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Specific interactions between arbuscular mycorrhizal fungi and plant growth-promoting bacteria—as revealed by different combinations

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Abstract

The interactions between two plant growth promoting rhizobacteria (PGPR), *Pseudomonas fluorescens* SBW25 and *Paenibacillus brasilensis* PB177, two arbuscular mycorrhizal (AM) fungi (*Glomus mosseae* and *G. intraradices*) and one pathogenic fungus (*Microdochium nivale*) were investigated on winter wheat (*Triticum aestivum* cultivar Tarso) in a greenhouse trial. PB177, but not SBW25, had strong inhibitory effects on *M. nivale* in dual culture plate assays. The results from the greenhouse experiment show very specific interactions; *e.g.* the two AM fungi react differently when interacting with the same bacteria on plants. *G. intraradices* (single inoculation or together with SBW25) increased plant dry weight on *M. nivale* infested plants, suggesting that the pathogenic fungus is counteracted by *G. intraradices*, but PB177 inhibited this positive effect. This is an example of two completely different reactions between the same AM fungus and two species of bacteria, previously known to enhance plant growth and inhibit pathogens. When searching for plant growth promoting microorganisms it is therefore important to test for the most suitable combination of plant, bacteria and fungi in order to get satisfactory plant growth benefits.

Introduction

A large number of different interactions between fungi and bacteria occur in association with plants and depending on the nature of the species involved the plant can be positively or negatively affected. One well-known example of a mutualistic association is that between arbuscular mycorrhiza (AM) fungi and plants (Finlay, 2007). This association is characterized by a flux of plant-produced carbon to the fungus and fungal-acquired nutrients to the plant. Some bacteria also form mutalistic associations with plants, for example by producing plant growth promoting hormones, by fixing nitrogen or by suppression of pathogens (Emmert & Handelsman, 1999; Bloemberg & Lugtenberg, 2001). There is also evidence that combined interactions between AM fungi and bacteria can enhance plant growth (see Artursson *et al.,* 2006, for a review), and that some of these interactions may be very specific (Artursson *et al.,* 2006).

Two examples of plant growth promoting rhizobacteria (PGPR) are *Pseudomonas fluorescens* SBW25 (Thompson *et al.*, 1995) and *Paenibacillus brasilensis* PB177 (von der Weid *et al.*, 2002). Both of these bacteria have previously been shown to attach to vital AM fungal hyphae (Toljander *et al.*, 2006) and *Pa. brasilensis* PB177 inoculation was shown to increase the extent of root colonization by the AM fungus *Glomus mosseae* on clover (Artursson, 2005).

The aim of this study was to investigate different combinations of PGPR and AM fungi with regard to promotion of wheat plant growth. In addition, the ability of these different microbial inoculants to suppress disease symptoms caused by *Microdochium nivale*, the causative agent of pink snow mold, was determined. *M. nivale* is an economically important plant pathogen in many temperate zones. Disease occurs whenever winter is characterized by heavy snow cover, especially on unfrozen ground, for a longer period of time (Smith, 1981). The hypothesis was that the optimized combinations of PGPR bacteria and AM fungi would counteract growth of *M. nivale* and thereby improve plant health.

Materials and methods

Microbial strains

Beneficial PGPR included in this study were *Pseudomonas fluorescens* SBW25 (Thompson *et al.*, 1995) and *Paenibacillus brasilensis* PB177 (von der Weid *et al.*, 2002). Arbuscular mycorrhizal fungal strains used were *Glomus mosseae* (Gerdemann & Trappe, 1974) and *G. intraradices* (Schenck & Smith, 1982). *Escherichia coli* DH5α (Hanahan, 1983) was included as a negative biocontrol strain in some experiments. For some treatments, wheat seeds (*Triticum aestivum* cultivar Tarso) naturally infested with *Microdochium nivale* (Smith, 1981) were used.

Fungal inhibition plate assay

A plate assay was performed to determine the possible antagonistic effects of *Ps. fluorescens* SBW25 and *Pa. brasilensis* PB177 towards *M. nivale*. Potato dextrose agar (PDA; Oxoid LTD, Basingstoke, England) plates were inoculated with bacteria and fungi at a distance of 7 cm from each other. SBW25 or PB177 were inoculated as a 2 cm streak at one end of the agar plate and *M. nivale* was inoculated as an agar-plug (5 mm diameter) from a growing fungal colony at the other end of the plate. *E. coli* DH5α was used as a negative control (the fungi grew over the whole plate, including the area inoculated with bacteria). After two weeks incubation at room temperature (20-22 ºC) the plates were evaluated for evidence of fungal growth inhibition compared to plates that were not inoculated with plant growth-promoting bacteria or with the control bacterial strain *E. coli* DH5α.

