Bioprotection and alternative fertilisation of petunia using mycorrhiza in a soilless production system

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Abstract Ornamental crop production is increasing worldwide and petunia is nowadays a major bedding plant with high economic impact. To meet growing demands, a high production of quality nursery plants is needed. However, production in greenhouses is confronted by two main problems: fertiliser-induced salinity and pathogen attack like by *Thielaviopsis basicola* which both cause high losses. Moreover, there is increasing consumer demand for crops produced by environment-friendly practices. The application of arbuscular mycorrhizal (AM) fungi can present an appropriate approach to avoid excessive use of fertilisers and pesticides. Here, we report studies of the interaction between *Petunia hybrida* and the AM fungus *Glomus mosseae* in abiotic and biotic stress situations. Root colonisation by *G. mosseae*, although very low, was able to compensate phosphate deficiency in the substrate and resulted in increased plant dry matter, water and phosphorus content in the shoots. However, these positive effects were attenuated at high salt concentrations (abiotic stress). In contrast, disease symptoms caused by the root pathogen *T. basicola* (biotic stress) were significantly reduced in *G. mosseae*-colonised plants. Such protective effects were not achieved with the AM fungi *Glomus intraradices* and *Gigaspora rosea*, and a negative effect of the pathogen on root colonisation by *G. intraradices* was observed. This shows for the first time under nursery practice conditions that *G. mosseae* can compensate the negative impact of lower levels of

phosphate fertilisation on plant growth in a soilless production system and induce bioprotection of petunia against *T. basicola*. Based on the different effects observed with the three AM fungi, petunia can provide an experimental system to define the molecular mechanisms underlying mycorrhiza-induced resistance against root pathogens.

Keywords Arbuscular mycorrhiza-induced resistance · *Gigaspora* · *Glomus* · Petunia · Salt stress · *Thielaviopsis basicola*

1 Introduction

World concern about the excessive use of fertilisers and pesticides on crops and the harmful side effects of chemical inputs in agriculture on human health and soil biodiversity has grown during the last decade. This has prompted research into the use of microbial inoculants as biological additives (Paulitz and Belanger 2001). Application of such ‘bioalternatives’ represents a promising strategy to counter chemical inputs in the development of sustainable crop production. In this context, most crop plants form a root symbiosis, arbuscular mycorrhiza (AM) with fungi of the phylum Glomeromycota (Smith and Read 2008). The benefits that AM fungi provide to plants can be summarised in three main activities: (1) biofertilisation by improving plant nutrient uptake, (2) bioregulation by modulating plant hormone production and (3) bioprotection by reducing the impact of abiotic and biotic stresses (Gianinazzi et al. 2010). In regard to these benefits, AM fungi could be classified as a biological means for natural long term, low-input plant production and disease control in different areas of agriculture, landscape regeneration, alleviation of desertification or bioremediation of contaminated soils (Jeffries et al. 2003).

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Ornamental crops are mainly produced as potted plants, and their marketability is greatly influenced by conditions used during their production such as substrate quality, drainage, irrigation, water quality and fertilisation (Chavez et al. 2008). Soilless culture substrates associated with rich fertiliser regimes are increasingly applied to meet present-day consumer demands for ornamentals and nursery plants (Gruda 2009). Whilst these offer significant advantages for high crop yield and product quality through complete control over water and nutrient supplies (Grillas et al. 2001), the use of substrates with no ion exchange capacity can lead to mineral nutrient losses and to short-term unintentional exposure of plants to high ion concentrations, because excess mineral ions cannot be bound or slowly delivered to the plant root. This in turn results in short periods of salt stress which are detrimental especially for young plants and, if not lethal, reduce vigour and yield (Rosendahl and Rosendahl 1990). Moreover, ornamental plant production is confronted with attack by root pathogens like *Fusarium oxysporum*, *Rhizoctonia solani* or *Thielaviopsis basicola* (Dreistadt 2001), which cause high losses in greenhouses.

