Trichoderma harzianum and *Glomus intraradices* Modify the Hormone Disruption Induced by *Fusarium oxysporum* Infection in Melon Plants

Ainhoa Martínez-Medina, Jose Antonio Pascual, Francisco Pérez-Alfocea, Alfonso Albacete, and Antonio Roldán

First, second, and fifth authors: CEBAS-CSIC, Department of Soil and Water Conservation and Organic Waste Management, and third and fourth authors: CEBAS-CSIC, Department of Plant Nutrition, Campus Universitario de Espinardo, Murcia, E-30100, Spain.
Accepted for publication 29 January 2010.

ABSTRACT

Martínez-Medina, A., Pascual, J. A., Pérez-Alfocea, F., Albacete, A., and Roldán, A. 2010. *Trichoderma harzianum* and *Glomus intraradices* modify the hormone disruption induced by *Fusarium oxysporum* infection in melon plants. Phytopathology 100:682-688.

The plant hormones salicylic acid (SA), jasmonic acid (JA), ethylene (ET), and abscisic acid (ABA) are known to play crucial roles in plant disease and pest resistance. Changes in the concentrations of these plant hormones in melon plant shoots, as a consequence of the interaction between the plant, the pathogen *Fusarium oxysporum*, the antagonistic microorganism *Trichoderma harzianum*, and the arbuscular mycorrhizal fungus *Glomus intraradices* were investigated. Attack by *F. oxysporum* activated a defensive response in the plant, mediated by the plant hormones SA, JA, ET, and ABA, similar to the one produced by *T. harzianum.* When inoculated with the pathogen, both *T. harzianum* and *G. intraradices* attenuated the plant response mediated by the hormones ABA and ET elicited by the pathogen attack. *T. harzianum* was also able to attenuate the SA-mediated response. In the three-way interaction (*F. oxysporum–T. harzianum–G. intraradices*), although a synergistic effect in reducing disease incidence was found, no synergistic effect on the modulation of the hormone disruption induced by the pathogen was observed. These results suggest that the induction of plant basal resistance and the attenuation of the hormonal disruption caused by *F. oxysporum* are both mechanisms by which *T. harzianum* can control Fusarium wilt in melon plants; while the mechanisms involving *G. intraradices* seem to be independent of SA and JA signaling.

Fusarium oxysporum f. sp. *melonis*, is one of the most destructive pathogens of melon crops worldwide and can reduce yield by up to 90% (7,39). The fungus enters the roots through penetration hyphae and colonizes the cortex by intercellular and intracellular growth. After reaching the vascular tissue, it spreads rapidly upwards through the xylem vessels, producing the typical wilt symptoms. Plants respond innately to *F. oxysporum* attack via a complex and integrated set of defenses, encompassing both constitutive and induced responses (4), which include production of signaling compounds such as salicylic acid (SA), jasmonic acid (JA), ethylene (ET), and abscisic acid (ABA), or reactive oxygen species. These signal transduction pathways control the expression of genes which have a role in the defense against *F. oxysporum*, such as the pathogenesis-related (PR) genes *PR1* and *PR5*, the protodermal factor (*PDF1*) gene, and the thionin (*Thi2.1*) gene (4).

A similar response is produced when the plant is colonized by certain nonpathogenic microorganisms, which trigger a wide range of defense mechanisms in plants that confer protection against pathogenic invasion. Three major signal molecules are known to be involved in the systemic defense responses of plants: SA, involved in the systemic acquired resistance (SAR), and JA and ET, involved in the induced systemic resistance (ISR) (1,32, 40). More recently, the ABA pathway has also been implicated in the defense response, through interaction with other pathways (37). *Trichoderma* species have been reported to induce systemic and localized resistance to a variety of plant pathogens (19,38, 41,43). Shoresh et al. (38) found that *T. asperellum* strain T-203 modulates the expression of genes involved in the jasmonate/ethylene signaling pathways of ISR. Similarly, alleviation of damage caused by soilborne pathogens such as *Phytophthora*, *Fusarium*, *Pythium*, *Rhizoctonia*, *Sclerotium*, and *Verticillium* has been reported widely in plants that harbor mycorrhizae (5,42). Systemic mechanisms of protection have been reported to be implicated in this protective effect (5). For example, colonization of tomato roots by the arbuscular mycorrhizal fungus *Glomus mosseae* protects the plant systemically against infection by *Phytophthora parasitica* (34).

Multiple-player cross-talk normally occurs in nature among plants, pathogens, and biocontrol agents (3,22,26,36). These interactions may result in a plant status different from that associated with the single interaction between the plant and each microorganism (14,15,25,43). This new plant status, a consequence of the multiple interactions, could have direct effects on the pathogenicity resistance processes mediated by the biocontrol agents and the pathogen, as observed by several authors (6,8,9,26,29).

Plant hormones which play a crucial role in the pathogenicity resistance process were analyzed to determine variation in their accumulation pattern associated with multiple-player interactions. Using a multi-analysis technique for hormone quantification, endogenous levels of SA, JA, ABA, and 1-aminocyclopropane-1-carboxylic acid (ACC), the ET precursor, were analyzed in melon plants inoculated with the mycorrhizal fungus *G. intraradices* and the fungus *T. harzianum* in the presence of the pathogen *F. oxysporum*. These studies are crucial, not only for the development of an integrated understanding of the plant–pathogen–biocontrol agent complex, but also for the development of rational strategies for managing Fusarium wilt disease.

MATERIALS AND METHODS

Host plant and fungal inocula. Melon plants (*Cucumis melo* cv. Giotto) were used as the host plant. The arbuscular mycorrhizal (AM) fungus *G. intraradices* from Centro de Edafología y

Corresponding author: A. Martínez-Medina;
E-mail address: ammedina@cebas.csic.es

doi:10.1094/PHYTO-100-7-0682

Biologia Aplicada del Segura-CSIC (Spain) was used in this experiment. *G. intraradices* was multiplied using *Sorghum vulgare* as trap plant. The AM fungal inoculum density was found to be 35 infective propagules per gram of soil. The isolate of *T. harzianum*, deposited in the Spanish Type Culture Collection (isolate CECT 20714) (30) by Centro de Edafología y Biologia Aplicada del Segura-CSIC (Spain), was chosen for this study owed to its high biocontrol capacity against *F. oxysporum* and its survival in peat (26). *T. harzianum* inoculum was produced using a specific solid medium, prepared by mixing commercial oats, bentonite, and vermiculite (1:2.5:5, wt/vol/vol) according to Martínez-Medina et al. (26). 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) and amended with streptomycin sulfate at 100 mg liter^{-1} 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 resuspended in sterile distilled water, recentrifuged, and filtered using quartz wool. The fungal spore suspension was adjusted to 1×10^8 conidia ml^{-1}.

