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Induction of Phenolic Compounds and Pathogenesis-Related Proteins by Mycorrhizal Fungal Inoculations against *Phytophthora capsici* Leonian in Pepper

H. Ozgonen, N. Yardimci and H. Culal Kilic
Department of Plant Protection, Faculty of Agriculture, Suleyman Demirel University,
32260, Isparta, Turkey

Abstract: In this study, the effects of mycorrhizal fungal inoculations of *Glomus mosseae*, *G. etunicatum*, *G. fasciculatus* and *Gigaspora margarita* species on phenolic compounds and pathogenesis related proteins were investigated in pepper - *Phytophthora capsici* plant pathosystem. Root colonization by mycorrhizal fungi increased after 15, 25 and 45 days after inoculation depending on the root developmental stage. *G. mossea* and *G. fasciculatus* reached the highest root colonization level. In addition, arbuscule formation, spores inside or outside the root cells were observed after 25 and 45 days, respectively. The disease development of pepper plants was decreased depending on the mycorrhizal fungi colonization compared to the pathogen inoculated plants. Total phenolic compounds increased in all treatments as compared to non mycorrized non pathogen treated control, but was highest when plants were inoculated with both, the mycorrhizal fungi and the pathogen. Overall, 12 different phenolic compounds were identified using thin layer chromatography according to Rf. In general, there was an increased in the activity of β-1.3-glucanases and chitinases after 3, 6 and 9 days as compared to control in stem extracts of mycorrhizal fungi and or *P. capsici* treatments. The enzyme activities were higher 6 days after inoculation, but decreased 3 days later in all treatments. In conclusion, increasing activity of phenolic compounds and enzymes were observed depending on simultaneous inoculations with mycorrhizal fungi and pathogen, that could be involved in disease resistance.

Key words: β-1,3-glucanase, *Capsicum annum*, chitinase, *Glomus mosseae*, *Glomus etunicatum*, *Glomus fasciculatus*, *Gigaspora margarita*

INTRODUCTION

Mycorrhizal fungi have an important role in the agroecosystem because of its ability to establish symbiotic interactions with roots of approximately 95% of plant species (Smith and Read, 1997). Mycorrhizal fungi contribute to plant development by means of enlarging absorbing surface root system via colonization of roots, increasing uptake of macro and micro nutrients initially phosphorus and providing liveliness of roots (Marschner and Dell, 1994). In addition, mycorrhizal fungi have an important role in protecting plant against pathogens via induction of some resistance mechanisms against them, particularly soil-borne pathogens such as *Pythium* sp., *Phytophthora* sp., *Fusarium* sp. and *Verticillium* sp. (Azcon-Aguilar and Barea, 1992; Trotta *et al*., 1996; Garmendia *et al*., 2004).

Some studies were conducted using disease reactions as well as microscopic and biochemical studies related with the effectiveness of mycorrhizal fungi against plant pathogens. Mycorrhizal fungi reduced disease severity of the disease by forming a mechanical barrier as a result of root colonization and or enhanced plant development (Dumas-Gaudot *et al*., 2000). Also, mycorrhizal fungi induce biochemical resistance mechanisms depending on the root colonization. In mycorrhized plants, protection against soil-borne fungal pathogens is due to the activation of defense plant responses such as production of Pathogenesis Related (PR) proteins (Lambais, 2000; Azcon-Aguilar *et al*., 2002). Dassi *et al*. (1998) reported that the amount of PR proteins were increased in *G. mosseae*-colonized tomato roots after inoculation with *Phytophthora parasitica*. Symbiotic microorganisms caused an increase in PR proteins especially chitinases, β-1,3-glucanases and peroxidases (Dumas-Gaudot *et al*., 1996; Pozo *et al*., 1998, 1999; Lambais *et al*., 2003). Garmendia *et al*. (2006) also reported that, the colonization of pepper roots by *G. deserticola* induced isoforms of asidic chitinases and peroxidases and mycorrhizal fungi plus inoculation with *Verticillium dahliae* inoculation slightly induced the peroxidases. Devi and Reddy (2002) reported that the amount of

Corresponding Author: H. Ozgonen, Department of Plant Protection, Faculty of Agriculture, Suleyman Demirel University, 32260, Isparta, Turkey

phenolic compounds were increased such as *trans-p*-coumaric asit, *trans*-ferulic acid and vanillic acid in *G. mosseae* and/or *Rhizobium* inoculated roots and upper part of peanut plants.

In this study, the amount of phenolic compounds and PR proteins were determined by mycorrhizal fungal inoculations against Phytophthora blight caused by *P. capsici* in pepper.

