Research Article

Received: 26 June 2008 Revised: 6 April 2009 Accepted: 29 April 2009 Published online in Wiley Interscience: 25 June 2009

(www.interscience.wiley.com) DOI 10.1002/jsfa.3660

Interactions between arbuscular mycorrhizal fungi and *Trichoderma harzianum* and their effects on Fusarium wilt in melon plants grown in seedling nurseries

Ainhoa Martínez-Medina, Jose A Pascual,* Eva Lloret and Antonio Roldán

Abstract

BACKGROUND: Biological control through the use of *Trichoderma* spp. and arbuscular mycorrhizal fungi (AMF) could contribute to a reduction of the inputs of environmentally damaging agrochemical products. The objective of this study was to evaluate the interactions between four AMF (*Glomus intraradices, Glomus mosseae, Glomus claroideum* and *Glomus constrictum*) and *Trichoderma harzianum* for their effects on melon plant growth and biocontrol of Fusarium wilt in seedling nurseries.

RESULTS: AMF colonisation decreased fresh plant weight, which was unaffected by the presence of *T. harzianum*. Dual inoculation resulted in a decrease in fresh weight compared with AMF-inoculated plants, except for *G. intraradices*. AMF colonisation level varied with the AM endophyte and was increased by *T. harzianum*, except in *G. mosseae*-inoculated plants. Negative effects of AMF on *T. harzianum* colony-forming units were found, except with *G. intraradices*. AMF alone were less effective than *T. harzianum* in suppressing disease development. Combined inoculation resulted in a general synergistic effect on disease control.

CONCLUSION: Selection of the appropriate AMF species and its combination with *T. harzianum* were significant both in the formation and effectiveness of AM symbiosis and the reduction of Fusarium wilt incidence in melon plants. The combination of *G. intraradices* and *T. harzianum* provided better results than any other tested.

Keywords: *Trichoderma harzianum*; arbuscular mycorrhizal fungi; *Glomus* sp.; *Fusarium oxysporum*; melon; seedling nurseries

INTRODUCTION

Arbuscular mycorrhizal fungi (AMF) that form symbiotic relationships with the roots of most terrestrial plants are known to improve the nutritional status of their host and to protect plants against several soil-borne plant pathogens.[1–3] In this way the use of AMF as inoculants to benefit plant growth and health could contribute to a reduction of the inputs of pesticides and other environmentally harmful agrochemical products currently required for optimal plant growth and health.[4] However, this symbiotic association is not a characteristic common to all plants, and the incidence of root colonisation varies depending on the plant species and the AMF.[5] The successful use of AMF in sustainable agriculture requires selection of the appropiate host/fungus combination, infectivity and efficacy being two of the criteria for the appropriateness.[6,7] A successful selection has a significant importance in certain plant species that show a poor ability to form this symbiosis. Roots of melon plants have been reported to show a degree of mycorrhizal colonisation of between 2.5 and 17%, compared with 41% reported in cucumber plants, 46–59% in tomato, 60% in lettuce and 50% in soy bean.[8–12]

Species of *Trichoderma*, a non-pathogenic saprophyte, have been reported to supress fungal diseases in a number of crop plants.[13] Some strains are able to colonise the root surface, causing changes in plant metabolism and inducing a localised or systemic resistance response. This root colonisation by *Trichoderma* spp. frequently enhances root growth and development, crop productivity, resistance to abiotic stresses and the uptake and use of nutrients.[14]

In semi-arid Mediterranean conditions, most growers establish their melon (*Cucumis melo* L.) crops by transplanting nursery-raised plantlets from seedling nurseries rather than by direct seeding.[15] Therefore this horticultural crop is usually grown in containers with soilless media that lack AMF and biocontrol fungal propagules, favouring the development of soil diseases. Fusarium wilt, caused by the fungus *Fusarium oxysporum* (Schlectend) f.sp. *melonis*, is one of the most destructive diseases of melon crops worldwide and can reduce yields by up to 90%.[15,16] Chemical methods used to control this vascular wilt are either not very efficient or cause negative effects on environmental and human health.[17,18] Research has demonstrated that biological control, through the

* *Correspondence to: Jose A Pascual, CSIC-Centro de Edafología y Biología Aplicada del Segura, Campus Universitario de Espinardo, E-30100 Murcia, Spain. E-mail: jpascual@cebas.csic.es*

CSIC-Centro de Edafología y Biología Aplicada del Segura, Campus Universitario de Espinardo, E-30100 Murcia, Spain

use of beneficial fungi such as *Trichoderma*, is a potentially feasible alternative to the use of fumigants or fungicides.[19,20]

The study of interactions between beneficial organisms associated with plant roots is important, because such interactions might either enhance or inhibit the beneficial effects of individual species. These interactions must be identified and characterised for their successful establishment in plants. AMF have been shown to cause changes in the rhizosphere that could affect microbial populations both qualitatively and quantitatively and to promote the activity of other micro-organisms that may compete with soil-borne pathogens.[21,22] It has also been demonstrated that some *Trichoderma* strains may influence AMF activity.[18,23] Little is known about the interactions between AMF and beneficial saprophytic fungi, and the few studies published on this topic do not provide any conclusive statements owing to the small number of fungi and host plants that have been studied so far.[24,25]

We hypothesise that the combined use of AMF and *Trichoderma harzianum* (Rifai) can improve plant growth and protection against the pathogen *F. oxysporum* within the context of a sustainable soil–plant system. To achieve this aim, dual inoculation with four AMF from the genus *Glomus* (*G. constrictum* (Trappe), *G. mosseae* (Nicol. & Gerd) Gerdemann & Trappe, *G. claroideum* (Schenck & Smith) and *G. intraradices* (Schenk & Smith)) and the fungus *T. harzianum* was evaluated, in the presence or absence of the pathogen *F. oxysporum*, with regard to (1) the capacity for root colonisation of each AMF in the presence or absence of *T. harzianum*, (2) the survival of *T. harzianum* populations in the rhizosphere in the presence or absence of the AMF, (3) the growth and nutrition of melon plants in seedling nurseries and (4) the biocontrol by the AMF and *T. harzianum* of Fusarium wilt in melon plants in seedling nurseries.