Petunia is an ornamental crop of high economic interest which is particularly sensitive to the accumulation of soluble salts caused by high fertiliser concentrations in the growing media (Kang and van Iersel 2009). It can be easily propagated from cuttings and has many advantages also as a plant model system (Gerats and Vandenbussche 2005). Forward and reverse genetic approaches for mutant screening are possible (Wegmueller et al. 2008), a large EST collection and microarray tools have recently become available (Breuillin et al. 2010) and genomic sequencing is currently on-going (Franken and Drüge, personal communication). This makes petunia a very useful plant for studies of the genetics and basic biology of the AM symbiosis as well as mechanisms underlying AM fungal benefits.

In the present work, we have investigated (1) the feasibility of applying the AM fungus *Glomus mosseae* to reduce phosphate fertiliser in a soilless medium (vermiculite/sand), (2) the benefits of mycorrhization on petunia growth under high salt concentrations, and (3) the efficiency of mycorrhiza-induced bioprotection of three different AM fungi against a root pathogen.

2 Material and methods

2.1 Cultivation of plants and application of mycorrhizal inoculum

Petunia hybrida Mitchell (W138) seeds were disinfected for 10 min with sodium hypochlorite (NaOCl, 10%), washed three times with sterile water and germinated in boxes containing autoclaved vermiculite for 4 weeks. The seeds were

incubated at 22/24°C with 16/8 h photoperiod under cool-white fluorescent light (four 18 W/865 New generation lamps, Philips, Hamburg, Germany). Seedlings were thereafter transplanted into 400 cm³ pots filled with a vermiculite/sand nutrient-free mix (1:1, v/v) and added (mycorrhizal, M) or not (non-mycorrhizal, NM) with 10% of a vermiculite-based inoculum from one of three AM fungi *G. mosseae* (Nicolson & Gerd.) Gerd. & Trappe BEG12 (IBG, Dijon, France), *Gigaspora rosea* Nicolson & N.C. Schenck BEG9 (IBG, Dijon, France) or *Glomus intraradices* Schenck & Smith (BEG141). All inocula were produced by Agrauxine (Quimper, France) with a minimal propagule number of 50,000 I⁻¹. Non-mycorrhizal control plants were watered with an inoculum filtrate (two times filtration using Whatmann n°1 filter paper; Schleicher and Schuell, Dassel, Germany) to introduce associated microorganisms. Plants were cultivated in a randomised block design in growth chambers (Vötsch, Balingen-Frommern, Germany day/night 22/20°C and 16/8 h, light intensity 200 µmol m⁻² s⁻¹, 60% relative humidity) for 5 weeks before harvest or further treatments. The plants were fertilised twice a week with a modified Hoagland nutrient solution (Oyarzun et al. 1993) containing 10% phosphate (0.1 mM KH₂PO₄; 15 ml/pot).

2.2 Comparison of effects of mycorrhiza and phosphate fertilisation

Mycorrhizal plants were produced as described above. In order to compare the effect of *G. mosseae* to phosphate fertilisation, control plants were divided into four sets with each three plants and fertilised with a Hoagland nutrient solution containing final concentrations of 0.1, 0.26, 0.51 or 1 mM KH₂PO₄. All plants were grown under same conditions described above and harvested after 5 weeks to measure phosphorus content and shoot biomass.

2.3 Abiotic and biotic stress treatments

Five weeks after inoculation with *G. mosseae*, salt stress was induced for 2 weeks by providing plants twice a week with a modified Hoagland nutrient solution (0.1 mM KH₂PO₄) containing 250 mM NaCl.

Four pathogenic fungi were tested for disease development in petunia roots: *Pythium aphanidermatum* (Edson) Fitzp., *F. oxysporum* Schlecht., *R. solani* Kühn and *T. basicola* (Berk. and Broome) Ferraris (syn. *Chalara elegans*). *P. aphanidermatum* isolated from cucumber was grown for 10 days on carrot agar medium (15 g carrots in 1.5% Agar-Agar, Roth, Karlsruhe, Germany). Twenty plugs of infected medium were agitated for 12 h in 1 L of sterile water. Petunia plants were inoculated by injecting 20 ml of this suspension into the substrate close to the stem base after adjusting the concentration with sterile water to 10⁶ conidia/