Experimental design and growth conditions. A completely randomized design was established with three factors (*G. intraradices*, *T. harzianum*, and *F. oxysporum*) and fivefold replication, giving a total of eight treatments: (i) uninoculated control; (ii) *G. intraradices*; (iii) *T. harzianum*; (iv) *G. intraradices* + *T. harzianum*; (v) *F. oxysporum*; (vi) *G. intraradices* + *F. oxysporum* (vii) *T. harzianum* + *F. oxysporum*, and (viii) *G. intraradices* + *T. harzianum* + *F. oxysporum*.

To prepare the treatments, sterilized peat (0.37 g kg^{-1} N total, 1.158 g^{-3} kg^{-1} available P, and 1.34 g^{-3} kg^{-1} available K) was amended with the different inocula. The AM inoculum was mixed with the peat at a rate of 5% (vol/vol), while *T. harzianum* inoculum was mixed at a rate of 1×10^6 conidia g^{-1} of peat (26). Nursery polystyrene plant containers with 10 individual wells (8 cm^3) were filled with the inoculated peat (7 g per well), and 10 melon seeds were sown (one seed per each container well) and covered with vermiculite. Each specific container was considered as a replicate, and five replicates of each treatment were used, giving a total of 40 containers.

The experiment was carried out in a seedling nursery. Seedlings were grown using standard nursery-culture conditions, which included germination in a dark germination chamber (20°C and 70% relative humidity). After 60 h, the individual containers with pregerminated seeds were moved and placed in a random design in nursery beds, which included irrigation without any fertilizer. Three weeks after planting, half of the experiment was inoculated with *F. oxysporum* to reach a final concentration of 1×10^4 conidia g^{-1} of peat. Three weeks after pathogen inoculation (six weeks after the start of the assay) plants were harvested and peat samples were stored at 4°C until use for biological analyses. The aerial part of the plant was immediately frozen in liquid N and stored at –80°C until required for biochemical analyses.

Hormone and ACC extraction and analysis. Extraction and purification of SA, JA, ACC and ABA, were performed according to Dobrev and Kaminek (12). Hormones and ACC were analyzed mostly as described previously by Albacete et al. (2). The analyses were carried out with an HPLC/MS system consisting of an Agilent 1100 Series HPLC (Agilent Technologies, Santa Clara, CA) equipped with an autosampler connected to an Agilent Ion Trap XCT Plus mass spectrometer (Agilent Technologies) using an electrospray interface. Previous to injection, 100 µl of each fraction was filtered through 13-mm-diameter Millex filters having a nylon membrane of 0.22-µm pore size (Millipore, Bedford, MA). For each sample, 8 µl, dissolved in mobile phase A, was injected onto a Zorbax SB-C18 HPLC column (5 µm, 150 × 0.5 mm, Agilent Technologies) at 40°C and eluted at a flow rate of 10 µl min^{-1}. Mobile phase A, consisting of water/acetonitrile/formic acid (94.9:5:0.1, vol/vol/vol), and mobile phase B, consisting of water/acetonitrile/formic acid (10:89.9:0.1, vol/vol/vol), were used for the chromatographic separation. The elution consisted of maintaining 100% A for 5 min, and 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 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 chromatograms were recorded at 280 nm with the DAD (diode-array detector) module (Agilent Technologies). Different control samples with known concentrations of each analyzed component (0.05, 0.075, 0.1, 0.2, and 0.5 mg liter^{-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 22,000 (m/z)/s from 50 to 500 m/z. The nebulizer gas (He) pressure was set to 30 psi, while the drying gas was set to a flow of 6 liter 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 and JA, calibration curves were constructed for each component analyzed using internal standards: [$^2H_6$]*cis,trans*-abscisic acid and [$^2H_5$](±)-jasmonic acid (Olchemin Ltd., Olomouc, Czech Republic) (0.05, 0.075, 0.1, 0.2, and 0.5 mg liter^{-1}) and corrected for 0.1 mg liter^{-1}. ACC and SA were quantified by the external standard method, using the same concentration range (purchased from Sigma-Aldrich Inc., St. Louis, MO). Recoveries ranged between 92 and 95%. All samples were run in triplicate.

Biological analyses. Roots were cleared with 10% KOH and stained with 0.05% trypan blue (31). The percent root length colonized by *G. intraradices* was calculated by the gridline intersect method (16). Positive counts for AM colonization included the presence of vesicles or arbuscules or typical mycelia within the roots.

Serial dilutions of the peat samples in sterile, quarter-strength Ringer solution (Oxoid, Madrid, Spain) were used for quantifying *T. harzianum* populations by a plate count technique, using PDA amended with 50 mg liter^{-1} of rose bengal and 100 mg liter^{-1} of streptomycin sulfate. Plates were incubated at 28°C for 5 days. After the incubation period, colony forming units (CFU) were counted and expressed per gram of dry soil (105°C). Komada medium (23) was used to quantify *F. oxysporum*. 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. Petri dishes with the melon stem segments 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.

Statistical analysis. Percent colonization and disease incidence were arcsine-transformed and the other data were log-transformed, to compensate for variance heterogeneity, before analysis of variance. The effects of the presence of *G. intraradices*, *T. harzianum*, and *F. oxysporum*, and their interactions on the measured variables were tested by a three-way analysis of variance and comparisons among means were made using Tukey's multiple-range test ($P \leq 0.05$). Statistical procedures were performed using SPSS software, version 15.0 (SPSS Inc., Chicago, IL).

RESULTS

AM root colonization and *T. harzianum* and *F. oxysporum* populations. Inoculation with *G. intraradices* produced a significant increase in the AM root colonization with respect to the uninoculated plants. There was a significant interaction between the factors *G. intraradices* and *T. harzianum* for AM root colonization; it was significantly increased in coinoculated plants

compared with plants inoculated with *G. intraradices* alone (Tables 1 and 2). A significant *G. intraradices–F. oxysporum* interaction was observed for AM colonization; in presence of the pathogen, AM root colonization was decreased significantly (Tables 1 and 2).

No differences in *T. harzianum* CFU were observed between treatments involving *T. harzianum* inoculated alone and those involving *G. intraradices–T. harzianum* coinoculation (Tables 1 and 2). There was a significant *T. harzianum–F. oxysporum* interaction for *T. harzianum* CFU; a decrease in the number of *T. harzianum* CFU was observed in the presence of the pathogen, compared with the corresponding treatments where it was absent (Tables 1 and 2).