MATERIALS AND METHODS

Host plant and fungal inocula

Giotto melon (*C. melo* L.) was used in all assays as the host plant. The AMF used were four isolates from the genus *Glomus* (*G. constrictum, G. mosseae, G. claroideum* and *G. intraradices*) obtained from the collection of CEBAS-CSIC (Murcia, Spain). The AM fungal inoculum consisted of a mixture of rhizospheric soil from trap cultures (*Sorghum vulgare*) containing 35 infective propagules g^{-1}. *Trichoderma harzianum* was isolated from agricultural soil and is deposited at the Spanish Type Culture Collection (CECT 20 714). It was characterised by its high *in vitro* capacity to control *F. oxysporum* and its survival on peat.[26] For the production of inocula, a specific solid medium was prepared by mixing commercial oat, bentonite, vermiculite and distilled water.[26] This blend was mixed by hand and sterilised by autoclaving for 1 h at 121 °C twice, once on each of two consecutive days. The substrate was inoculated with the isolated *T. harzianum* and incubated at 28 °C for 8 days in darkness, obtaining a fungal suspension containing 10^9 conidia g^{-1}. The concentration of conidia was determined with a haematocytometer.

Monoconidial *F. oxysporum* (Schlectend) f.sp. *melonis* race 1,2 was isolated from infected melon plants obtained from a seedling nursery. For the production of inocula, the pathogen was cultivated for 5 days in potato dextrose broth (Scharlau Chemie, Barcelona, Spain) amended with 100 mg L^{-1} streptomycin sulfate, at 28 °C in darkness, on a shaker at 120 rpm. After the incubation period the fungal culture was centrifuged at 193 × *g*, resuspended in sterilised water and re-centrifuged. The fungal suspension contained 10^8 conidia $2mL^{-1}$.

Experimental design and growth conditions

A factorial design of randomised blocks was established with three factors and fivefold replication. The first factor had five levels: non-inoculation and inoculation with four AMF (*G. constrictum, G. mosseae, G. claroideum* and *G. intraradices*). The second factor had two levels: non-inoculation and inoculation with *T. harzianum*. The third factor had two levels: non-inoculation and inoculation with *F. oxysporum* in the seedling nursery.

Specific nursery polystyrene plant containers with 10 individual wells were filled with the different treatments (7 g per well). Each specific container was considered as a unit, and five replicates of each treatment were used. Ten melon seeds were sown (one seed per well) in the polystyrene container and covered with vermiculite.

The different treatments were prepared by mixing commercial peat with the different inocula. The peat (0.37 g kg^{-1} total N, 1.158 mg kg^{-1} available P and 1.34 mg kg^{-1} available K) was sterilised by autoclaving for 1 h at 121 °C twice, once on each of two consecutive days. The AM inocula were mixed with the sterilised peat at a rate of 17 g kg^{-1}, while the *T. harzianum* inoculum was mixed with the sterilised peat to reach a population density of 10^6 conidia g^{-1} peat.

The experiment was carried out in a seedling nursery. Seedlings were grown using standard nursery culture conditions, which included germination in a controlled environment germination chamber until seedling emergence. After 60 h the individual containers with pre-germinated seeds were moved in a random design and placed in nursery beds that included irrigation without fertiliser.

Three weeks after planting, half of the experiment was inoculated with *F. oxysporum* to reach a final concentration of 10^4 conidia g^{-1} peat. Six weeks after planting, plants were harvested and substrate samples were taken from the wells and stored at 4 °C for biological and biochemical analyses.

Plant analyses

The fresh and dry (105 °C, 5 h) weights of shoots were recorded. Plant tissues were ground before chemical analysis. Phosphorus and potassium concentrations were determined after digestion in nitric acid/perchloric acid (2 : 1 v/v) for 2 h; phosphorus was determined by colorimetry and potassium by flame photometry.[27–29] Nitrogen concentration was determined by a modified Kjedahl method.[30]

Roots were cleaned with 100 g L^{-1} KOH and stained with 0.5 g L^{-1} trypan blue.[31] The percentage root length colonised by AMF was calculated by the gridline intersect method.[32] Positive counts for AM colonisation included the presence of vesicles or arbuscules or typical mycelia within the roots.

Substrate biological analysis

Substrate samples were suspended on sterilised Ringer solution (1 : 10 w/v), and 1/10 serial dilutions were done to calculate the number of colony-forming units (CFU) of *T. harzianum* by plating them on 1/10 potato dextrose agar (PDA) (Scharlau Chemie) amended with 50 mg L^{-1} rose bengal and 100 mg L^{-1} streptomycin sulfate. Komada medium was used as a selective medium for quantitative isolation of *F. oxysporum*.[33] Plates were incubated at 28 °C for 6 days. After the incubation period the CFU were counted.