MATERIALS AND METHODS

Plant, pathogen and mycorrhizal fungi: *Capcicum annum* L. cv. Charliston Bagci was used as plant material. *Phytophthora capsici* (Pc5 isolate) was isolated from naturally infected plants at Adana.

Four different mycorrhizal fungi species, increased in *Zea mays* L. were used: *G. mosseae* (GM), *G. etunicatum* (GE), *G. fasciculatus* (GF) and *Gi. margarita* (GiM).

Studies were conducted at the Mycology and Molecular Biology Laboratories, Department of Plant Protection, University of Suleyman Demirel during 2005 to 2008.

Seedling production with mycorrhizal fungal inoculation and establishment of pot trial: Pepper seedlings were produced in plastic containers (30×40 cm). Growing medium consisted of soil:sand:pumice (1:1:1/v:v:v) autoclaved twice at 121°C, 1 kPa. GM, GE, GF and GiM inocula consisted of soil+root fragments+spores+mycelia were placed 2-3 cm below seeds before planting (Menge and Timmer, 1982). Mycorrhizal inoculum densities in soil were, 188 spores 10 g^{-1} soil, 2.1 kg for GM; 268 spores 10 g^{-1} soil, 1.3 kg for GE; 229 spores 10 g^{-1} soil, 1.7 kg for GF and 235 spores 10 g^{-1} soil, 1.6 kg for GiM. Seedlings were produced without mycorrhizal fungal inoculations representing control group. Containers were placed in growth room at 25±2°C and a 12 h photoperiod. Plants were watered with deionized water during seedling production until the 3-4 leaves stage.

Pot trials were established when seedlings reached the stage of 3-4 leaves with or without mycorrhizal inoculation. Mycorrhizal inocula were applied in the planting hole when seedlings were transplanted to 15 cm diameter pots at a ratio of 50 g for GM, 35 g for GE, 45 g for GF and 40 g for GiM depending on the spore number in 10 g soil, to reach a final concentration of ˜1000 spores.

Treatments were as follows: *G. mosseae* (GM), *G. etunicatum* (GE), *G. fasciculatus* (GF) and *Gi. margarita* (GiM), *P. capsici* (Pc), Control (C) and the combination of each of the four mycorrhizal fungi species with *P. capsici*. Each treatment was replicated five times.

For *P. capsici* inoculation, the oomycete was grown on oatmeal agar plates at 28°C for 7 days and placed under fluorescent light for sporulation. Culture plates were incubated in sterile distilled water for 40 min at 4°C and then during 30 min at 25°C room temperature. Zoospore released from sporangia of *P. capsici* were collected by filtering through two-layers of cheesecloth and zoospore concentration was adjusted to 1×10^6 zoospores mL^{-1} using haemocytometer (Sunwoo *et al*., 1996). A 10 mL spore suspension was applied to soil around the root of the plant in each pot after 15 days.

One week after inoculation, symptoms were evaluated according to a 0-5 scale: where 0 = no visible disease symptoms; 1 = leaves slightly wilted with brownish lesions beginning to appear on stems; 2 = 30-50% of entire plant diseased; 3 = 50-70% of entire plant diseased; 4 = 70-90% of entire plant diseased and 5 = plant dead (Sunwoo *et al*., 1996).

Disease severity values were expressed as a percentage of affected tissue according to Tawsend-Heuberger transformation (Gomez and Gomez, 1983).

Microscopic observations: Roots from mycorrhizal inoculated plants were observed at three different stages of plant development:

- **Stage I:** (15 days after mycorrhizal fungal inoculation) Determination of hyphal development in or on roots
- **Stage II:** (25 days after mycorrhizal fungal inoculation) Formation of arbuscul or vesicle of mycorrhizal fungi
- **Stage III:** (45 days after inoculation) Formation of arbuscule, vesicle or spores

The roots were cleared and stained as described by Koske and Gemma (1989) and the percentage of root colonization was estimated by a gridline intersection method (Giovannetti and Mosse, 1980).

Determination of phenolic compounds: For determination of phenolic compounds, root extractions were performed for each treatment. Roots were harvested for each treatment and extracted with ethyl alcohol according to Mahadevan *et al*. (1965). Amount of total phenolic compounds were determined according to Bray and Thorpe (1954) and Johnson and Scholl (1957).