Inoculation with *G. intraradices* did not affect the *F. oxysporum* population significantly, while there was a significant *T. harzianum–F. oxysporum* interaction with respect to the *F. oxysporum* population, which was increased by *T. harzianum* inoculation (Tables 1 and 3).

Salicylic acid. The shoot SA concentration was significantly increased (by 75%) in *T. harzianum*-inoculated plants respect to uninoculated plants, the factor *T. harzianum* being significant for shoot SA concentration (Fig. 1A, Table 1). *G. intraradices* inoculation did not change the shoot SA concentration with respect to the uninoculated plants. A significant *G. intraradices–T. harzianum* interaction was found for the shoot SA concentration: *G. intraradices–T. harzianum* coinoculated plants showed an increased SA concentration compared with plants inoculated only with *G. intraradices*; however, the SA concentration was decreased in comparison with plants inoculated only with *T. harzianum* (Fig. 1A, Table 1). *F. oxysporum* mediated an increase in the SA concentration in all treatments except in *T. harzianum*-inoculated plants, the factor *F. oxysporum* being highly significant (Fig. 1A and B, Table 1). No differences in SA shoot concentration were observed between *G. intraradices*-inoculated plants in the presence of the pathogen and plants inoculated singly with the pathogen (Fig. 1B). Both *T. harzianum*-inoculated plants and *G. intraradices–T. harzianum* coinoculated plants showed a decrease shoot SA concentrations with respect to plants which did not involve either *T. harzianum* or *G. intraradices* inoculation in the presence of *F. oxysporum* (Fig. 1B). Plants coinoculated with *G. intraradices* and *T. harzianum* showed a similar shoot SA concentration to that of plants inoculated only with *T. harzianum,* in the presence of the pathogen, but it was decreased with respect to plants inoculated with *G. intraradices* alone.

Jasmonic acid. The shoot JA concentration was increased significantly in *T. harzianum*-inoculated plants relative to the uninoculated plants. The factor *T. harzianum* being significant for the shoot JA concentration (Fig. 2A, Table 1). *G. intraradices*

TABLE 1. Three-factor analysis of variance (*Glomus intraradices*, *Trichoderma harzianum*, and *Fusarium oxysporum*) and *P* significance values for all the parameters studied[z]

				Interaction			
Measured parameters	*G. intraradices* inoculation	*T. harzianum* inoculation	*F. oxysporum* inoculation	*G. intraradices* × *T. harzianum*	*G. intraradices* × *F. oxysporum*	*T. harzianum* × *F. oxysporum*	*G. intraradices* × *T. harzianum* × *F. oxysporum*
Arbuscular mycorrhizal root colonization	<0.001	NS	NS	0.01	0.03	NS	NS
T. harzianum population	NS	<0.001	NS	NS	NS	0.02	NS
F. oxysporum population	NS	NS	<0.001	NS	NS	<0.001	NS
Salicylic acid	NS	0.04	<0.001	0.039	<0.001	NS	NS
Jasmonic acid	NS	0.012	<0.001	0.01	<0.001	0.006	NS
Aminocyclopropane-1-carboxylic acid	0.01	0.01	<0.001	0.02	0.03	0.03	NS
Abscisic acid	0.04	<0.001	<0.001	0.002	<0.001	<0.001	<0.001
Disease incidence	NS	NS	<0.001	NS	<0.001	<0.001	<0.001

[z] NS = not significant.

TABLE 2. The arbuscular mycorrhizal (AM) root colonization (%) and *Trichoderma harzianum* population (colony forming units × 10^6 g^{-1} of peat) 6 weeks after planting, in the absence or presence of *Fusarium oxysporum*

	AM root colonization			*T. harzianum* population		
Treatment	Without *F. oxysporum*	With *F. oxysporum*	ANOVA	Without *F. oxysporum*	With *F. oxysporum*	ANOVA
–Glomus intraradices–T. harzianum	Nd[z]	Nd	–[z]	Nd	Nd	–
+G. intraradices–T. harzianum	67.33 ± 4.83 a	12.07 ± 3.76 a	*	Nd	Nd	–
–G. intraradices + T. harzianum	Nd	Nd	–	2.10 ± 0.97 a	1.13 ± 0.09 a	*
+G. intraradices + T. harzianum	84.72 ± 4.72 b	17.00 ± 7.44 a	*	1.90 ± 0.56 a	0.75 ± 0.11 a	*

[z] Abbreviations: Nd = not detected; and – = not determined. Data are means ± standard error of five replicates. Values in the same column with the same letters represent no significant difference between treatments according to Tukey's multiple range test ($P \leq 0.05$). * = significant differences within the same row ($P \leq 0.05$).

TABLE 3. The *Fusarium oxysporum* population (colony forming units × 10^4 g^{-1} of peat) and Fusarium wilt incidence (%) in melon plants inoculated with *Glomus intraradices* or *Trichoderma harzianum*, alone or in combination, 3 weeks after pathogen (*Fusarium oxysporum*) inoculation

	F. oxysporum population		Wilt incidence	
Treatment	Without *F. oxysporum*	With *F. oxysporum*	Without *F. oxysporum*	With *F. oxysporum*
–G. intraradices–T. harzianum	Nd[z]	1.17 ± 0.23 a	Nd	80 ± 11 a
+G. intraradices–T. harzianum	Nd	1.00 ± 0.17 a	Nd	60 ± 7 b
–G. intraradices + T. harzianum	Nd	7.45 ± 1.15 b	Nd	40 ± 5 c
+G. intraradices + T. harzianum	Nd	0.83 ± 0.47 a	Nd	13 ± 2 d

[z] Nd = not detected. Data are means ± standard error of five replicates. Values in the same column with the same letter represent no significant difference between treatments according to Tukey's multiple range test ($P \leq 0.05$).

inoculation did not alter the shoot JA concentration relative to uninoculated plants. There was a significant *G. intraradices–T. harzianum* interaction for shoot JA concentration; *G. intraradices–T. harzianum* coinoculated plants showed a decrease in comparison with plants inoculated with only *T. harzianum* and had a shoot JA concentration similar to that of uninoculated plants and that of plants inoculated with *G. intraradices* alone (Fig. 2A, Table 1). The factor *F. oxysporum* inoculation was highly significant, and it increased the JA concentration in all treatments (Fig. 2A and B, Table 1). Significant *G. intraradices–F. oxysporum* and *T. harzianum–F. oxysporum* interactions were found for the shoot JA concentration. In the presence of the pathogen, the shoot JA concentration in *G. intraradices*-inoculated plants was significantly higher than that in both in *T. harzianum*-inoculated plants and in *G. intraradices–T. harzianum* coinoculated plants (Fig. 2B, Table 1).