Greenhouse experimental set-up

Commercial planting soil (Hasselfors garden AB, Örebro, Sweden) was autoclaved at 121°C for 20 min. on two separate occasions to kill heat resistant bacterial spores. The sterile soil was mixed 1:1

with sand that was also previously sterilized by autoclaving. The soil mixture was added to 1 L pots and watered to field capacity before addition of *G. mosseae* and *G. intraradices* and treated wheat seeds as described below. Treatment combinations are shown in Table 1.

All treatments where mycorrhizal fungi (*G. mosseae* or *G. intraradices*) were included, received 100 g of an AM fungal inoculum containing a commercial mixture of chopped mycorrhizal root pieces, spores, and hyphae mixed together on a sand-based carrier (Biorize Sarl, Dijon, France). The inoculum was added to each pot before the wheat seeds were planted, to a depth of about 5 cm and subsequently mixed with adjacent soil. The non-mycorrhizal treatments received the same amount of autoclaved inoculum.

Winter wheat seeds naturally infested with *Microdochium nivale* were inoculated with bacteria by seed coating before sowing. In addition a second seed lot (same cultivar) that was not infested with *M. nivale* was used. The infection level of the infested seeds was 34-53 % as evaluated in a previous seed hygienic test (ISTA, International Seed Testing Association, www.seedtest.org). Bacteria (*Ps. fluorescens* SBW25 and *Pa. brasilensis* PB177) were grown overnight in 100 ml GB-medium (1 litre; 10 g glucose, 10 g peptone, 1 g yeast extract, 5 g sodium chloride). The cultures were centrifuged at 3500 rpm for 15 min, washed once with PBS (Phosphate Buffered Saline 1 L: 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4; pH=7.4) and resuspended to a final cell concentration of 10^9 cells ml^{-1} PBS. 10 g of wheat seeds were mixed with 10 ml bacterial suspension, and the seeds were soaked for 15 min and dried on absorbant paper for 10 minutes. This treatment resulted in approximately 10^8 cells $[10\ g]^{-1}$ wheat seeds as determined by selective agar plating of antibiotic resistant mutant bacterial strains assumed to have similar colonization capacity to the wild type strains. Control seeds without bacteria were soaked in PBS alone.

Pots seeded with three seeds each were replicated five times for each treatment. The pots were randomly mixed in the greenhouse and watered once a day with tap water to field capacity without fertilizer. Three weeks after sowing the length of the emerged seedlings were measured from the soil surface to the tallest plant part and the tallest

seedling was retained in each pot for the remainder of the experiment. At the end of the experiment, after 12 weeks, plant yields were evaluated by determining root and shoot dry weights after drying at 70 ºC for 3-5 days.

AM fungal root colonization assay

A fungal colonization assay was performed as described by Artursson & Jansson (2003). Ten 1-cm long sections from one randomly selected pot per treatment were analyzed (McGonigle *et al.*, 1990). Ten intercepts per root section = 100 intercepts per pot and treatment (12 treatments) = 1200 intercepts in total were viewed and analyzed for mycorrhizal colonization. The amount of arbuscules was also evaluated (McGonigle *et al.*, 1990).

Statistics

The significance of differences between treatments was evaluated by ANOVA (Analysis of Variance) and Tukey´s post test as well as evaluation of the least significance difference (LSD) set at 95 % confidence limits.

Results

Fungal inhibition plate assay

Paenibacillus brasilensis PB177 inhibited growth of *M. nivale* on agar plates (Fig. 1 and Fig. 2). By contrast, *Pseudomonas fluorescens* SBW25 and *E. coli* DH5α (negative control) did not inhibit growth of the fungus. Fungal sporulation however, was affected by the presence of all three bacterial species; *M. nivale* growing on plates containing bacteria sporulated with pigmented spores unlike the mycelia growing on plates without bacteria (Fig. 1). Growth patterns were similar until day 8 when fungi on plates inoculated with PB177 stopped growing, and the fungi on plates with SBW25 and *E. coli* DH5α continued, as well as *M. nivale* controls (Fig. 2).

