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The interaction with arbuscular mycorrhizal fungi or *Trichoderma harzianum* alters the shoot hormonal profile in melon plants

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ABSTRACT

Arbuscular mycorrhizal fungi (AMF) and *Trichoderma harzianum* are known to affect plant growth and disease resistance through interaction with phytohormone synthesis or transport in the plant. Cross-talk between these microorganisms and their host plants normally occurs in nature and may affect plant resistance. Simultaneous quantification in the shoots of melon plants revealed significant changes in the levels of several hormones in response to inoculation with *T. harzianum* and two different AMF (*Glomus intraradices* and *Glomus mosseae*). Analysis of zeatin (Ze), indole-3-acetic acid (IAA), 1-aminocyclopropane-1-carboxylic acid (ACC), salicylic acid (SA), jasmonic acid (JA) and abscisic acid (ABA) in the shoot showed common and divergent responses of melon plants to *G. intraradices* and *G. mosseae*. *T. harzianum* effected systemic increases in Ze, IAA, ACC, SA, JA and ABA. The interaction of *T. harzianum* and the AMF with the plant produced a characteristic hormonal profile, which differed from that produced by inoculation with each microorganism singly, suggesting an attenuation of the plant response, related to the hormones SA, JA and ethylene. These results are discussed in relation to their involvement in biomass allocation and basal resistance against Fusarium wilt.

1. Introduction

Beneficial rhizosphere microorganisms that improve plant nutrition and health include arbuscular mycorrhizal fungi (AMF) and *Trichoderma* spp. Alleviation of damage caused by soil-borne pathogens, such as *Phytophthora, Fusarium, Pythium, Rhizoctonia, Sclerotium* and *Verticillium* has been reported widely in mycorrhizal plants (Barea et al., 1997; Bi et al., 2007; Whipps, 2004). The AM establishment and functioning result from a complex molecular dialogue between the plant and the AM fungus (Harrison, 2005; Parniske, 2008; Paszkowski, 2006a; Requena et al., 2007). Some processes occurring in this dialogue are known to be mediated by phytohormones on the plant side (Hause et al., 2007). The establishment of the AM symbiosis has been reported to induce changes in the phytohormone balance in the roots of the host plants, with respect to cytokinins, gibberellins, ethylene, abscisic acid (ABA) and jasmonates (Allen et al., 1980, 1982; Drüge and Schönbeck, 1992; Hause et al., 2002, 2007; López-Ráez et al., 2010; Ludwig-Müller, 2000, Ludwig-Müller et al., 2002; Riedel et al., 2008), but there is only limited evidence about the systemic effects of this particular symbiosis in the shoots of AM plants (Pozo et al., 2009; Toussaint, 2007). In this regard, Taylor and Harrier (2003) observed that AM-tomato plants showed different gene expression patterns in leaf and root tissues, which could have been the result of an alteration of the hormonal balance in the host plants. There have been also several studies related to the induction of resistance by AMF, focusing especially on the activation of plant defence mechanisms in roots (Avis et al., 2008; Garcia-Garrido and Ocampo, 2002; Pozo and Azcón-Aguilar, 2007; Pozo et al., 2009). However, among the studies that have explored the role of phytohormones in AM–plant interactions, the results are inconsistent (Hause et al., 2007; Ludwig-Müller, 2000; Pozo and Azcón-Aguilar, 2007) and are focused on understanding the biology of the AM symbiosis, mainly at the root level. Therefore, there is still a lack of information on the physiological implications of the symbiosis in the shoot of the host plant (Toussaint, 2007). In this regard, accumulation of insect anti-feedant compounds (Gange, 2006) and transcriptional up-regulation of defence-related genes (Liu et al., 2007) have been described recently in the shoots of mycorrhizal plants.

Trichoderma (teleomorph *Hypocrea*) is a genus of asexual fungi found in the soils of all climatic zones. These fungi are opportunistic, avirulent plant symbionts and function as parasites and antagonists of many phytopathogenic fungi, thus protecting plants from diseases (Benítez et al., 2004; Harman et al., 2004; Howell, 2003; Vinale et al., 2008). Some strains can penetrate plant roots and colonise the epidermis and outer cortex, causing substantial changes in plant metabolism. It is well documented that some