Thin layer chromatography were performed on Whatman No. 1 chromatographic paper using concentrated root extracts with two dimension progression technique. Applied solvent systems were benzen-acetic acid-water (60:70:30, upper phase) and

sodium formate -formic acid-water (10:1:200), respectively. Points on chromatographic paper were examined under UV light and/or by spraying reagents (Sulphamidic asit and p-nitraline) and ferric chloride with 1% according to Rf values.

According to Rf, regions without sprayed point were dissolved in ethyl alcohol (90%) and extract was evaporated until dryness. Extract was mixed with 3 mL distilled water and 0.5 mL of Folin's reagent and shacked. After 3 min, 1 mL of diluted sodium carbonate was added and completed to 10 mL within 1 h. Measurements were performed using spectrophotometer at 725 nm after formation of blue color.

Enzyme analysis

Preparation of stem extracts: Stem extractions were performed for each sample from harvested plants according to Hwang *et al.* (1997). Stem pieces were homogenized in 0.5 M sodium acetate buffer with 15 mM of mercaptoethanol. Crude extracts were centrifuged at 4°C, 10.000 g for 60 min and then supernatant was centrifuged at 4°C, 20.000 g for 60 min.

Protein in collected supernatant was determined using as standard known bovine serum albumin.

Proteins in crude extract were mixed with 4 part acetone and precipitated at 20°C overnight. Precipitation was centrifuged at 15.000 g for 15 min and washed with cold acetone twice and dried.

Residue was suspended in 30 mM of sodium acetate buffer (pH 5.2) for cleaning and then samples were kept at -70°C until use.

Measurement of β-1,3-glucanase activity: β-1,3-glucanase activity was performed using a calorimetric assay method as described by Kauffman *et al.* (1987). For enzyme activity detection, laminarin was used as a substrate to determine the decreased sugar amount arising from laminarin in extract.

Substrate buffer included 0.1 M of sodium acetate buffer (pH 5.2) contain laminarin (1 mg mL^{-1} buffer). Reaction mixture was prepared using 0.9 mL of subtrate buffer and 0.1 mL of enzyme solution (stem extract).

Reaction tubes were kept at 37°C for 1 h and the decrease in sugar amount was determined according to Nelson (1944). Accordingly, glucose was used as standard and 1 kat (katal) defined as enzyme activity equivalent 1 mol catalyzed glucose.

Measurement of chitinase activity: Chitinase activity in crude extract was determined using calorimetric assay (Hwang *et al.*, 1997).

Reaction mixture was prepared as final volume of 0.5 mL of 0.1 M sodium acetate buffer (pH 5.2) including 0.5 mg of washed chitin and enzymatic solutions in different volume. Mixture was kept in water bath at 37°C for 1 h and incubated by shaking.

For determining the chitinase activity, 0.3 mL of supernatant was incubated at 37°C to hydrolyze chitin oligomers using 5 μL, 25% glucoronidase. To induce the formation of N-acetyl glucosamin after reaction, 0.6 M of 0.1 mL potassium tetraborat was added and kept in boiling water for 3 min and incubated with 1 mL diluted reagent and glacial acetic acid (1:2, v:v) at 37°C for 20 min after cooling. Reagent stock solution was prepared using glacial acetic acid and 11.5 M of HCl, 87.5 mL:12.5 mL (v/v) mixture contain 10% (w/v) 4 (dimethyl amine) benzaldehyde. Resulting Glc-Nac was determined according to Legrand *et al.* (1987).

Statistical analysis: The data were subjected to analysis of variance (F-test). Treatment means were compared using Fisher's Least Significant Difference (LSD) test at p = 0.05 (Gomez and Gomez, 1983).

RESULTS

Microscopic observation: The colonization percent of mycorrhizal fungi, vesicule, arbuscule and spores in and outside of the host cell were observed in different stages of seedling development. Results were shown in Table 1.

At seedling stage I, root colonization percentage after seedling emergence were 9.0, 5.0, 8.0 and 5.0% for GM, GE, GF and GiM, respectively. Structures of the mycorrhizal fungi were not observed at this stage.

At stage II, the colonization percentage of GM and GF found as 21.0 and 18.0%, respectively; whereas colonization percentage of GE and GiM were 15%. Mycorrhizal arbuscules were observed at this stage of seedling development.

The highest colonization rates were obtained from GM and GF treated plants by 53.0 and 50.0%, respectively at stage III. Arbuscules and spores were observed in all inspected roots at this final stage of seedling development.