Greenhouse experiment

After three weeks incubation in a greenhouse the wheat seeds germinated and no significant differences between treatments could be seen with respect to the number of plants that emerged or plant shoot height (data not shown). For the sake of simplification, the results described below (after 12 weeks) are discussed separately depending on whether the seeds were infested with *M. nivale* or not. The amounts of arbuscles found for the different treatments were highly correlated to the percent root colonization of AM fungi (data not shown). The numbers of arbuscles were also reduced in *G. mosseae* compared to *G. intraradices*, but no further differences due to treatment could be detected.

Non-infested seeds. There was a significant increase in the shoot dry weights of plants that germinated from non-infested wheat seeds treated with the SBW25 bacterial strain + *G. intraradices*. By contrast, there was a decrease in shoot dry weights in the treatments with the PB177 strain + *G. intraradices* or with SBW25 alone (Fig. 3).

There was no treatment that significantly increased the root dry weights from non-infested seeds compared to untreated controls, although there was a slight increase for treatments with *G. intraradices*. However, some treatments resulted in a significant decrease in root dry weights; namely SBW25, *G. mosseae* + PB177, *G. mosseae* + SBW25 and *G. intraradices* + PB177 (Fig. 3).

G. mosseae colonized wheat roots to a greater extent when either bacterial strain, PB177 or SBW25, were present (Fig. 4). By contrast, only the presence of the PB177 strain was correlated to a higher colonization of *G. intraradices*. In fact, *G. intraradices* colonization was significantly lower in plants treated with SBW25 than in plants that were not inoculated with bacteria.

When both root and shoot weights from non-infested seeds were added together to represent the total dry weight of the plant there was a significant reduction in plant yields in plants treated with the SBW25 bacterial strain alone, and with the PB177 strain in combination with either of the AM fungi.

M. nivale infested seeds. In general, the *M. nivale* infested plants had shoot and root dry weights that were significantly lower ($p < 0.05$) than those of non-infested plants. However, when the infested plants were treated with *G. intraradices* solely or in combination with the SBW25 bacterial strain, there was a significant increase in shoot dry weights ($p < 0.05$); the latter also significantly higher compared to healthy non-treated wheat shoots. All other treatments had no significant positive or negative effects on plant shoot dry weights (Fig. 3) compared to the *M. nivale* infested control plants.

When investigating the impacts of the different microbial combinations on plant root dry weights, we observed a clear tendency for treatments with *G. intraradices* and *G. intraradices* + SBW25 to increase root dry weights of infested plants, although this effect was not significant. In addition, root dry weights from *M. nivale* infested plants were higher from seeds treated with *G. mosseae* compared to controls without AM fungi (Fig. 3). The root dry weights in the presence of *G. mosseae* alone were higher than the other *G. mosseae* treatments and comparable to those of non-infested

plants, indicating some inhibition of *M. nivale* disease symptoms on roots by *G. mosseae* inoculation.

When the plants were infested with *M. nivale*, but not inoculated with bacteria, there was a decrease in *G. mosseae* colonization compared to non-infested wheat, whereas the amount of colonization by *G. intraradices* was the same in both cases (Fig. 4). By contrast, when the bacteria were also inoculated onto the *M. nivale* infested seeds, they caused different effects on the resulting AM fungal colonization frequencies. Neither of the bacteria affected *G. mosseae* colonization. But there was a tendency for seeds treated with *G. mosseae* + *M. nivale* + PB177 to result in AM fungal colonization levels comparable to *G. mosseae* alone. A different situation was observed for the other AM fungus in that both bacterial inoculations increased *G. intraradices* colonization and this increase was significant for the PB177 strain (Table 1; Fig. 4).