ACC. An increase in the shoot ACC concentration was observed in *T. harzianum*-inoculated plants as well as in *G. intraradices*-inoculated plants, with respect to uninoculated plants, both factors being significant for the shoot ACC content (Fig. 3A, Table 1). Coinoculation with *G. intraradices* and *T. harzianum* decreased the shoot ACC concentration, in comparison with plants inoculated with either agent singly, with coinoculated plants showing no differences from uninoculated plants. *F. oxysporum* infection increased the shoot ACC concentration in plants which did not involve either *T. harzianum* or *G. intraradices* inoculation and in *G. intraradices–T. harzianum* coinoculated plants, the factor *F. oxysporum* being highly significant for shoot ACC concentration (Fig. 3A and B, Table 1). Significant *G. intraradices–F. oxysporum* and *T. harzianum–F. oxysporum* interactions were found for shoot ACC concentration: a decrease was observed in *T. harzianum*-inoculated plants and in *G. intraradices*-inoculated plants in the presence of *F. oxysporum*, compared with the same treatments in the absence of the pathogen (Fig. 3A and B, Table 1). The shoot ACC concentration in the latter treatments was significantly lower than in plants which did not involve either *T. harzianum* or *G. intraradices* inoculation and in *G. intraradices–T. harzianum* coinoculated plants, in the presence of the pathogen (Fig. 3B).

Abcisic acid. *T. harzianum* inoculation increased the ABA concentration (by 150%) with respect to uninoculated plants, the factor *T. harzianum* inoculation being highly significant for shoot ABA concentration (Fig. 4A, Table 1). Inoculation with *G. intraradices* alone or in combination with *T. harzianum* did not alter the shoot ABA concentration relative to the uninoculated plants. The factor *F. oxysporum* was highly significant for shoot

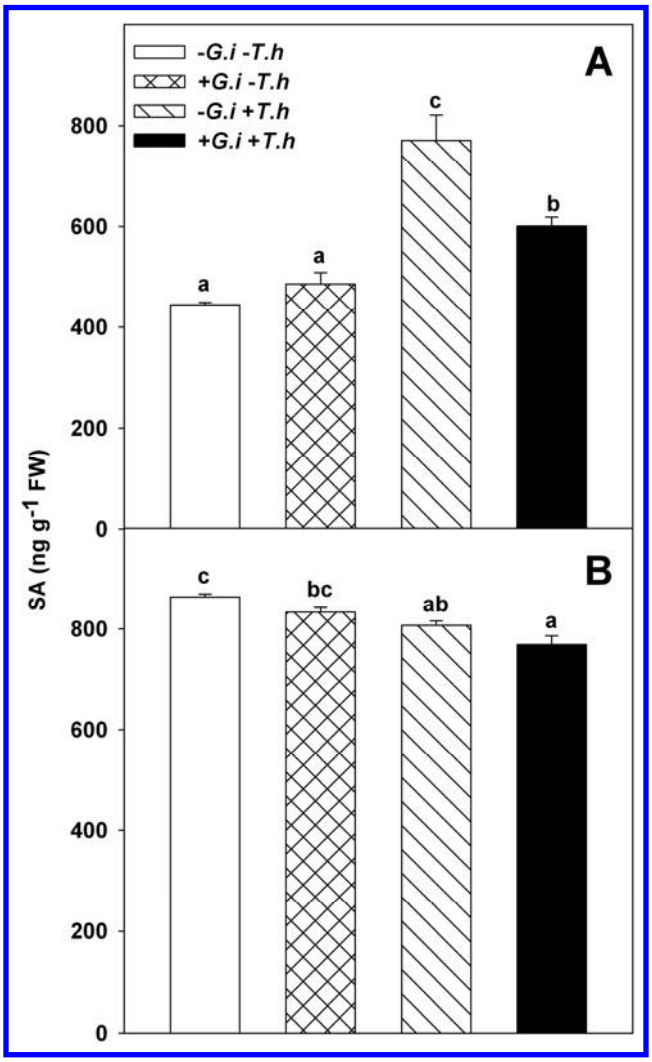

Fig. 1. Shoot salicylic acid (SA) concentration (in nanograms per gram fresh weight) in melon plants inoculated with *Glomus intraradices* (*G.i*) or *Trichoderma harzianum* (*T.h*), alone or in combination, in the **A,** absence or **B,** presence of *Fusarium oxysporum*. Bars indicate the standard error of five replicates. Columns with the same letter do not significantly differ according to Tukey's multiple range test ($P \leq 0.05$), $n = 5$.

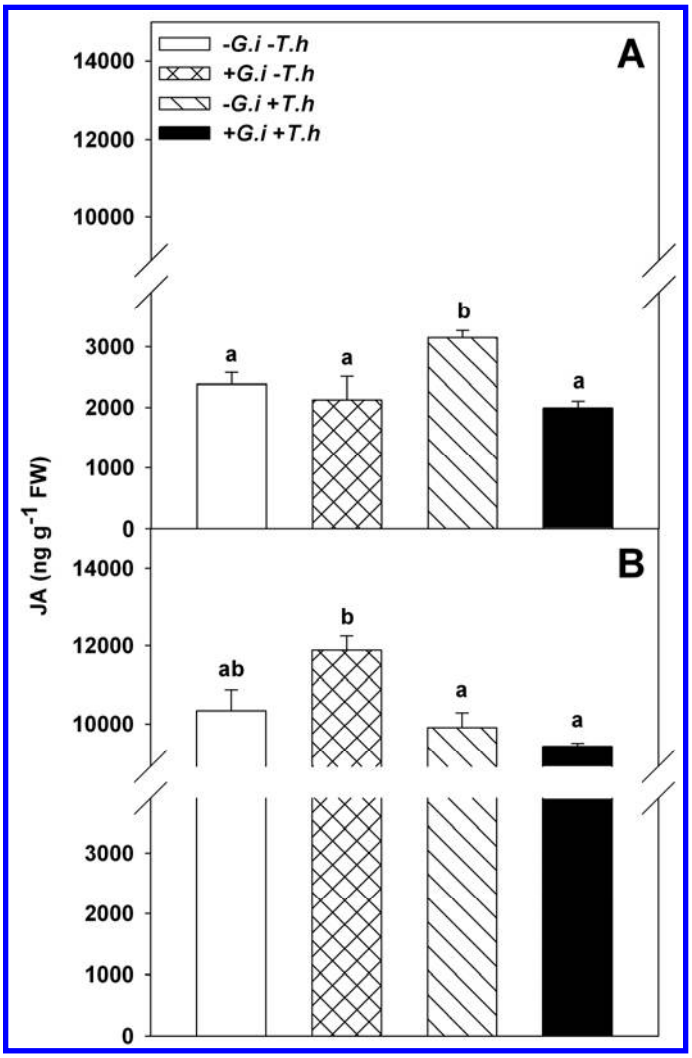

Fig. 2. Shoot jasmonic acid (JA) concentration (in nanograms per gram fresh weight) in melon plants inoculated with *Glomus intraradices* (*G.i*) or *Trichoderma harzianum* (*T.h*), alone or in combination, in the **A,** absence or **B,** presence of *Fusarium oxysporum*. Bars indicate the standard error of five replicates. Columns with the same letter do not significantly differ according to Tukey's multiple range test ($P \leq 0.05$), $n = 5$.