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strains promote plant growth, increase nutrient availability, improve crop production and enhance resistance to pathogens, even in the shoot (Elad, 2000; Harman et al., 2004; Korolev et al., 2008; Shoresh et al., 2005; Vinale et al., 2008; Yedidia et al., 2003). It has been proposed that the phytohormones jasmonic acid (JA), ethylene and salicylic acid (SA) play a major role in the resistance induced by several *Trichoderma* isolates. *Trichoderma asperellum* isolate T203 has been shown to induce resistance in cucumber to *Pseudomonas syringae* pv. *Lachrymans*, through the pathway mediated by the signal phytohormones JA and ethylene in the plant (Shoresh et al., 2005). Further evidence for a role of JA and ethylene in *Trichoderma*-induced resistance was provided by Korolev et al. (2008), who demonstrated that mutants of Arabidopsis with a defect in ethylene or JA signalling were unable to elevate their systemic resistance levels to *Botrytis cinerea* after colonisation by *Trichoderma harzianum* isolate T39. Moreover, a number of mechanisms have been proposed to explain the growth enhancement by *Trichoderma* spp. (Benítez et al., 2004; Harman et al., 2004), among them, fungal interactions with phytohormonal signalling and induction of resistance against pathogens (Vassilev et al., 2006). A possible role of indoleacetic acid (IAA) in the growth stimulation of tomato plants produced by inoculation with *Trichoderma aureoviride* was proposed by Gravel et al. (2007).

We hypothesised that this wide range of plant responses induced by each of these beneficial microorganisms could be modified by their simultaneous co-inoculation, and that this could be reflected in the overall metabolism of melon plants. This study was conducted to investigate the alteration of the hormonal profile of melon shoots as a consequence of the plant response to two AMF (*Glomus intraradices* and *Glomus mosseae*), which showed different colonisation patterns and functionalities in previous studies, and to the beneficial fungus *T. harzianum*, following their individual and co-inoculation. Such studies are crucial, not only to the development of an integrated understanding of complex plant-beneficial microorganism relationships, but also for the development of rational strategies for improving pathogen resistance using beneficial microorganisms.

2. Results

2.1. Plant growth

Inoculation with the AMF alone did not change shoot fr. wt. compared with control plants, while inoculation with *T. harzianum* alone increased shoot fr. wt. by 20% ($P < 0.001$) (Table 1). The combined application of *T. harzianum* with the AMF resulted in an increased shoot fr. wt. relative to plants inoculated with the AMF alone. Although no changes in the shoot fr. wt. due to AMF colonisation were observed, an increased shoot/root ratio was observed in AMF-inoculated plants (23–25% higher) compared to control plants (Table 1). The same effect was observed after inoculation with *T. harzianum*. A significant interaction between the factors AMF and *T. harzianum* was observed regarding the shoot/root ratio. The shoot/root ratio of *G. intraradices*–*T. harzianum* co-inoculated plants was higher than that for plants inoculated with these microorganisms separately.

2.2. Arbuscular mycorrhizal root colonisation, T. harzianum proliferation and disease incidence

A significant interaction between the factors AMF and *T. harzianum* was observed regarding AM root colonisation. The presence of *T. harzianum* increased AM root colonisation relative to plants inoculated with the AMF alone, co-inoculation with *G. intraradices* and *T. harzianum* producing a higher percentage of colonisation than any other treatment (Table 2). No differences in the number of *T. harzianum* colony forming units (CFU) recovered from the substrate were observed for the treatments involving co-inoculation with either *G. intraradices* or *G. mosseae*, with respect to inoculation with *T. harzianum* alone (Table 2). *T. harzianum* decreased the disease incidence (by 50%, $P < 0.001$) with respect to control plants (Table 2). Inoculation with *G. intraradices* or *G. mosseae* alone also reduced the disease incidence (by 25% and 50%, respectively, $P < 0.001$). Although a significant interaction was found between the factors AMF and *T. harzianum* regarding disease incidence, the combination of *T. harzianum* with the AMF produced different patterns of disease incidence. Plants co-inoculated with *T. harzianum* and *G. intraradices* showed a lower disease incidence than plants inoculated with either microorganism singly, while no differences in disease incidence were found following the combined inoculation with *T. harzianum* and *G. mosseae*, compared with either microorganism inoculated singly (Table 2).

Table 1
The shoot and root fr. wt. (g) and the shoot/root ratio of melon plants inoculated with *Trichoderma harzianum* and/or *Glomus intraradices* or *Glomus mosseae*, 6 weeks after planting.

