed (data not shown).

We tested whether *P. polymyxa* Sb3-1 was indeed capable of colonizing oilseed rape endophytically in

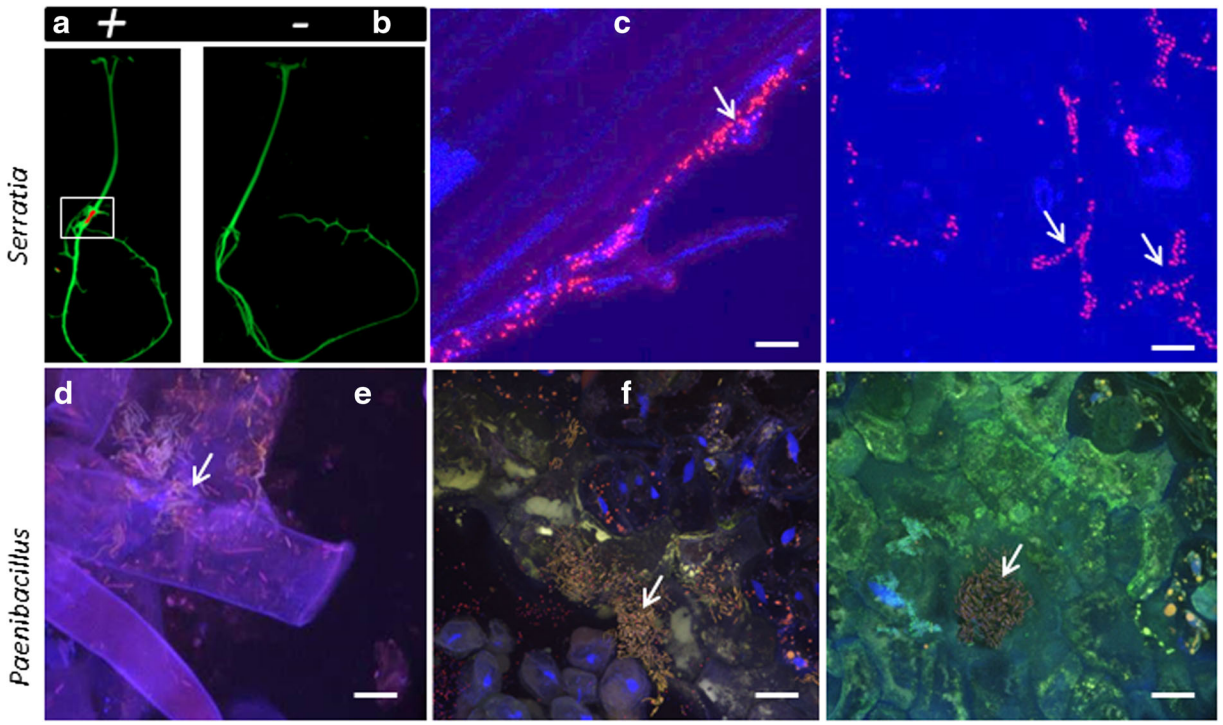


Fig. 2 Visualization of 14 day old oilseed rape seedlings grown in sterile germination pouches primed with either DsRed transformed *S. plymuthica* 3RP8 (labelled as "+") or with NaCl solution (labelled as "-") using universal hood transilluminator (**a**). The *red coloration* at the root base (highlighted with a *white square*) indicates a high saturation level of colonizing bacteria labelled with DsRed. Observations in (**b**) and (**c**) were made with CLSM and show DsRed transformed *S. plymuthica* 3RP8 colonizing the root base (**b**) and leaves of the oilseed rape seedling (**c**). *Paenibacillus* strains were visualized using FISH-CLSM using an equimolar ratio of the *Firmicutes*-specific FISH probes LGC354A, LGC354B and LGC354C labeled with the fluorescent dye FITC (**d-f**). *P. polymyxa* Mc2-9 colonies are denoted with *arrows*. The image (**d**) shows *P. polymyxa* Mc2-9 macrocolonies detected in the cavities of the damaged oilseed rape root. Images (**e-f**) show *P. polymyxa* Mc2-9 colonies detected in the cavities of the oilseed rape leafs. Images are a projection of 27–77 adjacent confocal optical sections. *Arrows* denote bacterial colonies. Bar represents 25 μm

order to confirm observations made by CLSM where the selected strains were found not only on the surfaces of plants but also in the plant tissues. The surface of the oilseed rape seedlings was sterilized followed by an extensive wash. Only 2.6 CFU per seedling were found in the final washing step in average, indicating an almost perfect sterilization of the plant surface. The homogenized tissues of the seedlings, on the other hand, contained 403 CFU per seedling which suggested that *P. polymyxa* Sb3-1 is capable of an endophytic lifestyle.