To determine colonisation by *F. oxysporum* in inoculated plants, stem segments (∼1.5 cm) immediately above crowns

Table 1. Fresh and dry weights of melon plants inoculated with *Trichoderma harzianum* and/or *Glomus constrictum, Glomus mosseae, Glomus claroideum* or *Glomus intraradices*, 42 days after planting

	Fresh weight (g)		Dry weight (g)	
AMF	No *T. harzianum*	*T. harzianum*	No *T. harzianum*	*T. harzianum*
No AMF	5.80 ± 0.19c	5.40 ± 0.04bc	0.72 ± 0.02g	0.59 ± 0.01cdef
G. constrictum	4.95 ± 0.25ab	4.41 ± 0.44a	0.56 ± 0.03bcde	0.52 ± 0.05abc
G. mosseae	4.24 ± 0.08a	4.44 ± 0.53a	0.47 ± 0.01a	0.49 ± 0.06ab
G. claroideum	5.80 ± 0.45c	5.32 ± 0.27bc	0.67 ± 0.05fg	0.61 ± 0.03def
G. intraradices	5.33 ± 0.38bc	4.26 ± 0.29a	0.57 ± 0.04bcde	0.44 ± 0.03a

Data are mean ± standard deviation of five replicates. For each analysis, values in the same row or column with the same letters represent no significant difference between treatments according to Tukey's multiple range test ($P \leq 0.05$).

were cut from inoculated plants, surface sterilised by soaking in 10 g L^{-1} sodium hypochlorite for 5 min and rinsed with sterilised water. The segments were incubated on PDA at 28 °C for 6 days and the appearance of *F. oxysporum* colonies was observed.

Statistical analysis

The data were subjected to analysis of variance (ANOVA) using (SPSS Inc, Chicago, IL, USA) for Windows, Version 15.0. The statistical significance of the results was determined by performing Tukey's multiple range test ($P < 0.05$).

RESULTS

Interactions between AMF and *T. harzianum* in absence of pathogen

Effects on plant weight

A significant decrease in both fresh and dry weights was observed in the treatments with the AMF alone compared with the control, except for *G. claroideum* (Tables 1 and 3). The lowest fresh and dry weights were observed in plants inoculated with *G. mosseae*. Inoculation with *T. harzianum* alone did not affect fresh weight compared with non-inoculated plants. A lower plant fresh weight was observed when the peat was co-inoculated with both AMF and *T. harzianum* than when each AMF was inoculated alone, except with *G. mosseae*. The treatment *G. intraradices* plus *T. harzianum* was the only treatment that significantly reduced fresh weight compared with AMF treatments alone. However, independently of *T. harzianum* inoculation, the latter AMF gave the highest weight of the AMF assayed.

Effects on nutrient content

Total plant nitrogen was affected by *T. harzianum* inoculation but not by AMF (Tables 2 and 3). Dual inoculation of *T. harzianum* with AMF did not show any significant difference in total nitrogen from *T. harzianum* inoculation alone. Combination of *T. harzianum* with *G. mosseae* or *G. claroideum* significantly decreased nitrogen levels compared with inoculation with *G. mosseae* or *G. claroideum* alone. The total phosphorus level was not affected by *T. harzianum* inoculation. The presence of AMF significantly decreased the

Table 2. Levels of nitrogen, phosphorus and potassium in melon plants inoculated with *Trichoderma harzianum* and/or *Glomus constrictum, Glomus mosseae, Glomus claroideum* or *Glomus intraradices*, 42 days after planting

	Nitrogen (g kg^{-1})		Phosphorus (g kg^{-1})		Potassium (g kg^{-1})	
AMF	No *T. harzianum*	*T. harzianum*	No *T. harzianum*	*T. harzianum*	No *T. harzianum*	*T. harzianum*
No AMF	1.01 ± 0.10c	0.82 ± 0.07ab	0.65 ± 0.05c	0.63 ± 0.02c	1.96 ± 0.07a	2.07 ± 0.01abc
G. constrictum	0.96 ± 0.05abc	0.85 ± 0.02abc	0.40 ± 0.04b	0.66 ± 0.02c	2.65 ± 0.04cd	2.76 ± 0.06d
G. mosseae	1.02 ± 0.03c	0.79 ± 0.04a	0.25 ± 0.05a	0.70 ± 0.02c	2.70 ± 0.21d	2.53 ± 0.18bcd
G. claroideum	0.99 ± 0.12c	0.80 ± 0.05a	0.63 ± 0.05c	0.68 ± 0.10c	2.25 ± 0.2abcd	2.41 ± 0.39bcd
G. intraradices	0.90 ± 0.06abc	0.88 ± 0.12abc	0.42 ± 0.04b	0.68 ± 0.05c	2.42 ± 0.08bcd	2.70 ± 0.21cd

Data are mean ± standard deviation of five replicates. For each analysis, values in the same row or column with the same letters represent no significant difference between treatments according to Tukey's multiple range test ($P \leq 0.05$).

Table 3. Two-factor ANOVA (AMF inoculation and *Trichoderma harzianum* inoculation) for parameters studied: *P* significance values

Factor	Plant fresh weight	Plant dry weight	N	P	K	AM root colonisation	*T. harzianum* population
AMF inoculation (AM)	*	**	NS	*	**	***	NS
T. harzianum inoculation (Th)	NS	*	*	NS	NS	NS	***
Interaction Th × AM	*	*	NS	NS	NS	***	*

Significance: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; NS, not significant.

phosphorus level compared with the control, except in plants inoculated with *G. claroideum*. The lowest level of phosphorus was observed in plants inoculated with *G. mosseae*. However, dual inoculation did not reduce the phosphorus level relative to the control. Total potassium was not affected by *T. harzianum*. Inoculation with AMF, except for *G. claroideum*, did significantly increase potassium levels; this was maintained when *T. harzianum* was co-inoculated with the AMF.

Effects on AMF and T. harzianum

The inoculation with the different AMF produced a significant increase in AM root colonisation, depending on the AMF inoculated (Fig. 1 and Table 3). The lowest percentage of AM colonisation was obtained in plants inoculated with *G. claroideum*. The presence of *T. harzianum* significantly increased AM colonisation, except in the treatment with *G. mosseae*. The number of *T. harzianum* colonies recovered from the substrates co-inoculated with AMF decreased significantly compared with no AMF treatment (inoculated with *T. harzianum*), except in the case of inoculation with *G. intraradices* (Fig. 2 and Table 3). *Trichoderma harzianum* was detected below 2.5 log CFU g^{-1} in the treatments that did not include inoculation with *T. harzianum*, showing no significant differences between the different AMF inocula (data not shown).