	Treatment	Shoot fr. wt. (g)	Root fr. wt. (g)	Shoot/root ratio
	Control	1.63 ± 0.08	0.66 ± 0.08	2.48 ± 0.10^{d}
	G. intraradices	1.45 ± 0.09	0.47 ± 0.10$^{¥}$	3.11 ± 0.07^{b}
	G. mosseae	1.63 ± 0.07	0.53 ± 0.06$^{¥}$	3.07 ± 0.09^{b}
	T. harzianum	1.96 ± 0.02$^{¥}$	0.67 ± 0.10	2.92 ± 0.13^{c}
	G intraradices + *T. harzianum*	1.77 ± 0.07	0.52 ± 0.06$^{¥}$	3.43 ± 0.12^{a}
	G. mosseae + *T. harzianum*	1.99 ± 0.10$^{¥}$	0.67 ± 0.12	2.98 ± 0.07^{b,c}
Two-way ANOVA				
	AMF inoculation	*	***	***
	T. harzianum inoculation	***	NS	**
	AMF × *T. harzianum*	NS	NS	*

The data are the means of five replicates (± SE).
¥ Means are significantly different from the control according to Dunnett's test ($P < 0.05$). Data not sharing a letter in common differ significantly ($P < 0.05$) according to Fisher's LSD test.
NS, not significant; * $P < 0.05$; ** $P < 0.01$. *** $P < 0.001$.

2.3. Hormonal profiling

Significant interactions between the factors AMF and *T. harzianum* were observed for all phytohormones except zeatin.

Table 2
The arbuscular mycorrhizal (AM) root colonisation (%) and the *Trichoderma harzianum* population (colony forming units × 10^6 g^{-1} of peat) 6 weeks after planting, and the Fusarium wilt incidence (% of infected plants) 3 weeks after pathogen inoculation in melon plants inoculated with *Glomus intraradices* or *Glomus mosseae*, alone or co-inoculated with *Trichoderma harzianum*.

	Treatment	AM root colonisation	*T. harzianum* colony forming units	Fusarium wilt incidence
	Control	<5	N.d.^{A}	80^{d}
	G. intraradices	67.33 ± 4.83^{b}	N.d.	60^{c}
	G. mosseae	56.03 ± 4.39^{c}	N.d.	41^{b}
	T. harzianum	<5	2.10 ± 0.97^{a}	40^{b}
	G intraradices + *T. harzianum*	84.72 ± 4.72^{a}	1.90 ± 0.56^{a}	13^{a}
	G. mosseae + *T. harzianum*	70.27 ± 3.42^{b}	1.02 ± 0.49^{a}	47^{b}
Two-way ANOVA				
	AMF inoculation	***	–	***
	T. harzianum inoculation	NS	***	***
	AMF × *T. harzianum*	*	–	**

The data are the means of five replicates (± SE).
A N.d., not detected. Data not sharing a letter in common differ significantly ($P < 0.05$) according to Fisher's LSD test.
NS, not significant; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Inoculation with *T. harzianum* or AMF increased the zeatin (Ze) content, by 30%, with respect to the non-inoculated control plants, both factors being significant ($P < 0.001$) (Fig. 1A). No difference in Ze content was observed between these treatments. Although no significant interaction between AMF and *T. harzianum* inoculation was found, an increased Ze content was observed in co-inoculated plants compared to singly-inoculated plants.

The IAA content was increased in *T. harzianum*-inoculated plants relative to the non-inoculated control ($P < 0.001$) (Fig. 1B). The factor AMF inoculation was significant for IAA ($P < 0.01$). However, AMF inoculation induced different patterns of change: *G. intraradices* increased the IAA content, while *G. mosseae* decreased it. Dual inoculation with *G. intraradices* and *T. harzianum* decreased the IAA content compared with inoculation with *G. intraradices* alone, while co-inoculation with *G. mosseae* and *T. harzianum* increased the content relative to inoculation with *G. mosseae* alone. Co-inoculation with an AM fungus and *T. harzianum* gave similar IAA levels, regardless of the AM fungus involved.

An increase in the ABA content was observed in *T. harzianum*-inoculated plants, with respect to control plants ($P < 0.001$) (Fig. 2A), while AMF inoculation did not alter the ABA content. Co-inoculation resulted in a decrease in ABA content relative to plants inoculated with *T. harzianum* alone, co-inoculated plants showing an ABA content similar to that of plants inoculated with each AMF alone, with no differences between co-inoculated plants and control plants.

The content of 1-aminocyclopropane-1-carboxylic acid (ACC), the ethylene precursor, was increased in *T. harzianum*-inoculated plants relative to the non-inoculated control plants ($P < 0.001$) (Fig. 2B). The factor AMF inoculation was significant for the ACC content ($P < 0.001$), however; whereas *G. intraradices* increased it, *G. mosseae* decreased it. Co-inoculation with *G. intraradices* and *T. harzianum* decreased the ACC content in comparison with plants inoculated with *G. intraradices* alone, while co-inoculation with *G. mosseae* and *T. harzianum* increased it relative to plants inoculated with *G. mosseae* alone.