ml, determined using a Thoma chamber; control plants received 20 ml of sterile water. *F. oxysporum* from infected petunia plants was inoculated by using three infected plugs of PDA fresh agar medium per plant added to the substrate (2 cm in depth, 1 cm from the stem base); non-infected agar plugs were used for control plants. *R. solani* (isolated from a diseased potato plant; identified as anastomosis group 3) was inoculated according to Schneider et al. (1997), using three infected barley seeds as inoculum (2 cm in depth, 1 cm from the stem base) and sterile barley seeds for control plants. *T. basicola* from the German Resource Centre for Biological Material (DSM no.: 63050) was grown on V-8 juice (Gemüsesaft, Penny, Germany) agar medium (1.5% Agar-Agar, Roth, Karlsruhe, Germany) for 1 week at 22°C with a 16/8 h photoperiod under cool-white fluorescent light. Fungal mycelium and conidia, collected from the agar surface, were diluted with sterile water to a concentration of 10^6 conidia/ml, determined using a Thoma chamber. *T. basicola* was inoculated by injecting 20 ml of the suspension per plant into the substrate close to the stem base; control plants received 20 ml of sterile water.

2.4 Disease assessment

Disease severity (DS) was visually assessed 2 and 4 weeks after pathogen inoculation on 30 root fragments/plant analysed under a binocular microscope. The degree of infection by the pathogenic fungi was rated in five classes based on the percentage of root length with brown lesions: 0, no infection; 1, <10%; 2, <50%; 3, >50%; and 4, >80%. The DS was calculated according to Fakhro et al. (2010) by: $\sum (n \times \text{times } x) / 30$ (n =number of fragments, x =each category from 0 to 4).

2.5 Determination of mycorrhizal colonisation, shoot biomass and phosphorus content

Petunia plants were harvested and roots were washed under running tap water, cleared in 10% KOH and stained with 0.05% trypan blue in glycerol (Phillips and Hayman 1970). Mycorrhizal colonisation was quantified on 30 root fragments/plant according to Trouvelot et al. (1986) and mycorrhizal parameters were calculated using the “Mycocalc” programme (<http://www2.dijon.inra.fr/mychintec/Mycocalc-prg/download.html>).

To determine dry matter and water content, shoots were weighed, subsequently dried for 3 days at 80°C and weighed again. Dry material of each shoot was dissolved in 5 ml of 65% nitric acid and ashed for 20 min at 200°C. Phosphorus was measured spectrometrically after addition of an ammonium–molybdate–vanadate solution with an EPOS-analyser 5060 (Eppendorf, Hamburg, Germany) according to the manufacturer’s instructions.

2.6 Statistical analysis

Statistical analyses of arcsin-transformed data (three or four biological replicates as indicated in the figure legends) were carried out using one-way or two-way analysis of variance (ANOVA) provided by the programme package Statistica (version 7.0; StatSoft Inc., Tulsa, OK, USA). Means were thereafter separated by Fisher’s test procedure at $P=0.05$. T test was used for comparing means of treatments where two-way ANOVA detected an interaction at $P=0.05$.

3 Results and discussion

3.1 Impact of *G. mosseae* on petunia biomass and P content

Parameters of root colonisation 5 weeks after inoculation with *G. mosseae* were 9.7 for mycorrhizal spread inside the root system (M%) and 2.8 for total arbuscule abundance (A %). In spite of this low level of mycorrhiza development, a significant positive effect on the plant was observed (Fig. 1). Biomass parameters were nearly doubled, P content increased up to 15%, and the AM fungus enhanced plant P uptake 2.5-fold. In the experiment where control plants were fertilised with a nutrient solution containing between 0.1 and 1 mM phosphate (P_i), only plants receiving 0.5 mM P_i showed similar biomass and P content as the plants inoculated with *G. mosseae* (Fig. 2). These results clearly show that colonisation of *P. hybrida* by *G. mosseae* positively affected growth parameters of 5-week-old plantlets in a soilless culture system. Such effects in an inert substrate without any ion exchange capacity are probably due to the efficient P uptake capacities of extraradical hyphae developing out from the roots (Smith and Read 2008) rather than the implication of P dissolving abilities of the AM fungus. The fact that the AM effect at 0.1 mM KH_2PO_4 fertiliser concentration was similar to that of 0.5 mM KH_2PO_4 in non-mycorrhizal plants indicates a fivefold economy of P