Discussion

The extent of arbuscular mycorrhizal fungal colonization of wheat roots in this study was dependent on the combinations of mycorrhiza and PGPR strains used. Clear preferences were observed for the microbial associations and beneficial effects of the bacterial strains on the two different AM fungi. For example in the absence of *M. nivale*, both PGPR strains resulted in increased wheat root colonization by *G. mosseae*, but only *Pa. brasilensis* PB177 resulted in a higher colonization by *G. intraradices*. These results suggest that the different arbuscular mycorrhizal fungi react differently when inoculated together with the same bacterium. In a previous study, Azcón (1989) investigated the impact of three AM fungi (*G. mosseae*, *G. fasciculatum* and another *Glomus* sp; E3 type) together with two bacterial strains (*Azotobacter* and *Enterobacteriaceae*) on tomato plants. They also found effects that were related to specific interactions between each AM fungus together with either of the bacteria. In addition, we previously observed differences in the attachment frequencies of *Pa. brasilensis* PB177 and *Ps. fluorescens* SBW25 cells to live and dead AM fungal hyphae (Toljander, *et al*., 2006), emphasizing fine-tuned interactions between the microorganisms according to their physiological status. These interactions could in turn impact the abilities of the AM fungi to subsequently colonize the plants.

The total plant yields (root and shoot dry weights) were lower for wheat infested with *M. nivale* than non-infested plants as would be expected due to the known disease symptoms caused by this fungus on wheat (Smith, 1981). However, there was a trend, although not significant, for *G. intraradices* to partly relieve growth inhibition caused by *M. nivale*. On the other hand, SBW25 together with *G. intraradices* on *M. nivale* infested wheat interacted synergistically, giving higher shoot dry weights than non-treated healthy plants. Synergistic effects have also been noted (Gamalero *et al.,* 2004) in evaluations of interactions between two strains of *Ps. fluorescens* and *G. mosseae* BEG12 on tomato plant growth. Also, co-inoculation of all three microorganisms on the same tomato plant gave an increase in plant growth compared to single inoculation.

The wheat plant yield was significantly negatively impacted by the presence of SBW25 on healthy plants. Strain SBW25 was originally isolated from the sugar beet phyllosphere (Thompson *et al.*, 1995) and later inoculated onto wheat plants in several greenhouse and field studies. In previous studies it was shown that SBW25 readily colonizes roots, shoots and seeds of wheat (Unge & Jansson, 2001, Jäderlund *et al.,* 2008). Especially, high numbers of bacteria were found on the seeds, where they also were most metabolically active, as assessed by bioluminescence expressed by *luxAB* marker genes (Unge & Jansson, 2001, Jäderlund *et al.,* 2008). This very high bacterial colonization level on seeds could potentially be the reason for the observed decrease in plant dry weight. In the previous study SBW25 inoculation did not impact the emergence of wheat seeds or plant shoot length after 28 days (Jäderlund *et al.,* 2008), but it has not been previously evaluated if this bacterium could have a negative impact on plant dry weight. However, this negative effect was not seen when SBW25 was used in combination with *G. intraradices*, pointing out the importance of careful evaluation of combinations of microorganisms used for promotion of the growth of different plants.

Interestingly, PB177 inoculation did not improve growth yields of *M. nivale* infested plants. This is in contrast to the strong inhibitory effect of PB177 on *M. nivale* that we observed in dual culture plate assays. These contradictory results suggest that PB177 does not suppress *M. nivale in planta*. The same result was obtained in a similar green house experiment where strain PB177 was included (Arthurson *et al.*, unpublished). There could be various reasons for this apparent dichotomy. To begin with, we do not know the reason for the suppression of fungal growth by PB177 on agar. One possibility is that PB177 produces some inhibitory compound or metabolite(s) *in vitro* that is not produced on the plant surface. An alternative explanation is that the bacteria were applied at sub-optimal cell densities to the seeds, either too high or too low, in relation to the *M. nivale* concentration. Another study performed in a similar way, with *G. mosseae* and 5 different *Paenibacillus* strains (including PB177) showed that 10^8 PB177 cells $[10\ g]^{-1}$ wheat seeds could be an inhibitory dose, as better plant yields were obtained when using a 100-fold lower bacterial inoculum dose (Arthurson *et*

al., unpublished). It could also be that the concentration and/or activity of the inhibitory substance is reduced after an optimum in the first incubation weeks. The stability of the numbers of PB177 cells on the wheat plants could not be measured due to lack of a specific detection technique, and thus it is not known if the cell concentration is reduced, unchanged or increased during these 12 weeks of incubation. It is also noteworthy that PB177 was isolated from a corn rhizosphere in Brazil (von der Weid, et al., 2002) and might not be suitable as a biocontrol agent on wheat. Further studies could help to clarify the mechanisms of inhibition of *M. nivale* by PB177 and why this effect is counteracted *in planta*. It would be useful to first examine the colonization pattern of PB177 on wheat plants over time and of course to identify potential inhibitory substances.