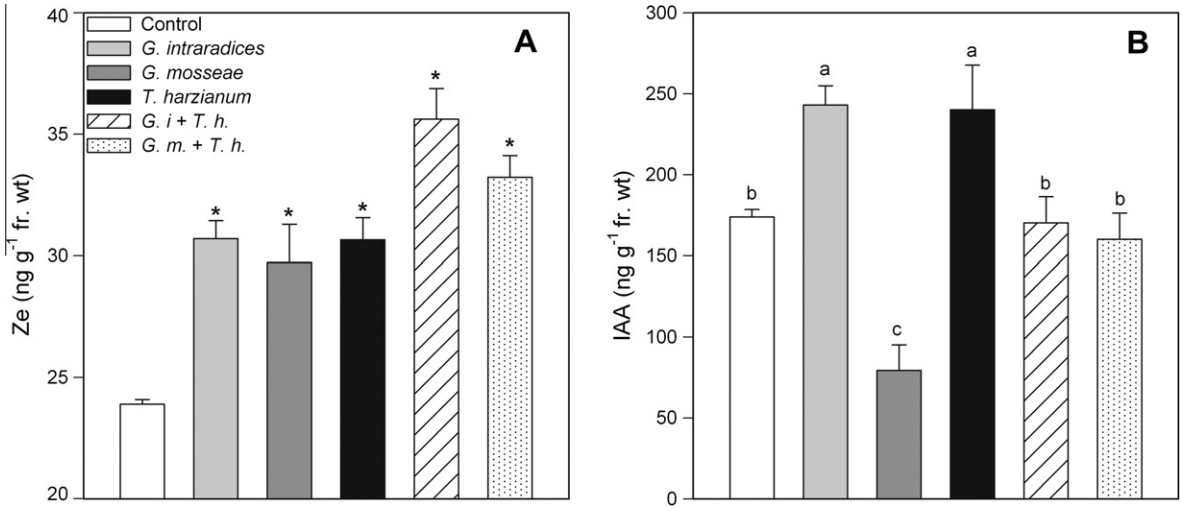

Fig. 1. Content of Ze and IAA in melon plant shoots. Content of zeatin (Ze) (A), and indole-3-acetic acid (IAA) (B), (in ng g^{-1} fr. wt.) in shoots of melon plants inoculated with *Trichoderma harzianum* and/or *Glomus intraradices* or *G. mosseae*. Bars indicate the SE of five replicates. *Means are significantly different from the control according to Dunnett's test ($P < 0.05$). Columns not sharing a letter in common differ significantly ($P < 0.05$) according to Fisher's LSD test.

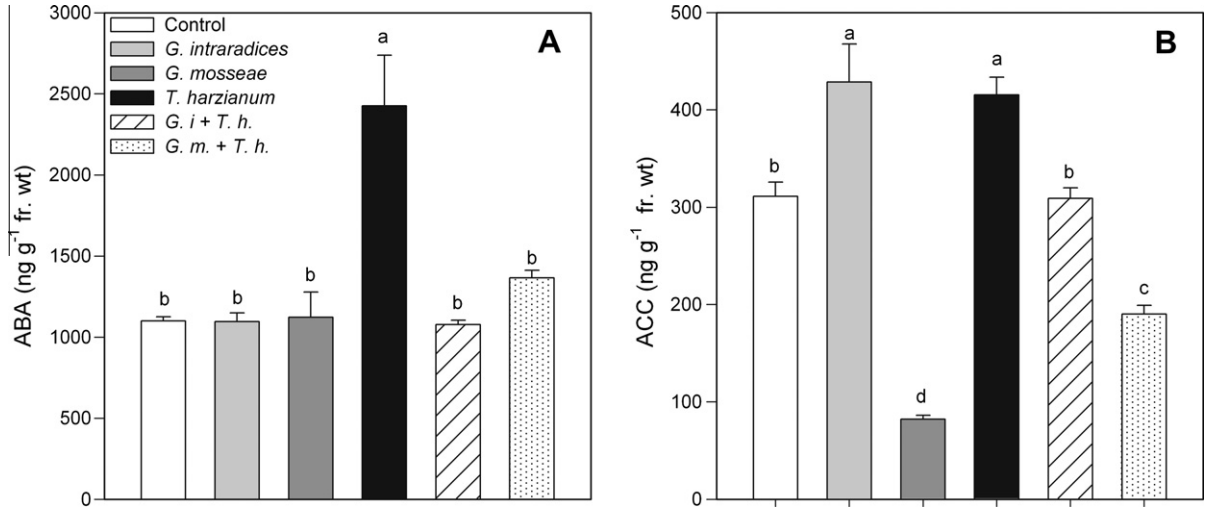

Fig. 2. Content of ABA and ACC in melon plant shoots. Content of abscisic acid (ABA) (A), and 1-aminocyclopropane-1-carboxylic acid (ACC) (B), (in ng g^{-1} fr. wt.) in shoots of melon plants inoculated with *Trichoderma harzianum* and/or *Glomus intraradices* or *G. mosseae*. Bars indicate the SE of five replicates. Columns not sharing a letter in common differ significantly ($P < 0.05$) according to Fisher's LSD test.