Fig. 1 *P. hybrida* shoot dry weight, water content, phosphorus content and uptake 5 weeks after inoculation with *G. mosseae* (M) and compared to corresponding control plants (NM). For all parameters, differences between treatments were significant according to t test ($P=0.05$, $n=3$)

Fig. 2 Shoot dry weight (A) and phosphorus content (B) of *P. hybrida* shoots 5 weeks after inoculation with *G. mosseae* and fertilised with 0.1 mM KH_2PO_4 (a), compared to control plants fertilised with 0.1 mM (b), 0.26 mM (c), 0.5 mM (d) or 1 mM KH_2PO_4 (e); *t* test analysis ($P=0.05$, $n=4$) indicates significant differences between mycorrhizal plants and all NM treatments except for plants fertilised with 0.51 mM KH_2PO_4 (d). nd Not enough material to determine the phosphorus content

fertilisation due to the use of mycorrhiza in the system. This shows that previous results by Gaur and Adholeya (2000) who calculated a 30% reduction in costs for mineral fertilisers in petunia production when using AM fungal inoculum can be also achieved in a soilless substrate.

3.2 Impact of *G. mosseae* on petunia tolerance against salt stress

When established mycorrhizal systems (5 weeks after inoculation) were challenged for 2 weeks with a high salt concentration, mycorrhization parameters increased (Table 1). In particular, colonisation of roots by *G. mosseae* (M%) was 2.5 times more intense and arbuscule abundance in root systems (A%) was more than fivefold higher compared to

Table 1 Mycorrhizal colonisation in *P. hybrida* roots 5 weeks after inoculation with *G. mosseae* and an additional 2 weeks without (M) and with salt stress (Ms: 250 mM NaCl)

	F%	M%	m%	a%	A%
M	96	10	10	25	2.8
Ms	99	25*	25*	58*	14.5*

F% colonisation frequency of the root system, M% colonisation intensity of the root system, m% colonisation intensity in mycorrhizal parts of the root system, A% arbuscule abundance in the root system, a% arbuscule abundance in mycorrhizal parts of the root system

* $P=0.05$, $n=3$, significant differences compared to the M treatment according to one-way ANOVA followed by Fisher's test

plants not grown under salt stress. This differs from previous observations where colonisation rates decreased under salt stress (Al-Karaki et al. 2001; Latef and Chaoxing 2011). Positive effects of the AM fungus were observed on plant biomass, water content and P content in non-stressed plants but all parameters showed a significant decline with salt stress which was more pronounced in mycorrhizal (46%, 71% and 29%, respectively) than non-mycorrhizal petunia (33%, 57% and 20%, respectively; Fig. 3). As expected, petunia turned out to be highly sensitive to salt stress. Inoculation with *G. mosseae* did not increase petunia tolerance and no positive interactions were observed between the factors 'mycorrhiza' and 'salt'. Elimination by the salt stress of the mycorrhizal responsiveness of petunia was not due to a negative effect on AM fungal development itself as mycorrhization parameters were enhanced in plants grown in the presence of salt, suggesting that mycorrhizal effectiveness rather than root colonisation had been affected. Salt

Fig. 3 Interaction between mycorrhiza and salt stress in *P. hybrida* 5 weeks after inoculation with *G. mosseae* and an additional 2 weeks in the absence and presence of salt stress (250 mM NaCl). Shoot dry weight (A), shoot water content (B) and phosphorus content (C) in plants inoculated with *G. mosseae* (M) compared to corresponding control plants (NM). Two-way ANOVA ($P=0.05$, $n=3$) revealed a significant interaction between the factors *G. mosseae* and salt stress for the parameters shoot dry weight and shoot water content. Letters above columns indicate significant differences between treatments using Fisher's test ($P=0.05$, $n=3$). Two-way ANOVA ($P=0.05$, $n=3$) revealed no significant interaction between the factors *G. mosseae* and salt stress for phosphorus content. Significant differences between mycorrhizal plants and controls are indicated by asterisks and significant differences between stressed and the corresponding non-stressed colonised or non-colonised plants are indicated by hash icons

stress tolerance induced by mycorrhiza has not been analysed in petunia before, but has in several other plants including the closely related species tomato where results indicated negative to positive interactions (e.g. Al-Karaki et al. 2001; Huang et al. 2010; Latef and Chaoxing 2011). Screening of other AM fungi to obtain mycorrhiza-induced salt tolerance in petunia will be useful. For example, a *Glomus* isolate from a saline soil was more efficient in ensuring growth of lettuce plants under salt-stressed conditions, as compared to a reference isolate (Ruiz-Lozano and Azcon 2000). In general, mechanisms of mycorrhiza-increased tolerance against salt stress seem to differ from those active against drought; the *G. mosseae* isolate used in the present study did not confer salt tolerance whilst it can clearly increased drought tolerance in petunia (Shamshiri et al. 2011).