Interactions between AMF and *T. harzianum* under pathogen pressure

Effects on plants

The percentage of plants infected by *F. oxysporum* in treatments involving inoculation with AMF alone was decreased significantly compared with the control, except in the case of plants inoculated with *G. claroideum* (Fig. 3). The lowest number of infected plants was observed in treatments involving inoculation with *G. mosseae*. Inoculation with *T. harzianum* significantly reduced the percentage of infected plants. Co-inoculation of *T. harzianum* and AMF was also effective in suppressing disease development relative to the control; furthermore, the infection percentages observed in

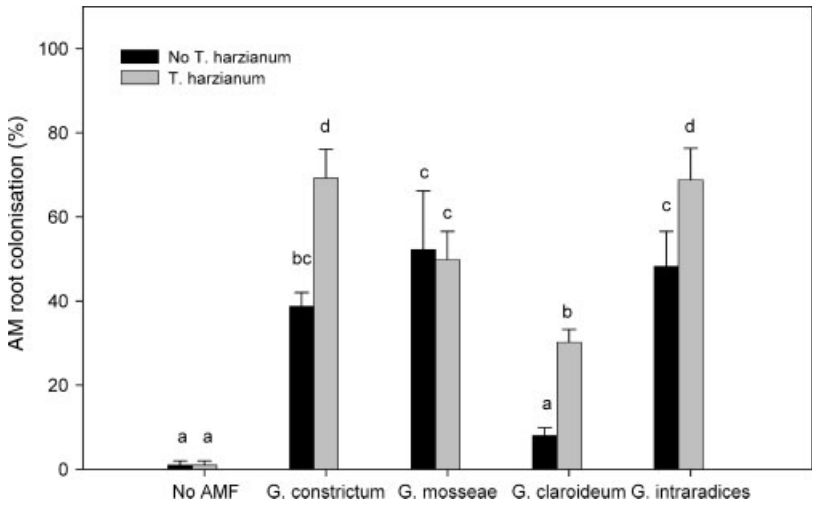

Figure 1. Percentage of root length colonised by *Glomus constrictum, Glomus mosseae, Glomus claroideum* and *Glomus intraradices* for melon plants co-inoculated or not with *Trichoderma harzianum*, 42 days after planting. Bars indicate standard deviation. Values with the same letters represent no significant difference between treatments according to Tukey's multiple range test at the $P = 0.05$ level ($n = 5$).

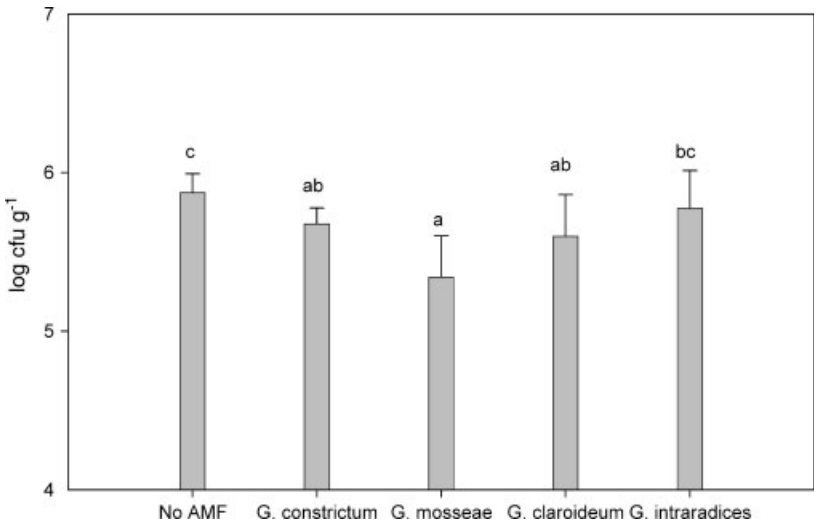

Figure 2. Number of *Trichoderma harzianum* colonies recovered from substrate in treatments co-inoculated with *T. harzianum* and *Glomus constrictum, Glomus mosseae, Glomus claroideum* or *Glomus intraradices*, 42 days after planting. Bars indicate standard deviation. Values with the same letters represent no significant difference between treatments according to Tukey's multiple range test at the $P = 0.05$ level ($n = 5$).

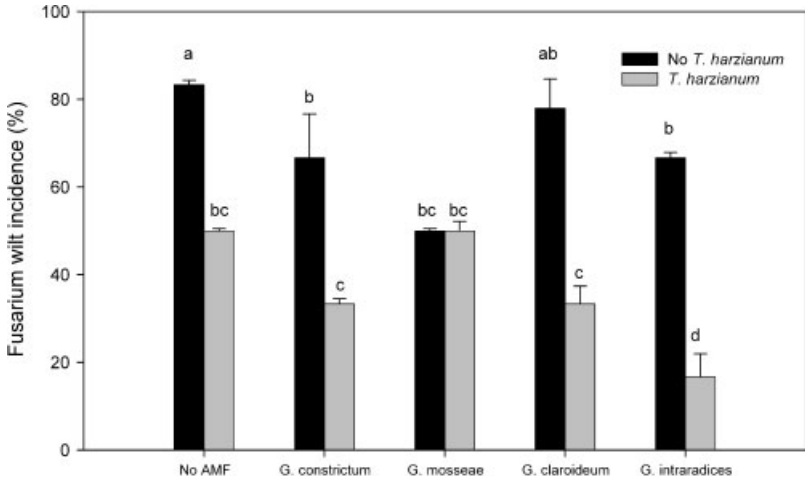

Figure 3. Percentage of plants infected by *Fusarium oxysporum* in treatments inoculated with *Trichoderma harzianum* and/or *Glomus constrictum*, *Glomus mosseae*, *Glomus claroideum* or *Glomus intraradices*, 42 days after planting. Bars indicate standard deviation. Values with the same letters represent no significant difference between treatments according to Tukey's multiple range test at the $P = 0.05$ level ($n = 5$).

the dual-inoculated plants were significantly lower ($P < 0.001$) compared with plants treated only with the AMF, except for the *G. mosseae* treatment.