AM fungal colonization, *per se*, was not correlated to an increase in wheat plant growth and the results varied depending on the bacterial inoculant used in combination with the different AM fungi studied here. These results are in accordance with some other studies that found no direct relationship between the extent of AM fungal colonization and plant growth (Akköprü & Demir, 2005; Azcón, 1989; Medina *et al*., 2003). There is the possibility that a large extent of AM fungal colonization in fact results in a decrease in plant dry weight due to an overload of the fungi on the plant system. Probably this phenomenon is very specific for each host plant and AM fungus and more studies are required to enlighten these issues. Colonization patterns of the different microorganisms on wheat plants infested with *M. nivale* looked quite different than on healthy plants. This could imply that the pathogenic fungus competes with the AM fungi and thereby reduce the AM fungal colonization. It also seems that the positive effect of the two bacterial strains on the AM fungal colonization is counteracted when *M. nivale* is present. However, two different seed lots were used, one that was infested with *M. nivale* and another one that was healthy. It is possible that some unknown differences between the seed lots affected the AM fungal colonization frequency independently of the presence of *M. nivale*.

The significant positive effects on plant dry weights that were measured in this study upon AM fungal inoculation were mostly detected in wheat infested with *M. nivale* and with *G. intraradices*.

Biocontrol effects of AM fungi on root pathogens have been previously reported (for a review see Borowicz, 2001) and may be due to several mechanisms, for example competition for colonization sites, improvement of nutrition uptake, activation of plant defence mechanisms or changes in microbial community composition (Azcón-Aguilar & Barea, 1996; Borowicz, 2001).

In conclusion, this study provides information about the specific interactions formed by two PGPR, one gram-negative and one gram-positive, two closely related AM fungi, one pathogenic fungus and wheat plants. Some combinations increased dry weights of *M. nivale* infested wheat plants in comparison to control plants. Both bacteria affected the colonization levels of the AM fungi on wheat roots, but in different ways, depending on which bacterium and AM fungi were applied and if *M. nivale* was present or not. However, these differences in AM fungal colonization were not directly related to plant yield *per se*, which instead was more dependent on which combinations of microorganisms were applied. Further studies are therefore necessary to elucidate the mechanisms behind disease suppression, fungal antagonism and plant growth stimulation by different bacteria and AM fungi.

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Table 1. Plant effects for different microbial treatments after 12 weeks of greenhouse incubation.

Treatments	Effect on AM fungal colonization	Effect on dry weight of shoots	Effect on dry weight of roots	Effect on dry weight of whole plant
Without M nivale[1]				
PB	n/a	0	0	0
SBW	n/a	-	-	-
G.m.		0	0	0
G.i.		0	0	0
G.m. + PB	+	0	-	-
G.m. + SBW	+	0	-	0
G.i. + PB	+	-	-	-
G.i. + SBW	-	+	0	0
Buffer treated control	n/a	n/a	n/a	n/a
With M nivale[2]				
PB + M. niv	n/a	0	0	0
SBW + M.niv	n/a	0	0	-
G.m. + M. niv	n/a	0	+	+
G.i. + M. niv	n/a	+	0	+
G.m. + PB+ M.niv	0	0	0	0
G.m. + SBW+ M.niv	-	0	0	0
G.i. + PB+ M.niv	+	0	0	0
G.i. + SBW + M.niv	0	+	0	+
Control M.niv	n/a	n/a	n/a	n/a

[1] Effects compared to buffer treated control plants

[2] Effects compared to control plants infested with *M. nivale* (or AM + *M. nivale* for AM colonization)

G.m. = *G. mosseae*, G. i.= *G. intraradices*, PB = *Pa. brasilensi*s PB177, M niv = *M. nivale*, SBW = *Ps. fluorescens* SBW5

n/a: not applicable

+: increase in AM fungal colonization or plant weight (according to 95 % CI)

-: decrease in AM fungal colonization or plant weight (according to 95 % CI)

0: unchanged AM fungal colonization or plant weight (according to 95 % CI)

Figure legends

Fig. 1 Test of bacterial inhibition of *M. nivale* on agar plates. (a) *M. nivale* alone and (b) *M. nivale* + PB177 (b) after 8 days incubation.