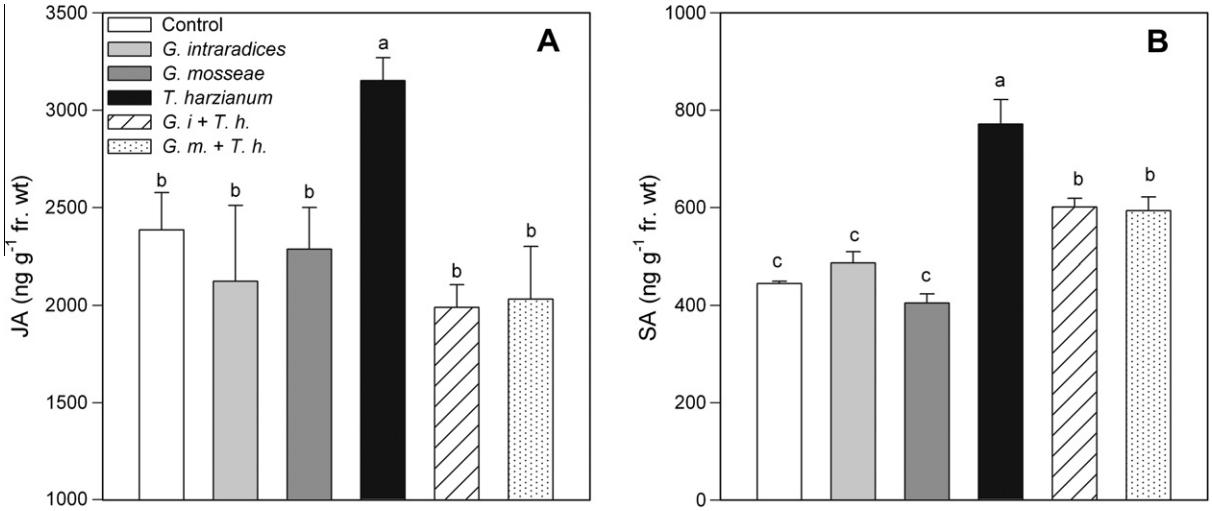

Fig. 3. Content of JA and SA in melon plant shoots. Content of jasmonic acid (JA) (A), and salicylic acid (SA) (B) (in ng g^{-1} fr. wt.) in shoots of melon plants inoculated with *Trichoderma harzianum* and/or *Glomus intraradices* or *G. mosseae*. Bars indicate the SE of five replicates. Columns not sharing a letter in common differ significantly ($P < 0.05$) according to Fisher's LSD test.

The JA content was increased in *T. harzianum*-inoculated plants with respect to uninoculated plants ($P < 0.05$) (Fig. 3A). The AMF inoculation did not alter the JA content with respect to control plants. AMF–*T. harzianum* co-inoculated plants showed a decrease in comparison with plants inoculated with only *T. harzianum* and had a JA content similar to that of uninoculated control plants (Fig. 3A).

The SA content was increased (by 75%) in *T. harzianum*-inoculated plants relative to uninoculated control plants ($P < 0.001$) (Fig. 3B). The AMF inoculation did not change the SA content with respect to the uninoculated plants. AMF–*T. harzianum* co-inoculated plants showed an increased SA content compared with plants inoculated only with the AMF whereas the content was decreased in comparison with plants inoculated only with *T. harzianum*.

3. Discussion

It is widely accepted that AM establishment induces hormonal changes in the roots of the host plant (Hause et al., 2002, 2007; López-Ráez et al., 2010; Riedel et al., 2008); however, there is only limited evidence about the systemic effects underlying plant–AMF interactions. The beneficial fungus *T. harzianum* has been reported to interact with plant hormonal signalling (Gravel et al., 2007; Korolev et al., 2008; Shoresh et al., 2005; Vassilev et al., 2006). Herein, plant systemic response to colonisation by the two AMF *G. mosseae* and *G. intraradices*, and *T. harzianum* was analysed in melon plants, following individual and co-inoculation with these beneficial microorganisms.