Effects on F. oxysporum

Inoculation with the AMF alone did not affect the *F. oxysporum* population; however, inoculation with *T. harzianum* significantly increased the pathogen population ($P < 0.001$) (Table 4). Co-inoculation with *T. harzianum* and the AMF resulted in pathogen populations similar to those of treatments inoculated with the AMF alone.

DISCUSSION

It is a well-known fact that the AM symbiosis improves the host growth and nutrition.[34,35] However, in our experiment, root colonisation by the AMF alone resulted in a decrease in plant growth. The fact that the lowest fresh and dry plant weights occurred in plants co-inoculated with AMF and *T. harzianum* seems to be a direct consequence of the action of the saprophytic fungus on AM root colonisation, as the saprophytic fungus increased the root colonisation. These findings may indicate that, although establishment of the symbiosis took place during the early stages (first 40 days), it was not functional until later stages.[36] An initial growth depression in AM plants followed by a growth enhancement has been reported in the association between alfalfa and *G. mosseae*.[37]

A similar decrease in phosphorus levels was observed in AMF-inoculated plants, but not when they were co-inoculated with *T. harzianum*. Several studies have demonstrated the capacity of *T. harzianum* to solubilise inorganic nutrients and to increase nutrient uptake.[38,39] Altomare *et al*.[38] hypothesised that phosphorus could be solubilised and stored in the *Trichoderma* biomass, to be released in a readily available form in close proximity to the roots after lyses of the aged mycelium.

Inoculation with *T. harzianum*, co-inoculated or not with AMF, produced a negative effect on nitrogen uptake. This result may be explained by competition between plant roots and *T. harzianum*, since, in many cases, micro-organisms are superior competitors.[40,41]

Endomycorrhizal fungi have been reported to lack a taxonomic specificity; however, plant species vary in the degree to which they respond to AM fungal species.[1,5] In our experiment, although all the AMF tested were able to colonise roots of *C. melo*, the AM colonisation level varied with the AM endophyte, reaching values around 40% in almost all cases except for *G. claroideum*, a significant increase in comparison with the usual level of mycorrhizal colonisation of melon roots.[8] A synergistic interaction regarding AM formation between the saprophytic fungus *T. harzianum* and the AMF was observed, as reported also by García-Romera *et al*.[42] and Fracchia *et al*.[43,44] In our work the presence of *T. harzianum* significantly increased root colonisation by *G. intraradices, G. constrictum* and *G. claroideum*, reaching values significantly higher than the most effective AMF inoculated alone (*G. mosseae*). Similar interactions have been proposed for other saprophytic fungi.[43,45–48] A role for soluble exudates and volatile compounds produced by saprophytic fungi has been proposed to explain these findings.[45] This result contrasts markedly with the absence of effects observed in other studies, such as that of Camprubí *et al*.[49] or Vázquez *et al*.[25] It contrasts also with the antagonistic effect observed by McAllister *et al*.[47] and Martínez *et al*.[23]

Table 4. *F. oxysporum* population ($\times 10^4$ CFU/g) recovered from the substrate in treatments inoculated with *T. harzianum*, and/or *G. constrictum*, *G. mosseae*, *G. claroideum* or *G. intraradices* 42, days after planting.

	F. oxysporum population	
AMF	No *T. harzianum*	*T. harzianum*
No AMF	1.02 ± 0.26ab	13.50 ± 1.22d
G. constrictum	2.35 ± 0.60bc	1.20 ± 0.98ab
G. mosseae	1.75 ± 0.63abc	2.65 ± 1.10bc
G. claroideum	2.05 ± 0.10abc	3.33 ± 1.46c
G. intraradices	1.07 ± 0.87ab	0.50 ± 0.06a

Data are mean ± standard deviation of five replicates. Values in the same row or column with the same letters represent no significant difference between treatments according to Tukey's multiple range test ($P \leq 0.05$).

A general antagonistic effect on *T. harzianum* populations due to the AMF was observed, except in the case of the co-inoculation with *G. intraradices*. Modifications of root exudates caused by the AMF may explain this finding.[50] Furthermore, substances released by the extrametrical mycelia of MA fungi may also influence microbial populations.[21] Competition between *T. harzianum* and AMF in the first stages could also explain these findings.[24,47] Similar results have been observed by Fracchia *et al*.,[48] while stimulation of the germination of *T. harzianum* conidia by *G. intraradices* has been reported by Filion *et al*.[21] This finding suggests a complex interaction between *T. harzianum* and the AMF.

A noteworthy fact is that the effects of the interaction between AMF and *T. harzianum* may be very different depending on the AMF and the saprophytic strain.[23] In our experiment a greater antagonistic effect on the *T. harzianum* population was observed in the presence of *G. mosseae*; interestingly enough, no synergistic effects on AM root colonisation by *G. mosseae* were observed in the presence of *T. harzianum*.