3.3 Effect of AM fungi on petunia interactions with root pathogens

In order to investigate the potential of mycorrhiza in controlling important root diseases in petunia nursery production, a pathosystem was established. Among the four pathogenic fungi tested, clear disease symptoms could not be observed on roots after inoculation of petunia with *F. oxysporum*, *P. aphanidermatum* or *R. solani*, whilst *T. basicola* caused typical browning of petunia roots that was obvious without any staining (Fig. 4). *T. basicola* is a common pathogen with a wide host range, including petunia, where the fungus causes root pruning, foliar stunting and severe yield losses (Johnson 1916). Resistant cultivars of petunia are not known which leaves fungicide application as the only current strategy to combat this pathogen (David 2008).

In order to assess the potential of AM fungi to reduce such symptoms, mycorrhizal and non-mycorrhizal plants were inoculated with *T. basicola*. In a preliminary

Fig. 4 Disease symptoms in a *P. hybrida* root 2 weeks after inoculation with *T. basicola*. The root browning (black arrows) is associated with the presence of chlamydospores and mycelium

experiment, *G. mosseae* development was unaffected two weeks after challenging 5-week-established mycorrhizal plants with the pathogen (data not shown) whilst DS in roots was significantly reduced from 0.86 in non-mycorrhizal plants to 0.22 in mycorrhizal plants. In a second experiment comparing the effectiveness of *G. mosseae*, *G. rosea* and *G. intraradices* against *T. basicola*, DS, mycorrhization, shoot biomass and P content were assessed 4 weeks after challenging 5-week-established mycorrhizal plants with the pathogen. At this time point (9 weeks after AM fungal inoculation), *G. mosseae* root colonisation was comparable to that observed at 5 weeks, but arbuscule abundance was considerably higher (Table 2 versus Table 1). Root colonisation and arbuscule development by *G. rosea* were very low as compared to *G. mosseae*, whilst *G. intraradices* reached the highest values for both parameters (Table 2).

Nine weeks after inoculation, shoot dry mass and water content were no longer significantly affected by mycorrhization with *G. mosseae* in the absence of *T. basicola*, but P content was still considerably higher (Fig. 5). *G. intraradices*, however, increased neither plant biomass nor P uptake compared to control plants. This observation is in agreement with the previously reported better capacity of *G. mosseae* compared to *G. intraradices* in supplying nutrients to petunia plants (Shamshiri et al. 2011). In contrast to the *Glomus* species, *G. rosea* had a negative influence on plant biomass

Table 2 Mycorrhizal colonisation and disease severity (DS) in *P. hybrida* roots 5 weeks after inoculation with three different AM fungi: *G. mosseae* (Gm), *G. rosea* (Gr) or *G. intraradices* (Gi) and an additional 4 weeks after inoculation with *T. basicola* (+Tb)

	F%	M%	m%	a%	A%	DS
Gm	100	13.0 ac	13 a	77 ad	10.0 ac	
Gm+Tb	96	18.7 a	19 ab	83 cd	15.7 a	0.065*
Gr	61 b	4.0 c	5 ac	46 ab	3.0 c	
Gr+Tb	37	3.0 c	2 c	31 b	2.0 c	0.240
Gi	100	32.0 b	32 b	92 cd	29.5 b	
Gi+Tb	97	19.0 a	13 ac	80 acd	14.0 ab	0.320
NM+Tb						0.490

Mycorrhizal colonisation parameters are compared between the 6 different treatments: Gm, Gr, Gi, Gm+Tb, Gr+Tb and Gi+Tb. ANOVA was used to determine the effect of the factor 'infection with *T. basicola*' (Tb) on mycorrhization parameters. Different letters indicate significant differences between values using Fisher's test ($P=0.05$, $n=3$). DS of *T. basicola* was compared between treatments in absence of the AM fungus (NM+Tb), or in plants colonised by *G. mosseae* (Gm+Tb), *G. rosea* (Gr+Tb) and *G. intraradices* Gi+Tb