Discussion

In our study we were able to evaluate the PGP potential of endophytic *Serratia* and *Paenibacillus* strains that were in vitro shown to be *Verticillium* antagonists. This potential was strain-specific and depended on the plants' growth conditions. However, a comparative assessment as well as additional experiments allowed a selection of optimal candidates for biocontrol agents against Verticillium wilt in oilseed rape and cauliflower. The results of this study could contribute to the development of an environmentally friendly seed treatment against the high risk pathogen *Verticillium* (Zeise and Steinbach 2004).

All tested strains except for *S. proteamaculans* SP1-3-1 showed different degrees of antagonism against *V. longisporum* and *V. dahliae*. This was consistent with the observations made by Zachow et al. (2013) where *S. proteamaculans* SP1-3-1 was reported not to be antagonistic towards *V. longisporum*. Among tested isolates all *Paenibacillus* strains scored better than the *Serratia* strains in the in vitro test. This was probably due to their strong antagonistic properties such as their ability to produce large amounts of soluble and volatile antifungal metabolites (reviewed by Raza et al. 2008; Rybakova et al. 2015).

The efficiency of the bacterial colonization of the environment is proposed to be a crucial factor with regard to the efficacy of microorganisms as suppressors of soil-borne diseases (Weller 1988). Therefore, the next step for our investigation was to compare the colonization properties of the preselected strains with each other. We noticed that *P. polymyxa* Pb71 did not sporulate under the conditions used for bio-priming, while the other four *Paenibacillus* strains showed high degrees of sporulation (data not shown). Further experiments with endospore-forming bacteria exhibiting different degrees of sporulation are necessary to prove whether the ability of a bacterial strain to produce spores may improve its capacity to attach to the seed.

We found that the average number of the *Paenibacillus* cells that attached to the seeds of both cauliflower and oilseed rape was almost 300 times less when compared to abundance of *Serratia* on seeds. This may in part be explained by a lower priming concentration used for *Paenibacillus* spp. Interestingly, the abundancy of bacterial cells isolated from the roots of the bio-primed seedlings was quite similar for both genera. On average, $\log_{10}$ 8.1 CFU g^{-1} roots fresh mass for *Paenibacillus* and $\log_{10}$ 8.6 g^{-1} roots fresh mass for *Serratia* were found on the oilseed rape and cauliflower roots. This suggests the existence of a saturation level for bacterial colonization of the roots of the plant grown under given conditions. This suggestion is consistent with the observations described by Müller and Berg (2008). The authors applied *S. plymuthica* HRO-C48 to the oilseed rape seeds with initial bacterial cell numbers ranging from $\log_{10}$ 3.0 to 7.0 CFU $seed^{-1}$ and observed no significant differences in the plate counts of bacteria re-isolated from the seedling roots. The difference between the final abundance of the bacteria on the roots described in this work ($\log_{10}$ 7.7–9.0 CFU g^{-1} roots fresh mass) and bacterial abundancy on the roots reported by Müller and Berg ($\log_{10}$ 4.7 CFU g^{-1} root fresh mass) is most probably due to the different plant growth conditions. In this study the bacterial abundancy on the roots was tested for plants that were grown in germination pouches, while in the experiments described by Müller and Berg (2008), plants were grown in non-sterile soil under greenhouse conditions.