Table 1: Root colonisation (%) and structures of *G. mosseae*, *G. etunicatum*, *G. fasciculatus* and *Gi. margarita*

	Time (No. of days after inoculation)											
	15					25				45		
AMF	C*	A	V	S	C	A	V	S	C	A	V	S
GM	9.0	-	-	-	21.0	+	-	-	53.0	+	-	+
GE	5.0	-	-	-	15.0	-	-	-	47.0	+	-	+
GF	8.0	-	-	-	18.0	+	-	-	50.0	+	-	+
GiM	5.0	-	-	-	15.0	+	-	-	45.0	+	-	+

*C: Colonisation (%); A: Arbuscule; V: Vesicule; S: Spore; +,-: Colonisation ratio are means of values

Table 2: Amounts of total phenolic compounds in *G. mosseae*, *G. etunicatum*, *G. fasciculatus*, *Gi. margarita* and/or *P. capsici* inoculations

Treatments	Total phenolic compounds (μg g^{-1} of fresh weight)
GM	72.0c[a]
GE	65.0d
GF	70.0c
GiM	65.0d
Pc	68.0cd
GM + Pc	115.0a
GE + Pc	92.0b
GF + Pc	110.0a
GiM + Pc	92.0b
Control	45.0e

[a]Means fallowed by different letters are significantly different (p = 0.05) according to Fisher's LSD test

Analysis of phenolic compounds: The amounts of total phenolic compounds (μg g^{-1} fresh weight) 15 days after inoculation are shown in Table 2.

The amounts of total phenolic compounds were increased in treated compared as compared to control plants. The amount of the total phenolic compounds was 45.0 μg g^{-1} of fresh weight in control compared to 68.0 μg g^{-1} fresh weight in Pc. Mycorhizal fungal inoculations individually provide increased the amount of phenolics compounds in root and the highest amount was obtained from GM treatment (72 μg g^{-1} of fresh weight). However, phenolic compounds concentration was highest in the combined treatment of mycorrhizal fungi and *P. capsici*. The amounts of phenolic compounds in GM + Pc and GF+Pc were 115.0 and 110.0 μg g^{-1} of fresh weight, respectively.

Several phenolic compounds were identified in treated plants: Caffeic acid, *trans*-coumaryl, capsaicin, *p*-aminobenzaldehyde, aspartic acid, chlorogenic acid, glutamic acid, linoleic acid, *cis*-feruloyl acid, stearic acid, capsicoside and an undefined F1.

Diseases severity (%) was determined in Pc inoculated plants when at the same time as phenolic compounds 15 days after inoculation. Disease symptoms consisted in root and crown rot. Mycorrhizal fungi reduced disease severity compared to that observed on non mycorrhized Pc infected plants (Table 3).

Enzyme analysis: Activities of β-1.3-glucanase and chitinase enzymes in the different treatments are shown in Table 4 and 5.

In all treatments, the β-1.3-glucanase and chitinase activities were maximum 6 days after inoculation, decreasing at the initial and final assessments 3 and 9 days after inoculation, respectively. The levels of both, β-1.3-glucanase and chitinase enzymes in control plants ranged 0-3 μ kat mg^{-1} of protein. The level of β-1.3-glucanase enzyme was higher in Pc infected plants (19 μ kat mg^{-1} of protein) and those with combined inoculation of mycorrhizal fungi and Pc treatments (19-25 μ kat mg^{-1} of protein), as compared to that at the single mycorrhizal treatments (10-16 μ kat mg^{-1} of protein) 6 days after inoculation (Table 4). Similarly, chitinase activity was higher in Pc infected plants (11 μ kat mg^{-1} of protein) and those with combined inoculation of mycorrhizal fungi and Pc treatments (9-14 μ kat mg^{-1} of protein), as compared to that at the single mycorrhizal treatments (5-7 μ kat mg^{-1} of protein) 6 days after inoculation (Table 5).

Table 3: Phytophthora root rot severity (%) in different inoculation of *G. mosseae*, *G. etunicatum*, *G. fasciculatus*, *Gi. margarita* and *P. capsici*

Treatments	Disease severity (%)
GM+Pc	15.0
GE+Pc	25.0
GF+Pc	20.0
GiM+Pc	35.0
Pc	85.0

Table 4: Activity of β-1.3-glucanase (μ kat mg^{-1} protein) in *G. mosseae*, *G. etunicatum*, *G. fasciculatus*, *Gi. margarita* and/or *P. capsici* inoculations

	Time (No. of days after inoculation)		
Treatments	3	6	9
GM	6c[a]	15c	5d
GE	6c	10d	4de
GF	6c	16c	5d
GiM	5c	10d	4e
Pc	8b	19b	8c
GM+Pc	11a	25a	18a
GE+Pc	8b	19b	15ab
GF+Pc	11a	22a	18a
GiM+Pc	8b	19b	15ab
Control	0d	3e	3e