Simultaneous quantification in the shoots of melon plants by LC–ESI-MS/MS revealed significant changes in the levels of several hormones in mycorrhizal plants, depending on the AM fungus involved. While the ABA, JA and SA levels remained unaltered in the shoots of mycorrhizal plants compared with uninoculated plants, Ze increased. In contrast, the levels of IAA and ACC were differentially altered by the two AMF assayed: they increased exclusively in the shoots of melon plants inoculated with *G. intraradices* but decreased in *G. moseeae*-inoculated plants. Differences in their colonisation patterns and in the hormonal and transcriptional changes brought about by *G. intraradices* and *G. mosseae* isolates have been reported previously in roots (Feddermann et al., 2008; López-Ráez et al., 2010; Martínez-Medina et al., 2009a; Pozo et al., 2002). These findings reinforce the idea that some of the hormonal changes in the host seem to be related to the AMF genotype. Despite the increase in the shoot/root ratio observed in mycorrhizal plants, no increase in shoot growth was observed. Therefore, the increase in the shoot/root ratio was due mainly to a decrease in the root fr. wt. It has been proposed that cytokinin plays an important role in root development and architecture (Hodge et al., 2009; Werner et al., 2003). The alteration in Ze synthesis or transport observed in AM plants may be responsible for the effects on root growth observed in our experiment (Hetrick, 1991; Hodge et al., 2009; Kuderová et al., 2008; Smith et al., 1997; Yao et al., 2009).

Inoculation with *T. harzianum* mediated systemic increases in the Ze, IAA, ACC and ABA contents, as well as an increase in shoot growth. Interaction with auxin signalling has been proposed as a mechanism of the plant growth promotion produced by *Trichoderma virens* (Contreras-Cornejo et al., 2009). It has been proposed that ethylene may affect shoot and root growth through the stimulation of auxin biosynthesis (Ruzicka et al., 2007). The strong correlation between the IAA and ACC levels ($r = 0.965$, $P < 0.01$) in the present study indicates the involvement of these two compounds in the promotion of shoot growth by *T. harzianum*; since both compounds changed in parallel, it is difficult to elucidate their independent effects and more functional work is needed. Inoculation with *T. harzianum* also modified strongly the shoot balance of stress- and defence-related hormones. In addition to the increases in the ACC and ABA contents, we observed systemic increases in the shoot content of the hormones JA and SA, for which a role in defence against plant pathogens has been proposed (Abeles et al., 1992; Pieterse et al., 2003; Sticher et al., 1997). There is evidence that *Trichoderma* spp. are able to induce systemic defence responses in a number of plant species (Hanson and Howell, 2004; Shoresh et al., 2005; Vinale et al., 2008). Shoresh et al. (2005) suggested the involvement of JA and ethylene in the protective effect conferred by *Trichoderma* on cucumber, but did not observe variation in the SA content. In contrast, Martinez et al. (2001) suggested that SA and ethylene together coordinate the activation of defence mechanisms via an interaction between the two signalling pathways, since SA caused an inhibition of ethylene production. Our results indicate that activation of both the JA and the SA pathways may be responsible for the enhanced resistance to *Fusarium oxysporum* conferred by *T. harzianum* in our experiment.

As mentioned above, the JA, SA and ACC levels remained unaltered in the shoots of mycorrhizal plants; however, a reduction

in the disease incidence was found. The JA content was increased several-fold in the roots of mycorrhizal barley, cucumber, *Medicago truncatula* and soybean plants (Hause et al., 2002; Meixner et al., 2005; Stumpe et al., 2005; Vierheilig and Piche, 2002), indicating a role of JA in the establishment and functionality of the AM symbiosis (Hause et al., 2007; Hause and Schaarschmidt, 2009). However, the levels of JA were not altered in tomato shoots (López-Ráez and Pozo, unpublished). In the same way, accumulation of SA or pathogenesis-related proteins or expression of marker genes associated with systemic acquired resistance has not been reported in systemic tissues of AM-plants (Pozo et al., 2009). Therefore, it has been suggested that priming is the main mechanism operating in the induction of resistance by AMF (Pozo and Azcón-Aguilar, 2007; Pozo et al., 2010).