AM symbiosis has been shown to reduce the damage caused by soil-borne plant pathogens.[51] In our experiment, lower percentages of plants infected by *F. oxysporum* were observed in AM plants. Competition for host photosynthates or site and microbial changes in the mycorhizosphere due to AM have been proposed.[52,53] A negative effect of AMF on the development of *F. oxysporum* populations has been reported.[21,54] However, no reduction of the *F. oxysporum* population was observed in the organic substrate in the presence of AMF. Therefore other mechanisms could explain the lower incidence of *F. oxysporum* on melon plants observed in our experiment, such as improved resistance and/or tolerance against the pathogen. Improvement of plant nutrition has been reported as one of the mechanisms by which AM association could control root pathogens, since increased nutrient uptake results in more vigorous plants.[51]

Trichoderma harzianum itself was more effective than the AMF with regard to suppressing disease development. Several pieces of work report the biocontrol capacity of *Trichoderma* spp.[13] Various mechanisms of biocontrol have been reported, such as mycoparasitism, antibiotic production or competition.[14] Interestingly, in this work we observed an increase in the pathogen population in the substrate. Ros *et al*.[55] reported a similar effect for *F. oxysporum* colonies in a substrate when green compost was added. Therefore other mechanisms, rather than a direct interaction of the biocontrol agent and the pathogen, can explain the suppression of the disease development by *T. harzianum*, such as an induction of plant defence.[56]

Combined inoculation of *T. harzianum* and AMF resulted in a general synergistic effect on disease control. This finding supports the hypothesis of Linderman[57] that AMF and *T. harzianum* function in tandem to control root diseases. The majority of studies considered so far concern a single pathogen and a single biocontrol agent in the rhizosphere. However, one way of improving biocontrol in the rhizosphere may be to add combinations of biocontrol agents, particularly if they exhibit different or complementary modes of action; such multiple interactions are the normal situation in the rhizosphere.[58] Several combinations have been studied: for example, *Trichoderma koningii* combined with *Pseudomonas chlororaphis* 30–84 or *Pseudomonas fluorescens* Q2-87 gave higher suppression of take-all in wheat than *T. koningii* alone.[59] *Trichoderma* (*Glicocladium*) *virens* GI-3 combined with *Burkholderia cepacia* provided greater protection to pepper seeds than either antagonist inoculated alone in the presence of four soil-borne pathogens.[60] Datnoff *et al*.[61] reported a higher suppressive effect against Fusarium crown and root rot of tomato with the combination of *T. harzianum* and *G. intraradices* than with either agent applied alone.[61] However, there are several examples of combinations of different biocontrol agents providing no better or, in some cases, worse biocontrol than the isolates used singly.[62,63]

In conclusion, since the melon plants were susceptible to AM root colonisation, initial growth and seedling nurseries seem to be a convenient time and place respectively to perform AM inoculation, as a high percentage of root colonisation was achieved, although a general increase in plant growth and nutrition was not observed in the greenhouse nursery. Selection of the appropriate AM fungal species and its combination with other fungi such as *T. harzianum* seem to be important in the effectiveness of AMF, enhancing formation of the symbiosis. Furthermore, combinations of AMF and *T. harzianum* were able to control Fusarium wilt more effectively than either agent applied alone. The combination of *G. intraradices* and *T. harzianum* provided better results than any other tested in this work. Finally, a commercial formulation of *G. intraradices* and *T. harzianum* strain CECT 20 714 could be effective in greenhouse nurseries, with a double objective: (a) to prepare melon plants with a high level of mycorrhization and (b) to reduce the incidence of Fusarium wilt in melon plants. Interactions between these two microbial agents should be researched deeply to understand the mechanisms involved and with the aim of directing inoculations to achieve the proposed objectives.

REFERENCES

1 Smith SE and Read DJ, *Mycorrhizal Symbiosis* (2nd edn). Academic Press, London (1997).
2 Harrison MJ, Molecular and cellular aspects of the arbuscular mycorrhizal symbiosis. *Annu Rev Plant Physiol Plant Mol Biol* **50**:361–389 (1999).
3 Bi HH, Song YY and Zeng RS, Biochemical and molecular responses of host plants to mycorrhizal infection and their roles in plant defense. *Allelopath J* **29**:15–27 (2007).
4 Barea JM, Azcón-Aguilar C and Azcón R, Interactions between mycorrhizal fungi and rhizosphere microorganisms within the context of sustainable soil–plant systems, in *Multitrophic Interactions in Terrestrial Systems*, ed. by Gange AC and Brown VK. Blackwell Science, Cambridge, pp. 65–77 (1997).
5 Jeffries P and Barea JM, Arbuscular mycorrhiza – a key component of sustainable plant–soil ecosystems, in *Fungal Associations*, Vol. IX of *The Mycota*, ed. by Hock B. Springer, Berlin, pp. 95–113 (2001).
6 Tarbell TJ and Koske RE, Evaluation of commercial arbuscular mycorrhizal inocula in a sand/peat medium. *Mycorrhiza* **18**:51–56 (2007).
7 Haas JH and Krikun J, Efficacy of endomycorrhizal-fungus isolates and inoculum quantities required for growth response. *New Phytol* **100**:613–621 (1985).
8 Akiyama K, Matsuoka H and Hayashi H, Isolation and identification of a phosphate deficiency-induced *C*-glycosylflavonoid that stimulates arbuscular mycorrhiza formation in melon roots. *Mol Plant–Microbe Interact* **15**:334–340 (2001).
9 Chandanie WA, Kubota M and Hyakumachi M, Interactions between plant growth promoting fungi and arbuscular mycorrhizal fungus *Glomus mosseae* and induction of systemic resistance to anthracnose disease in cucumber. *Plant Soil* **286**:209–217 (2006).
10 Edathil TT, Manian S and Udaiyan K, Interaction of multiple VAM fungal species on root colonization, plant growth and nutrient status of tomato seelings (*Lycopersicon esculentum* Mill.). *Agric Ecosyst Environ* **59**:63–68 (1996).
11 Kohler J, Caravaca F, Carrasco L and Roldan A, Interactions between a plant growth-promoting rhizobacterium, an AM fungus and a phosphate-solubilising fungus in the rizosphere of *Lactuca sativa*. *Appl Soil Ecol* **35**:480–487 (2007).
12 Garcia-Garrido JM, Tribak M, Rejon-Palomares A, Ocampo JA and Garcia-Romera I, Hydrolytic enzymes and ability of arbuscular mycorrhizal fungi to colonize roots. *J Exp Bot* **51**:1443–1448 (2000).