ABA concentration: the presence of the pathogen increased the concentration in plants which did not involve either *T. harzianum* or *G. intraradices* inoculation and in *G. intraradices–T. harzianum* coinoculated plants with respect to the same treatments in the pathogen's absence. *F. oxysporum* decreased the shoot ABA concentration in *T. harzianum*- and in *G. intraradices*-inoculated plants with respect to the same treatments in the absence of the pathogen (Fig. 4A and B). The shoot ABA concentration in plants which did not involve either *T. harzianum* or *G. intraradices* inoculation was higher than in any other treatment assayed in the presence of the pathogen (Fig. 4B). *G. intraradices–T. harzianum* coinoculated plants showed a higher shoot ABA concentration than singly-inoculated plants (Fig. 4B).

Biocontrol effect. The interactions *G. intraradices–F. oxysporum*, *T. harzianum–F. oxysporum*, and *G. intraradices–T. harzianum–F. oxysporum* were significant for disease incidence (Table 1). The treatments involving inoculation with *T. harzianum* had significantly decreased the disease incidence (by 50%) with respect to the control (Table 3). *G. intraradices* inoculation also significantly reduced the disease incidence. Coinoculation with *T. harzianum* and *G. intraradices* resulted in a disease incidence significantly lower than for plants inoculated with either agent separately.

DISCUSSION

In this work, we analyzed changes in the hormonal profile of melon plants associated with *F. oxysporum* attack and the alteration of the accumulation pattern caused by multiple player interactions. As expected, infection with *F. oxysporum* strongly modified the balance of stress- and defense-related hormones. We observed an increase in shoot SA, JA, ACC, and ABA, concentrations in diseased plants, when not inoculated with either *T. harzianum* or *G. intraradices*. Similarly, signaling pathways mediated by SA, JA, ethylene, and ABA are activated upon *F. oxysporum* recognition by Arabidopsis (4,28). Our findings suggest the involvement of both the SA and JA pathways in the *F. oxysporum*–melon plant interaction. Glazebrook (17) reported that resistance against necrotrophic pathogens is based on JA-related mechanisms while resistance against biotrophic ones is associated with activation of SA-dependent signaling. In contrast with that, Mauch-Mani et al. (27) observed that *F. oxysporum* induced systemic acquired resistance (SAR) and pathogenesis-related (PR) proteins in *Arabidopsis*, indicating that the SA pathway plays a major role in plant resistance against *F. oxysporum*. Moreover, treatment of *Arabidopsis* plants with SA has

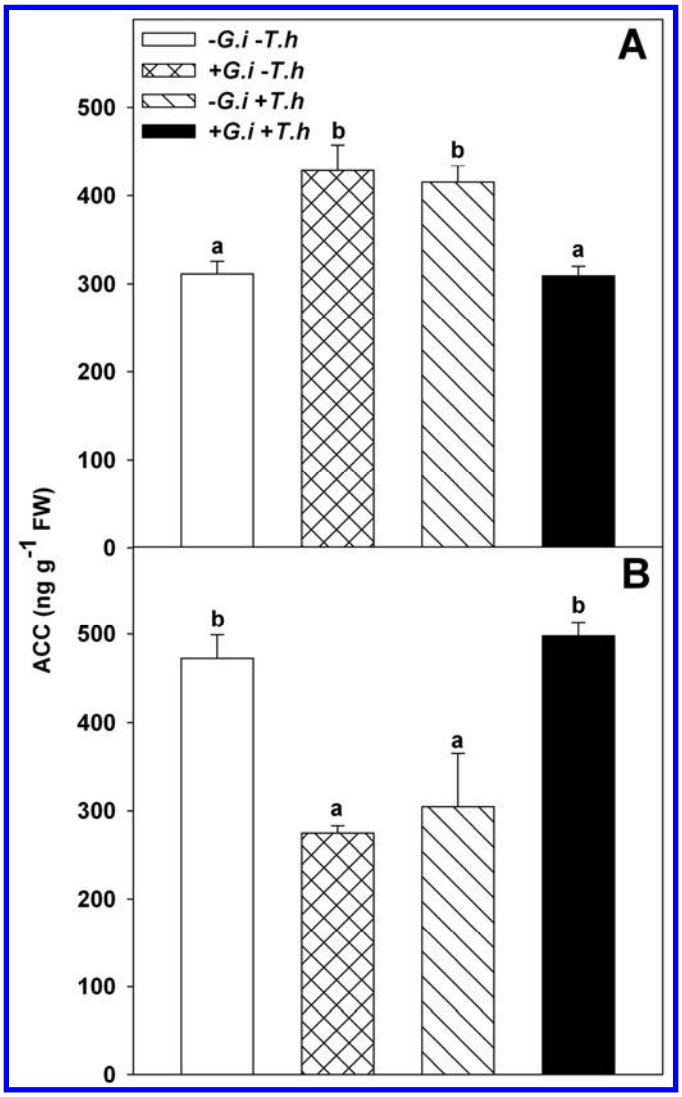

Fig. 3. Shoot 1-aminocyclopropane-1-carboxylic acid (ACC) concentration (in nanograms per gram fresh weight) in melon plants inoculated with *Glomus intraradices* (*G.i*) or *Trichoderma harzianum* (*T.h*), alone or in combination, in the **A,** absence or **B,** presence of *Fusarium oxysporum*. Bars indicate the standard error of five replicates. Columns with the same letter do not differ significantly according to Tukey's multiple range test ($P \leq 0.05$), $n = 5$.