The plant response to *T. harzianum* and AMF co-inoculation produced a characteristic hormonal profile, which differed from that produced by inoculation with each microorganism singly. An additive effect on shoot Ze resulted from the co-inoculation with the AMF and *T. harzianum*, such that co-inoculated plants had higher shoot Ze contents than singly-inoculated plants. However, there was no synergistic effect on the shoot levels of IAA, ACC or ABA, which remained at a lower level than in plants inoculated with *T. harzianum* alone. In co-inoculated plants, the shoot SA and JA levels were lower than in *T. harzianum*-inoculated plants, indicating an attenuation of the *T. harzianum*-induced systemic response related to these hormones. As obligate biotrophs, the AMF have similarities to biotrophic pathogens (Guimil et al., 2005; Paszkowski, 2006b). Thus, their sensitivity to SA-regulated defence mechanisms is likely. It has been reported that exogenous SA application delays mycorrhizal colonisation (Medina et al., 2003). Moreover, altered SA accumulation in mycorrhizal plants indicates that SA might be involved in the susceptibility of plants to AMF. In mycorrhiza-defective (Myc−) mutants of *Pisum sativum*, the SA accumulation was enhanced, whereas in Myc+ plants it was low (Blilou et al., 1999) or only transient (Blilou et al., 2000a,b). Exogenous JA may also contribute to the suppression of colonisation (Ludwig-Müller et al., 2002). Therefore, modification of plant defences is required for the establishment of the symbiosis (Pozo and Azcón-Aguilar, 2007). According to our own data, it is plausible that AMF repress SA- as well as JA-dependent systemic pathways stimulated by *T. harzianum* in the host, in order to achieve a compatible interaction.

In conclusion, it is shown that the establishment of mycorrhiza and the interaction of the plant with *T. harzianum* imply systemic changes in the contents of several phytohormones, which may have physiological implications for plant growth and resistance. Additionally, differing responses of the shoot to colonisation by different isolates of AMF have been demonstrated, which may underlie the differences in the physiological effects of different AMF. Furthermore, a characteristic hormonal profile due to the co-inoculation with *T. harzianum* and AMF, which differed from that produced by inoculation with either microorganism applied singly, was observed. The interaction of both microorganisms with the plant produced a general attenuation of the plant systemic response related to the hormones SA and JA, when compared with plants inoculated only with *T. harzianum*.

4. Experimental

4.1. Plants and fungal inocula

Melon plants (*Cucumis melo* cv. Giotto) were used as the host plants. The AMF used were *G. intraradices* and *G. mosseae*, obtained from the Centro de Edafología y Biologia Aplicada del Segura-CSIC (Spain). The density of the AM fungal inocula was found to be 35 infective propagules per gram of inocula. The isolate of *T. harzianum* used is deposited in the Spanish Type Culture Collection, isolate CECT 20714. *T. harzianum* inoculum was produced using a specific solid medium, prepared according to Martínez-Medina et al. (2009b). Monoconidial *F. oxysporum* was isolated from infected melon plants from a seedling nursery. For the production of the pathogen inoculum, *F. oxysporum* was cultivated for 5 days on potato dextrose broth (Scharlau Chemie, Barcelona, Spain), amended with 100 mg l^{-1} streptomycin sulphate, at 28 °C in darkness, on a shaker at 120 rpm. After the incubation period, the conidia were recovered by centrifugation (193 *g*, 10 min); the pellet was re-suspended in sterile distilled water, re-centrifuged and filtered using quartz wool.

4.2. Experimental design, biological treatments and growth conditions

A completely-randomised design was established with two factors (AMF inoculation and *T. harzianum* inoculation) and 10-fold replication giving a total of six treatments: (1) uninoculated control; (2) *G. intraradices*; (3) *G. mosseae*; (4) *T. harzianum* (5) *G. intraradices* + *T. harzianum*; and (6) *G. mosseae* + *T. harzianum*. All plants used were grown in nursery polystyrene plant containers with ten individual wells (8 cm^3), in a seedling nursery. The individual wells were filled with the inoculated peat. The AM inocula were added at a rate of 5% (v/v), while the *T. harzianum* inoculum was mixed with the peat, reaching a population density of 1×10^6 conidia per g of peat. Each specific container was considered as a replicate and 10 replicates of each treatment were used, giving a total of 60 containers. Six weeks after planting, half of the experiment was harvested and plant and substrate samples were taken. The remaining plants were infected by *F. oxysporum*, to reach a final content of 1×10^4 conidia g^{-1} of peat, in order to study the disease incidence. Three weeks after pathogen inoculation, the remaining plants were harvested and disease incidence was evaluated.