F%, M%, m%, A%, a%, see Table 1 for explanation, DS disease severity

* $P=0.05$, $n=3$, significant difference of *G. mosseae*-colonised roots to the control according to *t* test

Fig. 5 Effects of interactions between three AM fungi and *T. basicola* on *P. hybrida* shoot dry weight (A) shoot water content (B) and phosphorus content (C) 5 weeks after inoculation with each AM fungus and a further 4 weeks after infection with *T. basicola*: control, inoculated with *G. mosseae*, *G. rosea* or *G. intraradices* and infected with the pathogen (+Tb) or not (-Tb). Two-way ANOVA ($P=0.05$, $n=3$) revealed no interaction between the factors AM fungus (*G. mosseae*, *G. rosea* or *G. intraradices*) and *T. basicola*. Significant differences between mycorrhizal plants and the corresponding controls among the pathogen-inoculated or the pathogen-free plants are indicated by asterisks. Among the mycorrhizal plants or the corresponding controls, significant differences between pathogen-inoculated plants and pathogen-free plants are indicated by hash icons (Fisher's test, $P=0.05$, $n=3$)

(Fig. 5). Such negative effects of this fungus have been observed before and are probably based on its higher carbohydrate sink strength (Lerat et al. 2003).

In *G. mosseae*-colonised petunia plants inoculated with *T. basicola*, mycorrhization was barely affected, but DS was significantly reduced by the presence of this AM fungus (Table 2) and to a greater extent (7.5-fold) 4 weeks after pathogen infection as compared to after 2 weeks in the preliminary experiment (3.9-fold). Infection by *T. basicola* significantly reduced dry mass in petunia but to a lesser extent in *G. mosseae*-colonised plants than in controls. Shoot water and mycorrhizal-enhanced P content were unaffected by pathogen development in roots (Fig. 5). Neither *G. rosea* nor *G. intraradices* reduced DS following inoculation with *T. basicola* (Table 2) and the presence of the pathogen significantly reduced the development of *G.*

intraradices within petunia roots (Table 2). Such negative effects on AM fungal development have been observed before in the interaction between pea and *Aphanomyces euteiches* where also no correlation between mycorrhiza colonisation levels and mycorrhiza-induced resistance was observed (Bodker et al. 2002). The present results showed that the induced resistance associated with bioprotection was specific for *G. mosseae*. Although mycorrhiza-induced resistance has been reported for different AM fungi in different plants (Whipps 1997), effects of *G. rosea* have not previously been investigated and direct comparisons have always shown that *G. mosseae* is the most effective for members of the Solanaceae (e.g. Pozo et al. 1999; Garmendia et al. 2004). The basis for differences between AM fungi is largely unknown, but the level of mycorrhization at the moment of pathogen inoculation does not appear to play a role because this was higher for *G. intraradices* in the present study. Differences in mycorrhiza-induced resistance could be based in the specific expression of particular plant genes or proteins. For example, a β -1,3-glucanase was only expressed after *Phytophthora* inoculation of tomato roots colonised by *G. mosseae*, but not in with the presence of *G. intraradices* (Pozo et al. 1999).

4 Conclusion

G. mosseae BEG12 is suitable for reducing phosphate fertiliser levels in petunia production, at least during early growth periods, and its use can be envisaged to increase resistance against root pathogens. The present study shows for the first time that this AM fungal isolate has potential as a biological agent for sustainable petunia production in soilless substrates, so meeting consumers' demands for ecologically produced ornamental crops. *G. mosseae* BEG12 is, however, not efficient in inducing salt tolerance of petunia and screening of other isolates is necessary to cover this facet of bioprotection by AM. In addition, the differential interaction of *G. mosseae* and *G. intraradices* with petunia can provide a novel system to analyse the molecular basis of different activities of AM fungi against plant pathogens. Profiling of petunia gene expression in *G. mosseae* versus *G. intraradices* mycorrhiza will help to identify specific functional markers for breeding novel cultivars adapted to sustainable plant production systems.

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