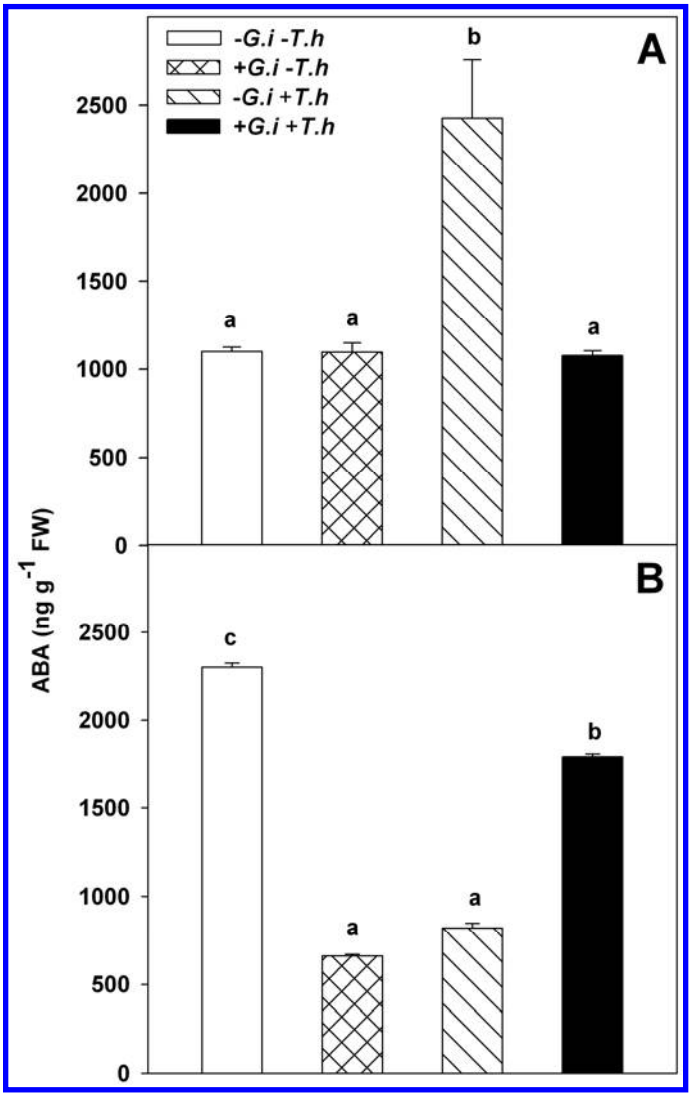

Fig. 4. Shoot abscisic acid (ABA) concentration (in nanograms per gram fresh weight) in melon plants inoculated with *Glomus intraradices* (*G.i*) or *Trichoderma harzianum* (*T.h*), alone or in combination, in the **A,** absence or **B,** presence of *Fusarium oxysporum*. Bars indicate the standard error of five replicates. Columns with the same letter do not significantly differ according to Tukey's multiple range test ($P \leq 0.05$), $n = 5$.

been reported to reduce *F. oxysporum* disease symptoms (13). In spite of the activation of the defense pathway in melon plants as a consequence of *F. oxysporum* attack, our results show a susceptible plant–pathogen interaction when neither *T. harzianum* nor *G. intraradices* were present. Susceptible interactions have been suggested to result either from the failure of the host plant to recognize and respond to the pathogen appropriately or from the responses themselves being ineffective (24). Indeed, several pathogens have also developed the ability to manipulate the defense-related regulatory network of plants. This results in hormonal imbalance and activation of inappropriate defense responses (35). For example, *Pseudomonas syringae* has been shown to hijack the ABA-signaling pathway in Arabidopsis to cause disease (10), suggesting a negative function of ABA as a mechanism used by *F. oxysporum* to suppress plant basal resistance. Moreover, our data reveal a similarity in the shoot hormonal profiling, involving plant–*F. oxysporum* and plant–*T. harzianum* interactions. Similar to what was observed in diseased plants, increases in the shoot SA, JA, ACC, and ABA concentrations were observed in nondiseased, *T. harzianum*-inoculated plants, indicating the involvement of similar pathways in both interactions. There is extensive evidence that *Trichoderma* spp. are able to elicit the defense systems of a number of plant species (19,38,41). These findings, together with the increased *F. oxysporum* populations observed in the current study with treatments involving *T. harzianum* inoculation, indicate that the protective effect against Fusarium wilt conferred on melon plants by *T. harzianum* was not only due to direct antagonism but rather was a plant-mediated phenomenon (20,38).

In the two-way interactions, the presence of the beneficial fungi *T. harzianum* as well as *G. intraradices* clearly modified the response of the plant hormonal profile to pathogen attack, which may be related to the control of the disease observed in this experiment. In fact, ACC and ABA accumulation in plant shoots was lower when *T. harzianum* or *G. intraradices* and the pathogen were interacting with the plant, compared with the pathogen alone, confirming that the presence of antagonists modifies the effect of a pathogen on the hormonal profile of a plant. Moreover, when *T. harzianum* was interacting with the pathogen, a decrease in SA was observed in diseased plants. In accordance with our findings, Marra et al. (25), studying the changes in the proteome of bean plants as a consequence of the interaction between the plant, the pathogen *Rhizoctonia solani* or *Botrytis cinerea*, and *Trichoderma*, reported a change in the expression pattern of plant genes as a response to pathogen attack, when the beneficial fungus was present. These authors observed that, when both *Trichoderma* and the pathogen were interacting with the plant, several PR-proteins were up-regulated less than by the pathogen alone. Our results suggest that the presence of *T. harzianum* or *G. intraradices* attenuated the ethylene- and ABA-dependent plant response to *F. oxysporum* attack, but did not interfere in the JA-dependent response. Unlike the *T. harzianum–F. oxysporum* interaction, *G. intraradices* was unable to interfere with the SA-dependent plant response to pathogen attack. In contrast with our results, Garmendia et al. (15) found that the greatest induction of phenylalanine ammonia lyase (PAL), the first enzyme in the phenylpropanoid biosynthesis pathway, which provides the precursor for biosynthesis of SA (40), by the pathogen *Verticillium dahliae* in pepper plants, was in plants associated with the presence of AM fungi. Similarly, Dehne and Schöbeck (11) found that the simultaneous infection of tomato with *G. mosseae* and *F. oxysporum* increased PAL activity more than did the mycorrhizal fungus or the pathogen alone. Several pieces of work suggest that the suppression of defense-related properties could be associated with the successful establishment of the mycorrhizal symbiosis (18,33). However, this was unlikely to have been the case in our experiment, as at the time of the infection with the pathogen, the symbiosis was already well established. Kapoor (21) reported increased PAL and JA concentrations in tomato plants infected with the pathogen *F. oxysporum*, when they were mycorrhizae-infected, and attributed the enhanced disease control to these increases.