13 Howell CR, Mechanisms employed by *Trichoderma* species in the biological control of plant diseases: the history and evolution of current concepts. *Plant Dis* **87**:4–10 (2003).
14 Harman GE, Howell CR, Viterbo A, Chet I and Lorito M, *Trichoderma* species opportunistic, avirulent plant symbionts. *Nature Rev Microbiol* **2**:34–56 (2004).
15 Soriano ML, Porras-Piedra A, Marcilla Goldaracena I, Porras Soriano A, Martín-Sánchez D, León-Egido M, *et al*, Microwave energy supplied by a prototype oven prevents the spread of Fusarium wilt during the propagation of melon plantlets by seeds. *Spanish J Agric Res* **4**:207–212 (2006).
16 Champaco ER, Martyn RD and Miller ME, Comparison of *Fusarium solani* and *Fusarium oxysporum* as causal agents of fruit rot and root rot in muskmelon. *HortScience* **28**:1174–1177 (1993).
17 Hoitink HAJ and Boehm MJ, Biocontrol within the context of soil microbial communities: a substrate-dependent phenomenon. *Annu Rev Phytopathol* **37**:427–446 (1999).
18 Brimner T and Boland GJ, A review of the non-target effects of fungi used to biologically control plant diseases. *Agric Ecosyst Environ* **100**:3–16 (2003).
19 De Cal A, Pascual S, Larena I and Melgarejo P, Biological control of *Fusarium oxysporum* f.sp. *lycopersici*. *Plant Pathol* **44**:909–917 (1995).
20 Pascual JA, García C, Hérnandez MT, Lerma S and Lynch JM, Effectiveness of municipal waste compost and its humic fraction in suppressing *Pythium ultimum*. *Microb Ecol* **44**:59–68 (2002).
21 Filion M, St-Arnaud M and Fortin JA, Direct interaction between the arbuscular mycorrhyzal fungus *Glomus intraradices* and different rhizosphere microorganisms. *New Phytol* **141**:525–533 (1999).
22 Barea JM, Azcón R and Azcón-Aguilar C, Mycorrhizosphere interactions to improve plant fitness and soil quality. *Antonie Leeuwenhoek* **81**:343–351 (2002).
23 Martínez A, Obertello M, Pardo A, Ocampo JA and Godeas A, Interactions between *Trichoderma pseudokoningii* strains and the arbuscular mycorrhizal fungi *Glomus mosseae* and *Gigaspora rosea*. *Mycorrhiza* **14**:79–84 (2004).
24 Green H, Larsen J, Olsson PA, Jensen DF and Jakobsen I, Suppression of the biocontrol agent *Trichodemra harzianum* by mycelium of the arbuscular mycorrhizal fungus *Glomus intraradices* in root-free soil. *Appl Environ Microbiol* **65**:1428–1434 (1999).
25 Vázquez MM, César S, Azcón R and Barea JM, Interactions between arbuscular mycorrhizal fungi and other microbial inoculants (*Azospirillum, Pseudomonas, Trichoderma*) and their effects on microbial population and enzyme activities in the rizosphere of maize plants. *Appl Soil Ecol* **15**:261–272 (2000).
26 Pascual J, Navarro N, Hernandez MT, García C, Bernal A, Lopez R, *et al*, Producto sólido eficaz para el control biológico de la fusariosis vascular del melón, su procedimiento de obtención y método de aplicación del mismo. Spanish Patent OPPM 200603290 (2007).
27 Plank CO, Plant analysis reference procedures for the southern region of the United States. *Southern Cooperative Ser. Bull. No. 368*, pp. 27–29 (1992).
28 Murphy J and Riley JP, A modified single solution method for the determination of phosphate in natural waters. *Anal Chim Acta* **27**:31–36 (1962).
29 Schollenberger CJ and Simon RH, Determination of exchange capacity and exchangeable bases in soils. *Soil Sci* **59**:13–24 (1945).
30 Bremner JM and Mulvaney CS, Nitrogen-total, in *Methods of Soil Analysis*, ed. by Page AL, Miller RH and Keeney DR. American Society of Agronomy, Madison, WI, pp. 595–624 (1982).
31 Phillips JM and Hayman DS, Improved procedures for clearing roots and staining parasitic and vesicular-arbuscular mycorrhizal fungi for rapid assessment of infection. *Trans Br Mycol Soc* **55**:158–161 (1970).
32 Giovannetti M and Mosse B, An evaluation of techniques for measuring vesicular-arbuscular mycorrhizal infection in roots. *New Phytol* **84**:498–499 (1980).
33 Komada H, Development of a selective medium for quantitative isolation of *Fusarium oxysporum* from natural soil. *Rev Plant Protect Res* **8**:114–124 (1975).
34 Koide RT, Nutrient supply, nutrient demand and plant response to mycorrhizal infection. *New Phytol* **117**:365–368 (1991).
35 Kohler J, Caravaca F, Carrasco L and Roldán A, Contribution of *Pseudomonas mendocina* and *Glomus intraradices* to aggregate stabilization and promotion of biological fertility in rizosphere soil of lettuce plants under field conditions. *Soil Use Manag* **22**:298–304 (2006).
36 Koide R and Elliot G, Cost, benefit and efficiency of the vesicular-arbuscular mycorrhizal symbiosis. *Funct Ecol* **3**:252–255 (1989).
37 Goicoechea N, Antolin MC and Sanchez-Diaz M, The role of plant size and nutrient concentrations in associations between *Medicago*, and *Rhizobium* and/or *Glomus*. *Biol Plant* **43**:221–226 (2000).
38 Altomare C, Norvell WA, Björkman T and Harman GE, Solubilization of phosphates and micronutrients by the plant-growth promoting and biocontrol fungus *Trichoderma harzianum* Rifai 1295-22. *Appl Environ Microbiol* **65**:2926–2933 (1999).
39 De Silva A, Patterson K, Rothrock C and Moore J, Growth promotion of highbush blueberry by fungal and bacterial inoculants. *HortScience* **35**:1228–1230 (2000).
40 Kaye JP and Hart SC, Competition for nitrogen between plants and soil microorganisms. *Trends Ecol Evol* **12**:139–143 (1997).
41 Hodge A, Stewart J, Robinson D, Griffiths BS and Fitter AH, Competition between roots and soil micro-organisms for nutrients from nitrogen-rich patches of varying complexity. *J Ecol* **88**:150–164 (2000).
42 García-Romera I, García-Garrido JM, Martín J, Fracchia S, Mujica MT, Godeas A, *et al*, Interactions between saprotrophic *Fusarium* strains and arbuscular mycorrhizas of soybean plants. *Symbiosis* **24**:235–246 (1998).
43 Fracchia S, Garcia-Romera I, Godeas A and Ocampo JA, Effect of the saprophytic fungus *Fusarium oxysporum* on arbuscular mycorrhizal colonization and growth of plants in greehouse and field trials. *Plant Soil* **223**:175–184 (2000).
44 Fracchia S, Sampedro I, Scervino JM, Garcia-Romera I, Ocampo JA and Godeas A, Influence of saprobe fungi and their exudates on arbuscular mycorrhizal symbiosis. *Symbiosis* **36**:169–182 (2004).
45 Calvet C, Barea JM and Pera J, *In vitro* interactions between the vesicular-arbuscular mycorrhizal fungus *Glomus mosseae* and some saprophytic fungi isolated from organic substrates. *Soil Biol Biochem* **8**:775–780 (1992).
46 Calvet C, Pera J and Barea JM, Growth response of marigold (*Tagetes erecta* L.) to inoculation with *Glomus mosseae, Trichoderma aureoviride* and *Pythium ultimum* in a peat–perlite mixture. *Plant Soil* **148**:1–6 (1993).
47 McAllister CB, García-Romera I, Godeas A and Ocampo JA, Interactions between *Trichoderma koningii, Fusarium solani* and *Glomus mosseae*: effects on plant growth, arbsucular mycorrhizas and the saprophyte inoculants. *Soil Biol Biochem* **26**:1363–1367 (1994).
48 Fracchia S, Mujica MT, García-Romera I, García-Garrido JM, Martin J, Ocampo JA, *et al*, Interactions between *Glomus mosseae* and arbuscular mycorrhizal sporocarp-associated saprophytic fungi. *Plant Soil* **200**:131–137 (1998).
49 Camprubí A, Calvet C and Estaún V, Growth enhacement of *Citrus reshni* after inoculation with *Glomus intraradices* and *Trichoderma aureoviride* and associated effects on microbial population and enzyme activity in potting mixes. *Plant Soil* **173**:233–238 (1995).
50 McAllister CB, Garcia-Romera I, Martín J, Godeas A and Ocampo JA, Interaction between *Aspergillus niger* van Tiegh and *Glomus mosseae* (Nicol and Gerd) Gerd and Trppe. *New Phytol* **129**:309–316 (1995).
51 Azcón-Aguilar C and Barea JM, Arbuscular mycorrhizas and biological control of soil-borne plant pathogens – an overwiew of the mechanisms involved. *Mycorrhiza* **6**:457–464 (1996).
52 Linderman RG, Vesicular-arbuscular mycorrhizae and soil microbial interactions, in *Mycorrhizae in Sustainable Agriculture (Special Publication No. 54)*, ed. by Bethlenfalvay GJ and Linderman RG. Agronomy Society of America, Madison, WI, pp. 45–70 (1992).
53 Azcón-Aguilar C and Bago B, Physiological characteristics of the host plant promoting and undisturbed functioning of the mycorrhizal symbiosis, in *Impact of Arbuscular Mycorrhizas on Sustainable Agriculture and Natural Ecosystems*, ed. by Gianinazzi S and Schepp H. Birkhäuser, Basel, pp. 47–60 (1994).
54 Caron M, Potential use of mycorrhizae in control of soil-borne diseases. *Can J Plant Pathol* **11**:177–179 (1989).
55 Ros M, Hernandez MT, Garcia C, Bernal A and Pascual JA, Biopesticide effect of green compost against Fusarium wilt on melon plants. *J Appl Microbiol* **98**:845–854 (2005).
56 De Meyer G, Bigirimana J, Elad Y and Höfte M, Induced systemic resistance in *Trichoderma harzianum* T39 biocontrol of *Botrytis cinerea*. *Eur J Plant Pathol* **104**:279–286 (1998).