4.3. Vegetative growth assessment and biological analyses

For each plant, the shoot and roots were harvested separately and their fr. wt. determined. Roots were cleared with 10% KOH and stained with 0.05% trypan blue (Phillips and Hayman, 1970). The percentage root length colonised by AMF was calculated by the gridline intersect method (Giovannetti and Mosse, 1980). Serial dilutions of the peat samples in sterile, quarter-strength Ringer solution (Oxoid, Madrid, Spain) were used for quantifying *T. harzianum* proliferation by a plate count technique, using potato dextrose agar (PDA) amended with 50 mg l^{-1} rose bengal and 100 mg l^{-1} streptomycin sulphate. Plates were incubated at 28 °C for 5 days (Martínez-Medina et al., 2009b). The disease incidence was determined by surface disinfecting stem segments (~1.5 cm, from immediately above the crown) for 5 min in 1% sodium hypochlorite, rinsing with sterile water and plating onto PDA medium. Plates were incubated at 28 °C for 6 days, and the appearance of *F. oxysporum* colonies around the stems was considered to be indicative of infected plants. The percentage of infected plants was used to determine the disease incidence.

4.4. Hormone and ACC extraction

Zeatin, indole-3-acetic acid, 1-aminocyclopropane-1-carboxylic acid, abscisic acid, jasmonic acid and salicylic acid were extracted and purified according to the method of Dobrev and Kaminek (2002).

4.5. Hormone and ACC analysis

Hormones and ACC were analysed mostly as described previously (Albacete et al., 2008). The analyses were carried out on an HPLC/MS system consisting of an Agilent 1100 Series HPLC (Agilent Technologies, Santa Clara, CA, USA), equipped with a μ-wellplate autosampler and a capillary pump, connected to an Agilent Ion Trap XCT Plus mass spectrometer (Agilent Technologies) using an electrospray (ESI) interface. Prior to injection, 100 μl of each fraction were filtered through 13-mm-diameter Millex filters having a 0.22-μm pore-size nylon membrane (Millipore, Bedford, MA, USA). Mobile phase A consisting of water/acetonitrile/formic acid (94.9:5:0.1) and mobile phase B consisting of water/acetonitrile/formic acid (10:89.9:0.1) were used for the chromatographic separation. Of each sample, 8 μl, dissolved in the mobile phase A, was injected onto a Zorbax SB-C18 HPLC column (5 μm, 150 × 0.5 mm, Agilent Technologies), thermostated at 40 °C and eluted at a flow rate of 10 μl min^{-1}. The elution consisted of maintaining 100% A for 5 min, then a linear gradient from 0% to 6% B in 10 min, followed by another linear gradient from 6% to 100% B in 5 min and finally 100% B was maintained for another 5 min. The column was equilibrated with the starting composition of the mobile phase for 30 min before each analytical run. The UV chromatogram was recorded at 280 nm with the DAD (diode-array detector) module (Agilent Technologies). Different control samples with known concentrations of each analysed component (0.05, 0.075, 0.1, 0.2 and 0.5 mg l^{-1}) were also run in the same conditions. The mass spectrometer was operated in the positive mode, with a capillary spray voltage of 3500 V and a scan speed of 22000 (*m/z*)/s from 50 to 500 *m/z*. The nebulizer gas (He) pressure was set to 30 psi and the drying gas was set to a flow of 6 l min^{-1} at a temperature of 350 °C. Mass spectra were obtained using the DataAnalysis program for LC/MSD Trap Version 3.2 (Bruker Daltonik, GmbH, Germany). For quantification of ABA, IAA, JA and Ze, calibration curves were constructed for each component analysed (0.05, 0.075, 0.1, 0.2 and 0.5 mg l^{-1}) and corrected for 0.1 mg l^{-1} internal standards: [$^2H_5$]*trans*-zeatin, [$^2H_6$]*cis,trans*-abscisic acid and [$^2H_5$](±)-jasmonic acid (Olchemin Ltd., Olomouc, Czech Republic) and [$^{13}C_6$]indole-3-acetic acid (Cambridge Isotope Laboratories Inc., Andover, MA, USA). The ACC and SA were quantified by the external standard method using the same concentration range (purchased from Sigma–Aldrich Inc., St. Louis, MO, USA). Recovery percentages ranged between 92% and 95%. All samples were run in triplicate.

4.6. Statistical analysis

Simple correlations between the different variables were performed using SPSS software, version 15.0 (SPSS Inc., Chicago, IL, USA). The data were also subjected to two-way analysis of variance (ANOVA). When appropriate, Fisher's LSD test was applied. Dunnett's test was performed to compare shoot fr. wt., root fr. wt. and Ze content of each treatment with the control. For the AM root colonisation and *T. harzianum* colony forming units, the non-inoculated treatments were excluded from the analyses because neither root colonisation nor *T. harzianum* CFU were detected in any of the non-inoculated treatments.

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