Our findings suggest that the disease control observed in *T. harzianum* plants may have been due not just to the enhancement of the basal resistance, as increased SA and JA concentrations were observed in healthy *T. harzianum*-inoculated plants, but also to the capacity of *T. harzianum* to modulate the plant ABA-, ethylene-, and SA-dependent response to pathogen attack, resulting in the activation of a more appropriate response. In contrast, the resistance against *F. oxysporum* mediated by *G. intraradices* in this experiment seems to include mechanisms independent of SA and JA signaling, although *G. intraradices* was also able to attenuate the plant defense response to the pathogen attack, resulting in attenuated shoot ACC and ABA accumulation.

A synergistic effect on biocontrol capacity was observed when *T. harzianum* and *G. intraradices* were coinoculated. However, in the three-way interaction (*T. harzianum–G. intraradices–F. oxysporum*), JA and SA showed accumulation patterns similar to those of diseased plants inoculated with *T. harzianum*. Furthermore, when applied in combination, the microorganisms were unable to modify the ACC- and ABA-dependent response to pathogen attack as well as when they were applied singly, resulting in higher levels of ACC and ABA. We did not observe any synergistic effect on the hormone mediated plant response as a consequence of the interaction between the two microorganisms, since no synergistic effect on SA, JA, or ACC was observed in healthy plants coinoculated with both microorganisms when compared with healthy plants inoculated with each microorganism alone. Therefore, the synergistic effect observed in plants coinoculated with *G. intraradices* and *T. harzianum* seems to have been due to a positive interaction between different mechanisms of plant protection mediated by the two agents, which may have included an improvement in plant nutrition, damage compensation, competition for colonization sites or photosynthates, changes in the root system, changes in rhizosphere microbial populations, and activation of plant defense mechanisms (33).

Taken together, our data indicate that the mechanisms involved in the control of Fusarium wilt by *T. harzianum* in melon plants, seem to be related not only to an induction of plant resistance, but also to its capacity to attenuate the hormonal disruption caused by the disease. However, the resistance mediated by *G. intraradices* against *F. oxysporum* seems to include mechanisms independent of SA and JA signaling. In the three-way interaction (*T. harzianum–G. intraradices–F. oxysporum*), a synergistic effect on disease control was found; however, no synergistic effect was observed neither on the attenuation of the hormone disruption induced by the pathogen, nor in the SA, JA, or ACC mediated plant response. Therefore, the synergistic effect regarding biological control capacity seems to be the result of numerous modes of action exhibited by each microorganism, which differ from the plant-mediated mechanism.

LITERATURE CITED

1. Abeles, F. B., Morgan, P. W., and Saltveit, M. E., Jr. 1992. Ethylene in Plant Biology. 2nd ed. Academic Press, San Diego.
2. Albacete, A., Ghanem, M. E., Martínez-Andújar, C., Acosta, M., Sánchez-Bravo, J., Martínez, V., Lutts, S., Dodd, I. C., and Pérez-Alfocea, F. 2008. Hormonal changes in relation to biomass partitioning and shoot growth impairment in salinized tomato (*Solanum lycopersicum* L.) plants. J. Exp. Bot. 59:4119-4131.
3. Barea, J. M., Azcón, R., and Azcón-Aguilar, C. 2002. Mycorrhizosphere interactions to improve plant fitness and soil quality. Antonie Leeuwenhoek 81:343-351.
4. Berrocal-Lobo, M., and Molina, A. 2007. Arabidopsis defense response against *Fusarium oxysporum*. Trends Plant Sci. 13:145-150.
5. Bi, H. H., Song, Y. Y., and Zeng, R. S. 2007. Biochemical and molecular