While, similar to other studies (Kurze et al. 2001; Müller and Berg 2008), *Serratia* strains either promoted the growth of the seedlings or had no effect on the seedlings growth under all tested conditions, this effect was only statistically significant for seedlings grown in sterile soil. On the other hand, we found significant differences in the effects of the tested *Paenibacillus* strains on the plant growth depending on the growth conditions. We found that all *Paenibacillus* strains tested damaged roots when plants were grown in the germination pouches. *P. polymyxa* Sb3-1 did not have a significant effect on plant growth in non-sterile soil, however, they significantly promoted plant growth in the sterile soil. The alterations in the initial priming concentration of *P. polymyxa* Sb3-1 ranging from $\log_{10}$ 4 to $\log_{10}$ 7 did not have any significant effects on plant

growth when plants were grown in non-sterile soil and on the filter paper. These results indicated that the choice of the optimal plant growth condition is more crucial for evaluation of the PGP effect of microorganisms than the choice of a bacterial concentration to be used for bio-priming. We have to notice that in all *ad planta* systems addressed in this study the PGP effects were evaluated at early seedling stage, and we do not know the effects of the BCAs on the later stage of the plants development yet.

The question as to why *Paenibacillus* may have a deleterious effect on the plants has been thoroughly studied by Timmusk et al. (2005 and 2015) and was recently discussed in detail by Rybakova et al. (2015). *Paenibacillus* applied to the roots of *A. thaliana* ecotype C24 seedlings was previously reported to damage plants that were grown in gnotobiotic conditions by degrading plant root cells as shown by CLSM (Timmusk et al. 2005). Indeed, the roots of seedlings grown from seeds bio-primed with each of the five *Paenibacillus* strains evaluated in this study appeared stunted and were significantly reduced in weight and length (Fig. 1). Moreover, the CLSM images showed that the roots of the plants were highly damaged and large macro-colonies of *Paenibacillus* cells were found in the cavities remaining after cell degradation both in roots and the leaves (Fig. 2). A similar effect of *Paenibacillus* on the *A. thaliana* seedlings was reported by Timmusk et al. (2005); however, in that case no *Paenibacillus* cells were detected in the leaves. Furthermore, we detected considerably more *Serratia* and *Paenibacillus* cells at the base of the seedlings' roots than in the root tips. The colonization patterns of *Serratia* strains observed by CLSM were similar to those observed for *Serratia* strains in the sugar beet rhizosphere (Zachow et al. 2010). Timmusk and co-workers, on the other hand, observed bacterial accumulation of *P. polymyxa* cells mainly around the root tip of *A. thaliana.* This difference may be due to the different plant cultivars used in these studies. The other possible explanation for the different character of spreading of bacteria to the plant tissues observed in this study and by Timmusk et al. (2005) is the use of different methods of applying bacteria to the plant. In our study seeds were submersed in a suspension of bacterial cells (bio-priming), while Timmusk and coworkers dipped the roots of the *A. thaliana* seedlings into the bacterial suspension. We speculate that it is easier for bacterial cells to spread to the green parts of the plant from the inoculated seed than in the case where bacterial cultures were applied to the roots of the seedlings. The accumulation of the bacterial cells at the root base may also be linked to its spatial proximity to the area of bio-priming.

It has been suggested that a paradoxical *Paenibacillus*-plant relationship may occur when the balance between *Paenibacillus* spp. and the soil microbiome in gnotobiotic conditions is upset. As a result, instead of protecting plants from pathogens, *Paenibacillus* spp. degrades root cells probably using the released metabolites as a nutrition source (Rybakova et al. 2015). *P. polymyxa* Sb3-1 was shown to be an endophytic bacterium for oilseed rape by both CLSM and seedlings' surface-sterilization following by cell count analysis. The ability of endophytic bacteria to destroy plant cell walls in order to enter into plant was linked to their ability of degrading pectin (Anand et al. 2006). It has been speculated that this is the way how endophytic bacteria may avoid cell defense mechanisms as the breakdown products of cell wall components, like pectin, induce systemic disease responses in plants (Anand et al. 2006). It is plausible that the plants grown artificially under gnotobiotic conditions in germination pouches without soil are weakened, and their defense system is impaired. This may result in a shift of the balance between the plant and endophytic bacteria in favor of bacteria resulting in damage of plant cells. This theory is also supported by the different effects of the same endophytic strain observed in oilseed rape and cauliflower seedlings in which cauliflower seedlings were much more strongly affected by the *Paenibacillus* spp. than oilseed rape seedlings. It has also been reported that morphological changes of the root have been associated with auxin production and excretion by PGP bacteria like *Paenibacilus* spp. Auxin has been shown to promote the sensitization of the host towards the bacterial pathogen and results in the development of disease symptoms (reviewed by Ludwig-Müller 2014 and Rybakova et al. 2015). Additionally, the non-ribosomal peptide/ polyketide synthases originated compounds produced by *P. polymyxa* has recently been shown to be partly or even fully responsible for its deleterious influence (Timmusk 2015). The local oversaturation of *Paenibacillus*-derived secondary metabolites in the rhizosphere of the seedlings grown in germination pouches may result in the observed deleterious effect.