[a]Means within each column fallowed by different letters are significantly different (p = 0.05) according to Fisher's LSD test

Table 5: Activity of chitinase (μ kat mg^{-1} protein) in *G. mosseae*, *G. etunicatum*, *G. fasciculatus*, *Gi. margarita* and/or *P. capsici* inoculations

	Time (No. of days after inoculation)		
Treatments	3	6	9
GM	4bc[a]	7c	4b
GE	4bc	6cd	3b
GF	3c	7c	3b
GiM	3c	5d	3b
Pc	5a	11b	8a
GM+Pc	6a	14a	6a
GE+Pc	6a	9c	4b
GF+Pc	6a	12ab	6a
GiM+Pc	3c	9c	4b
Control	2c	3e	0c

[a]Means within each column fallowed by different letters are significantly different (p = 0.05) according to Fisher's LSD test

DISCUSSION

In this study, the formation of root structures of mycorrhizal fungi (*G. mosseae*, *G. etunicatum*, *G. fasciculatus* and *Gi. margarita*) as well as the level of phenolic compounds and the pathogenesis related proteins β-1.3-glucanase and chitinase were determined.

The root colonization of the host plant by mycorrhizal fungi were increased with the stage of seedling. GM and GF showed the highest root colonization level. Mycorrhizal arbuscule formation was observed at the Stage II of host development, while both, arbuscule and spores were observed at stage III.

Severity of Phytophthora root rot symptoms were reduced on the combined mycorrhizal fungi and Pc treatments as compared to Pc inoculations alone. At the same time, the amount of total phenolic compounds were higher in all inoculated treatments than in the control treatment. However, the levels of phenolic compounds were much higher in the combined mycorrhizal fungi and pathogen inoculation treatments. Twelve different phenolic compounds were determined in the different treatments, except for one that could not be identified. Rabie (1998) reported that the amount of total phenolic compounds were increased by mycorrhizal fungus and nodule bacteria in fababean and *Botrytis fabae* pathosystem.

In present study, mycorrhizal fungi plus Pc inoculation resulted in an increase in the level of β-1.3-glucanase and chitinase compared to control treatment. The enzymes activities were the highest on the 6th day after inoculation in all treatments and decreased on the 3rd and 9th days after inoculation. Mycorrhizal fungal inoculations individually resulted in increasing levels of the both enzymes that were maximum for the combination of mycorrhizal fungi and Pc. Pozo *et al.* (1999) reported that *G. mosseae* and *G. intraradices* inoculation combined with *Phtophthora parasitica* inoculation of tomato resulted in an increase of β-1,3 glucanase activity 4 weeks after inoculation. Acibenzolar-S-methyl an abiotic inducer resulted in increasing level of enzymes activity as found by Suo and Leung (2002), the reported that ASM at 50 μM induced accumulation of extra cellular polysaccharides in rose against the rose scab pathogen *Diplocarpon rosae*. On the other hand, Garmendia *et al.* (2006) reported that in *G. deserticola* inoculated pepper plants, peroxidases activity slightly increased when plants were also inoculated with the Verticillium wilt pathogen.

A reduction of the severity of soil borne diseases by mycorrhizal fungi has been demonstrated in some plant-pathogens systems (Caron *et al.*, 1986; Dar *et al.*, 1997; Ozgonen *et al.*, 2001). The known preventing effects of mycorrhizal fungi against infection and/or colonization of plant roots by soil borne pathogens as well as an increase in uptakes of plant nutrients have been reported. However, physical protection against the plant pathogens by enhancing some of the biochemical mechanisms have also been reported (Dumas-Gaudot *et al.*, 2000).

Dugassa *et al.* (1996) investigated the effects of *G. intraradices* against *Fusarium oxysporum* f.sp. *lini* in flax by physiological and biochemical methods and resulted in increased resistance to this pathogen due to the colonization of plant roots by mycorrhizal fungi. These effects are reported to be related with the highest root colonization and increased concentrations of phytohormones, as gibberellins and oxin in shoots and ethylene in roots.

Plant hydrolytic enzymes (chitinases, β-1,3-glucanases) are among those antifungal compounds which have potential roles in protection against plant pathogens (Dumas-Gaudot *et al.*, 1996).

This findings showed that mycorrhizal symbiosis could work as signalling compounds in this system during the colonization process. It can be concluded that the amount of phenolic compounds and increased level of enzymes activity were related the specific mycorrhizal fungi and likely could be involved in protection of pepper plant against *P. capsici*.

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