responses of host plants to mycorrhizal infection and their roles in plant defense. Allelopathy J. 29:15-28.
6. Calvet, C., Pera, J., and Barea, J. M. 1993. 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.
7. Champaco, E. R., Martyn, R. D., and Miller, M. E. 1993. Comparison of *Fusarium solani* and *Fusarium oxysporum* as causal agents of fruit rot and root rot muskmelon. HortScience 28:1174-1177.
8. Chandanie, W. A., Kubota, M., and Hyakumachi, M. 2006. 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.
9. Datnoff, L. E., Nemec, S., and Pernezny, K. 1995. Biological control of *Fusarium* crown and root rot of tomato in Florida using *Trichoderma harzianum* and *Glomus intraradices*. Biol. Control 5:427-431.
10. De Torres-Zabala, M., Truman, W., Bennett, M. H., Lafforgue, G., Mansfield, J. W., Egea, P. R., Bogre, L., and Grant, M. 2007. *Pseudomonas syringae* pv. *tomato* hijacks the *Arabidopsis* abscisic acid signaling pathway to cause disease. EMBO J. 26:1434-1443.
11. Dehne, H. W., and Schönbeck, F. 1979. Investigations on the influence of endotrophic mycorrhiza on plant diseases. II. Phenol metabolism and lignification. Phytopathol. Z. 95:210-216.
12. Dobrev, P. I., and Kaminek, M. 2002. Fast and efficient separation of cytokinins from auxin and abscisic acid and their purification using mixed-mode solid-phase extraction. J. Chromatogr. A 950:21-29.
13. Edgar, C. I., McGrath, K. C., Dombrecht, B., Manners, J. M., Maclean, D. C., Schenk, P. M., and Kazan, K. 2006. Salicylic acid mediates resistance to the vascular wilt pathogen *Fusarium oxysporum* in the model host Arabidopsis thaliana. Australas. Plant Pathol. 35:581-591.
14. Gallou, A., Cranenbrouck, S., and Declerck, S. 2009. *Trichoderma harzianum* elicits defense response genes in roots of potato plantlets challenged by *Rhizoctonia solani*. Eur. J. Plant Pathol. 124:219-230.
15. Garmendia, I., Aguirreolea, J., and Goicoechea, N. 2005. Defense-related enzymes in pepper roots during interactions with arbuscular mycorrhizal fungi and/or *Verticillium dahliae*. Biocontrol 51:293-310.
16. Giovannetti, M., and Mosse, B. 1980. An evaluation of techniques for measuring vesicular-arbuscular mycorrhizal infection in roots. New Phytol. 84:498-499.
17. Glazebrook, J. 2005. Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. Annu. Rev. Phytopathol. 43:205-227.
18. Guenoune, D., Galili, S., Phillips, D. A., Volpin, H., Chet, I., Okon, Y., and Kapulnik, Y. 2001. The defense response elicited by the pathogen *Rhizoctonia solani* is suppressed by colonization of the AM-fungus *Glomus intraradices*. Plant Sci. 160:925-932.
19. Hanson, L. E., and Howell, C. R. 2004. Elicitors of plant defense responses from biocontrol strains of *Trichoderma virens*. Phytopathology 94:171-176.
20. Harman, G. E., Howell, C. R., Viterbo, A., Chet, I., and Lorito, M. 2004. *Trichoderma* species opportunistic, avirulent plant symbionts. Nat. Rev. Microbiol. 2:34-56.
21. Kapoor, R. 2008. Induced resistance in mycorrhizal tomato is correlated to concentration of jasmonic acid. Online J. Biol. Sci. 8:49-56.
22. Kohler, J., Caravaca, F., Carrasco, L., and Roldán, A. 2007. Interactions between a plant growth-promoting rhizobacterium, an AM fungus and a phosphate-solubilising fungus in the rhizosphere of *Lactuca sativa*. Appl. Soil Ecol. 35:480-487.
23. Komada, H. 1975. Development of a selective medium for quantitative isolation of *Fusarium oxysporum* from natural soil. Rev. Plant Prot. Res. 8:114-124.
24. Ludwig-Müller, J., Prinsen, E., Rolfe, S. A., and Scholes, J. D. 2009. Metabolism and plant hormone action during clubroot disease. J. Plant Growth Regul. 28:229-244.
25. Marra, R., Ambrosino, P., Carbone, V., Vinale, F., Woo, S. L., Ruocco, M., Ciliento, R., Lanzuise, S., Ferraioli, S., Soriente, I., Gigante, S., Turrà, D., Fogliano, V., Scala, F., and Lorito, M. 2006. Study of the three-way interaction between *Trichoderma atroviride*, plant and fungal pathogens by using a proteomic approach. Curr. Genet. 50:307-321.
26. Martínez-Medina, A., Pascual, J. A., Lloret, E., and Roldán, A. 2009. Interactions between arbuscular mycorrhizal fungi and *Trichoderma harzianum* and their effects on Fusarium wilt in melon plants grown in seedlings nurseries. J. Sci. Food Agric. 89:1843-1850.
27. Mauch-Mani, B., and Slusarenko, A. J. 1994. Systemic acquired resistance in *Arabidopsis thaliana* induced by a predisposing infection with a pathogenic isolate of *Fusarium oxysporum*. Mol. Plant-Microbe Interact. 7:378-383.
28. McDowell, J. M., and Dangl, J. L. 2000. Signal transduction in the plant immune response. Trends Biochem. Sci. 25:79-82.
29. Nemec, S., Datnoff, L. E., and Strandberg, J. 1996. Efficacy of biocontrol agents in planting mixes to colonize plant roots and control root diseases of vegetables and citrus. Crop Prot. 15:735-742.
30. Pascual, J., Navarro, N., Hernandez, M. T., García, C., Bernal, A., Lopez, R., Martinez, A., and Antón, A. I. 2007. An efficient solid product for the biological control of melon fusarium wilt, its production procedure and its application method. Spanish Patent 200603290.
31. Phillips, J. M., and Hayman, D. S. 1970. Improved procedures for clearing roots and staining parasitic and vesicular-arbuscular mycorrhizal fungi for rapid assessment of infection. Trans. Brit. Mycol. Soc. 55:158-161.
32. Pieterse, C. M. J., Van Pelt, J. A., Verhagen, B. W. M., Ton, J., van Wees, S. C. M., Léon-Kloosterziel, K. M., and van Loon, L. C. 2003. Induced systemic resistance by plant growth promoting rhizobacteria. Symbiosis 35:39-54.
33. Pozo, M. J., and Azcón-Aguilar, C. 2007. Unraveling mycorrhiza-induced resistance. Curr. Opin. Plant Biol. 10:393-398.
34. Pozo, M. J., Cordier, C., Dumas-Gaudot, E., Gianinazzi, S., Barea, J. M., and Azcón-Aguilar, C. 2002. Localized versus systemic effect of arbuscular mycorrhizal fungi on defence responses to *Phytophthora* infection in tomato plants. J. Exp. Bot. 53:525-534.
35. Robert-Seilaniantz, A., Navarro, L., Bari, R., and Jones, J. D. G. 2007. Pathological hormone imbalances, Curr. Opin. Plant Biol. 10:372-379.
36. Saldajeno, M. G. B., Chandanie, W. A., Kubota, M., and Hyakumachi, M. 2008. Effects of interactions of arbuscular mycorrhizal fungi and beneficial saprophytic mycoflora on plant growth and disease protection. Pages 211-226 in: Mycorrhizae: Sustainable Agriculture and Forestry. Z. A. Siddiqui, M. S. Akhtar, and K. Futai, eds. Springer Sciences, Netherlands.
37. Schmidt, K., Pflugmacher, M., Klages, S., Mäser, A., Mock, A., and Stahl, D. J. 2008. Accumulation of the hormone abscisic acid (ABA) at the infection site of the fungus *Cercospora beticola* supports the role of ABA as a repressor of plant defence in sugar beet. Mol. Plant Pathol. 9:661-673.
38. Shoresh, M., Yedidia, I., and Chet, I. 2005. Involvement of jasmonic acid/ethylene signaling pathway in the systemic resistance induced in cucumber by *Trichoderma asperellum* T203. Phytopathology 95:76-84.
39. Soriano, M. L., Porras-Piedra, A., Marcilla Goldaracena, I., Porras Soriano, A., Martín-Sánchez, D., León-Egido, M., Porras-Soriano, M. L., and Porras-Soriano, R. 2006. Microwave energy supplied by a prototype oven prevents the spread of Fusarium wilt during the propagation of melon plantlets by seeds. Span. J. Agric. Res. 4:207-212.
40. Sticher, L., Mauch-Mani, B., and Métraux, J. P. 1997. Systemic acquired resistance. Annu. Rev. Phytopathol. 35:235-270.
41. Vinale, F., Sivasithamparam, K., Ghisalberti, E. L., Marra, R., Woo, S. L., and Lorito, M. 2008. *Trichoderma*-plant-pathogen interactions. Soil Biol. Biochem. 40:1-10.
42. Whipps, J. M. 2004. Prospects and limitations for mycorrhizas in biocontrol of root pathogens. Can. J. Bot. 82:1198-1227.
43. Yedidia, I., Shoresh, M., Kerem, Z., Benhamou, N., Kapulnik, Y., and Chet, I. 2003. Concomitant induction of systemic resistance to *Pseudomonas syringae* pv. *lachrymans* in cucumber by *Trichoderma asperellum* (T-203) and accumulation of phytoalexins. Appl. Environ. Microbiol. 69:7343-7353.