57 Linderman RG, Mycorrhizal interactions with the rhizosphere microflora: the mycorrhizosphere effect. *Phytopathology* **78**:366–371 (1988).
58 Whipps JM, Microbial interactions and biocontrol in the rizosphere. *J Exp Bot* **52**:487–511 (2001).
59 Duffy BK, Simon A and Weller DM, Combination of *Trichoderma koningii* with fluorescent pseudomonads for control of take-all on wheat. *Phytopathology* **86**:188–194 (1996).
60 Mao W, Lewis JA, Lumsden RD and Hebbar KP, Biocontrol of selected soilborne diseases of tomato and pepper plants. *Crop Protect* **17**:535–542 (1998).
61 Datnoff LE, Nemec S and Pernezny K, Biological control of Fusarium crown and root rot of tomato in Florida using *Trichoderma harzianum* and *Glomus intraradices*. *Biol Control* **5**:427–431 (1995).
62 Larkin RP and Fravel DR, Efficacy of various fungal and bacterial biocontrol organisms for control of Fusarium wilt of tomato. *Plant Dis* **82**:1022–1028 (1998).
63 De Boer M, van der Sluis I, van Loon LC and Bakker PAHM, Combining fluorescent *Pseudomonas* spp. strains to enhance suppression of Fusarium wilt of radish. *Eur J Plant Pathol* **105**:201–210 (1999).