Fig. 2 Results from agar plate assays of bacterial inhibition of *M. nivale*. Treatments were: *M. nivale* control (grey filled diamonds), *M. nivale* + SBW25 (grey filled triangles), *M. nivale* + PB177 (black filled squares) and *M. nivale* + DH5α (non-filled squares). Mean values of three replicate plates are shown, error bars represent 95 % CI (confidence interval).

Fig. 3 Shoot (light bars) and root (dark bars) dry weights of wheat plants (a) non-infested or (b) infested with *M. nivale*. G.m. = *G. mosseae*, G. i.= *G. intraradices*, PB = *Pa. brasilensis* PB177, M niv = *M. nivale*, SBW = *Ps. fluorescens* SBW5. Error bars represent 95 % CI (confidence interval).

Fig. 4 Wheat root colonization of *G. mosseae* (dark bars) and *G. intraradices* (light bars). AM = *G. mosseae* or *G. intraradices* respectively, PB = *Pa. brasilensis* PB177, M niv = *M. nivale*, SBW = *Ps. fluorescens* SBW5. Error bars represent 95 % CI (confidence interval).

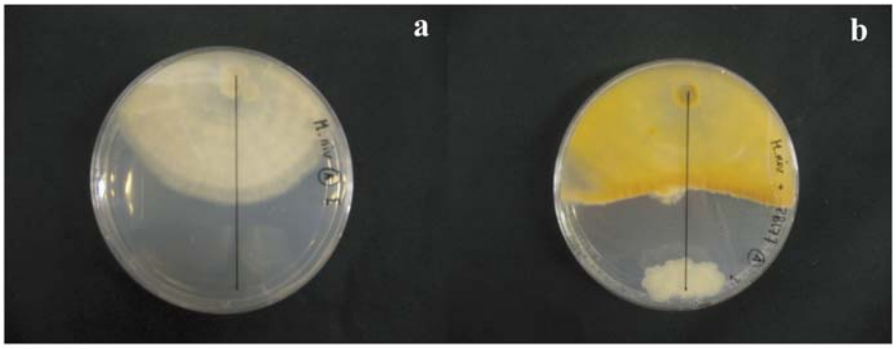


Figure 1

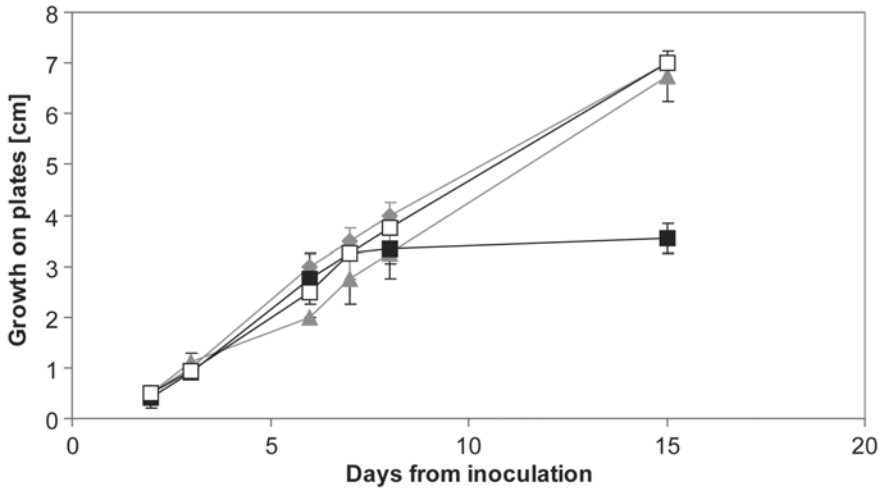


Figure 2

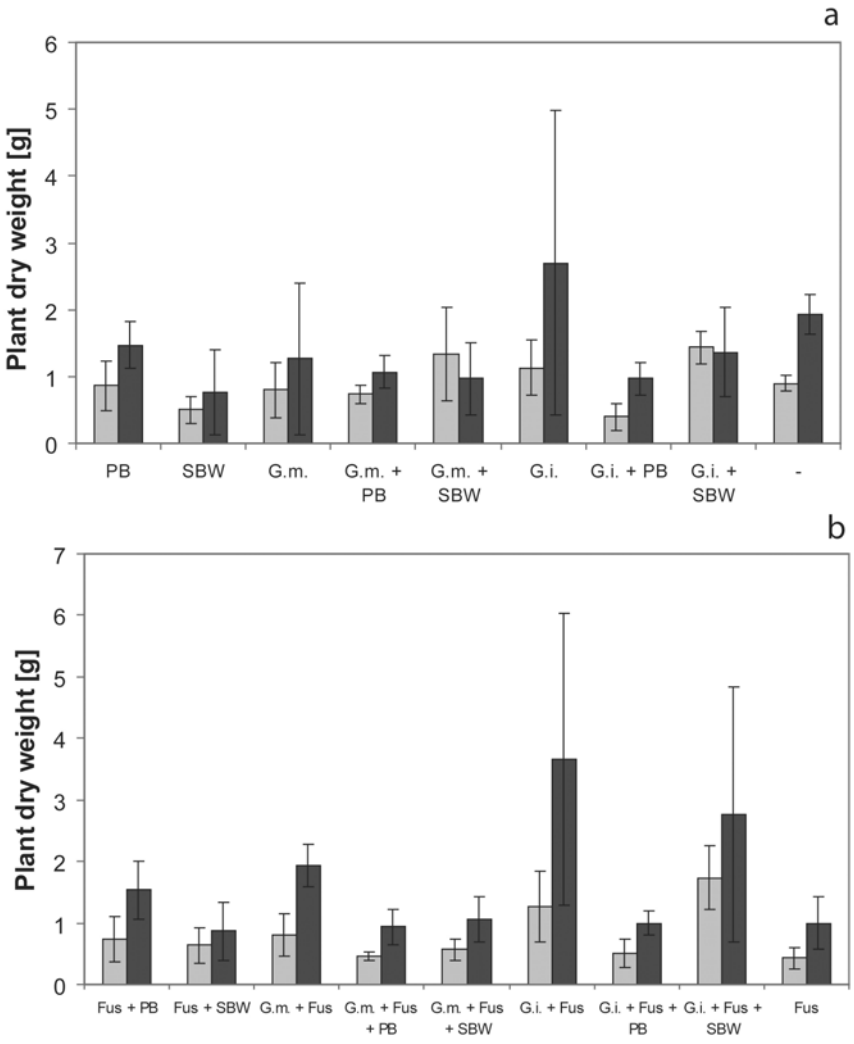

a
Plant dry weight [g]
0
1
2
3
4
5
6
PB
SBW
G.m.
G.m. + PB
G.m. + SBW
G.i.
G.i. + PB
G.i. + SBW
-
b
Plant dry weight [g]
0
1
2
3
4
5
6
7
Fus + PB
Fus + SBW
G.m. + Fus
G.m. + Fus + PB
G.m. + Fus + SBW
G.i. + Fus
G.i. + Fus + PB
G.i. + Fus + SBW
Fus

Figure 3

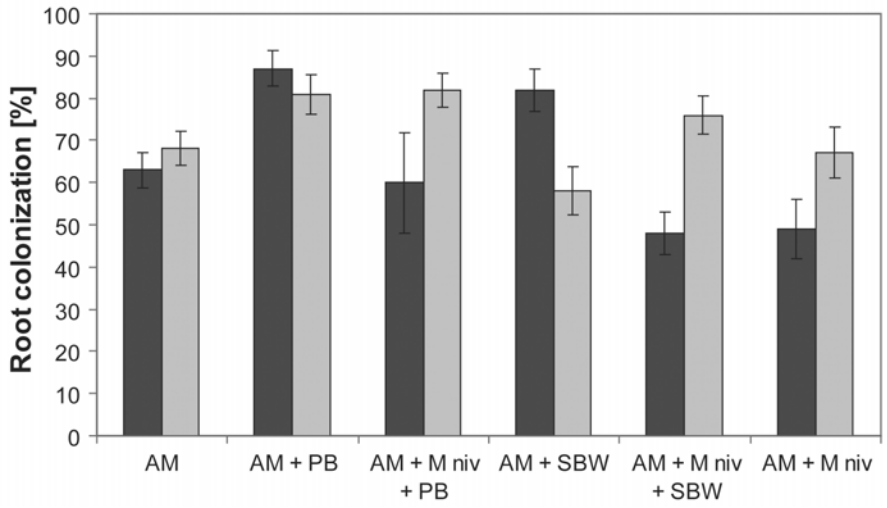

Root colonization [%]
100
90
80
70
60
50
40
30
20
10
0
AM
AM + PB
AM + M niv + PB
AM + SBW
AM + M niv + SBW
AM + M niv

Figure 4