When plants are grown under less artificial conditions, for example in non-sterile soil, *Paenibacillus* spp. can produce soluble and volatile metabolites that inhibit the growth of pathogens and also induce plants' defense

mechanisms resulting in changes in plant gene expression (Timmusk and Wagner 1999). They also build a biofilm around the roots that functions as a protective layer to prevent access by pathogens (Timmusk et al. 2005). These interactions may result in a PGP and a biocontrol effect of the *Paenibacillus* spp. as it was described for several *Paenibacillus* strains (Rybakova et al. 2015, and references therein). The question arises as to why the significant PGP effect of *P. polymyxa* Sb3-1 compared to the untreated control occurred only in sterile soil and not in the non-sterile soil. The PGP effect of some *P. polymyxa* spp. and diverse microbial communities on the host plants like Tobacco (*Nicotiana tabacum*) or *A. thaliana* grown in sterile soil has been shown by several groups (e.g., Phi et al. 2010 and Carvalhais et al. 2013). The situation where a PGP effect occurred in sterile soil while it was not observed in the non-sterile soil has to the best of our knowledge not been documented before. On the contrary, Kloepper and Schroth (1980) compared the PGP effects of several rhizobacteria on radish seedlings grown under gnotobiotic conditions in germination pouches, in sterile soil, and in non-sterile soil. The authors found that the same rhizobacteria increased plant growth in non-sterile conditions, while no significant effect on plant grown in sterile soil was observed. Of interest, some of the rhizobacteria studied by the authors also damaged roots of the seedlings grown in germination pouches. Another study (Li et al. 2012) describes an endophytic actinobacterial strain that stunted the root development of *Artemisia annua* seedlings grown under sterile conditions when inoculated at higher concentrations. The same strain did not show effects on the growth of *A. annua* under greenhouse conditions. One possible answer is that *Paenibacillus* as a facultative soil bacterium can easily survive in soil by using nutrients present in the soil for its own metabolism. Plant cells do not have to be degraded in order for the released metabolites to be used as a nutrition source for *Paenibacillus*. In this case, the positive effects of the *Paenibacillus* on the plant override possible negative effects and so the plant can profit from this relationship. The PGP is probably achieved by the production of plant growth hormones by the *Paenibacillus* rather than its biocontrol properties because possible pathogens are missing in the sterile soil conditions. The differences observed in PGP effect of *P. polymyxa* Sb3-1 on plants grown in sterile and non-sterile soil is most probably linked to the shift in the soil microbiome that occurs when other microorganisms are present, as in the case of non-sterile soil (Erlacher et al. 2014). For example, it is possible that the existing community in the non-sterile soil is sufficient for the optimal growth of the plant so that no effect of the treatment with the BCA can be observed.

In our study we were able to confirm some observations from previous studies, like the in vitro antagonistic effect towards *Verticillium* of the nine out of ten selected strains and the deleterious effect of *P. polymyxa* on the roots of the plants grown in gnotobiotic soil-free conditions (Timmusk et al. 2005). Moreover, we found that in contrast to other published bacteria-plant relationships (for example, Kloepper and Schroth 1980), *P. polymyxa* Sb3-1 enhanced the growth of oilseed rape seedlings in sterile soil conditions, while no effect was observed in the non-sterile soil.

Our results have shown that in the search for an ideal biocontrol strain it is not sufficient to perform only the in vitro tests or to exclusively study the interaction of the plant with the bacterium in artificial gnotobiotic conditions. This study suggests that the natural non-sterile soil is the best medium for studying plant-bacterium interaction as it reflects the field conditions on the best way. It is also apparent that further testing of the selected strains for their biocontrol effects against *Verticillium* wilt in *Brassica* spp. as well as further greenhouse experiments and field trials are necessary in order to fully evaluate the biocontrol effect of the selected strains. In conclusion, our study has not only contributed to the development of a sustainable and environmentally friendly solution to the as yet untreatable disease on *Brassica* plants, it has also allowed provided greater insight into a specific and controversial plant-endophyte interaction.

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