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Interaction Between Vasicular Arbuscular Mycorrhizae and Antagonistic Biocontrol Micro-organisms on Controlling Root-Rot Disease Incidence of Geranium Plants

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ABSTRACT: The individual and interaction of vasicular arbuscular mycorrhiza (VAM), *Glomus mosseae* and antagonistic biological control agents (BCAs) i. e. *Trichoderma harzianum, Penicillium oxalicum* and *Bacillius subtilis* were studied on normal *root –rot disease* incidence of geranium plants . Application of combined VAM and BCAs reduced root-rot caused by *Fusarium solani* and *Machrophomina phaseolina* either in artificial or in natural infested soils compared with individual treatments. VAM was the most effective plant colonization in the presence of BCAs while, biocontrol microorganisms were much higher in soil rhizosphere in mycorrhizal plants over 90 days of plant growth compared with non-mycorrhizae. Furthermore, different biocontrol mat enhanced VAM growth and chitinase production in dual culture. The plants inoculated with combined VAM and BCAs showed increased in chitinase induction in plant roots as well as plant growth, Phosphorous concentration and oil percentage were also increased. The plant which inoculated with *G. mosseae* showed higher in the presence of either *T. harzianum* or *P. oxalicum*. Combined BCAs and VAM had a synergistic effect on disease control.

Key words: Antagonistic biocontrol agents (BCAs), *Glomus mosseae* , antagonistic microorganism, *root –rot disease* and Vasicular arbuscular mycorrhizae (VAM).

Introduction

Geranium (*Pelargonium graveolens* L.) have been an important part of green house potted plant and bedding plant production for almost a century . Root- rot disease are considered as the most serious disease affecting that foliage crop in different countries including Egypt (Kalra *et al.*, 1992 and El-Gamal, Nadia, 1995).Several studies have demonstrated that the application of selected VAM fungi can benefit crop protection and production (Gianinazzi- Pearson *et al* .1996 ,Sharma and Dohroo ,1996 and Slezack *et al.*, 2000) . VAM are an integral part of the root system of most plants and constitute an important group of organisms in the soil microbial community (Ravnskov and Jakobsen ,1999) .

VAM associations have been shown to be effective in the biological control of soil–borne plant pathogens (Pozo *et al*. 1996, Torres–Barragan *et al .,*1996, BØdker *et al.,*1998 and Slezack*etal*.,2000).Investigations of mechanisms related to increased resistance to pathogen in mycorrhizal plants indicate that these are probably complex (Pozo *et al*., 1996),nutrient uptake include phosphorus, microbial changes in the rhizosphere and competition with the pathogens for nutrients and infection sites (BØdker *et al.,* 1998 and Kim *et al*., 1998) .

Root colonization by VAM fungi induces important physiological and biochemical changes in the host plant, enabling it to better overcome biotic and abiotic stresses (Azcon-Aguilar and Barea, 1998). Molecular and biochemical molecules analyses show the elicitation of defence- related molecules during the establishment of the symbiosis (Gianinazzi- Pearson *et al.*, 1996). Increases of certain hydrolyse activities, mainly chitinase, β-1,3-glucanases have been reported for BCAs (Krol, 1999) as well as mycorrhizal plants (Pozo *et al* ., 1998 and 1999) , and possible role of these enzymes in the regulation of the symbiosis , as well as in plant protection against root pathogens , has been reviewed (Azcon-Aguilar and Barea , 1996) . Fungi like *Trichoderma spp., Peneillium oxalicum* and bacteria as *Bacillus subtilis* are known to be regarded as beneficial (Andrade *et al*.,1998).These microorgansims can antagonize plant pathogens by competition , parasitism, antibiosis or by a synergistic combination of these modes of action (Whipps, 1992) . The ability of BCAs to consistently colonize the roots and survive for long period when introduced into rhizosphere of plants is of considerable importance, because it can remain viable for successful inoculation under a wide range of conditions (Podile and Prakash, 1996). Because of importance of beneficial antagonistic micro-organisms in the soil ecosystems a better of the interaction between VA M fungi and the BCAs is essential to ensure the safe release of microbial inoculants used as bio fungicides.

The objective of this study was to examine the effect of interaction of VAM, *G. mosseae* fungal and BCAs include *T. harzianum , P. oxalicum* and *B.subtilis* on the root-rot disease and growth of geranium plants under their interactions.

Materials and Methods

Inoculants and pot experiments: The root-rot pathogens , *F.solani* and *M. phaseolina* were isolated from diseased plants grown in Elklubia Governorate. Isolates of *T. harzianum , P. oxalicum* and *B. subtilis* were isolated from rhizophere of geranium plants and examined for antagonistic effects against pathogens. Cultures were identified at Plant Pathology Department National Research Centre, Dokki, Egypt. Fungi were grown on malt extract liquid medium for 15 days at 25^0C with gentle shaking, subsequent suspension in with $2x\ 10^4$ colony-forming units (cfu). Bacteria were grown on Nutrient broth medium overnight at $28\ ^0$C and cell suspension adjusted to 10^7 cfu/ml^{-1}.

Inoculum of VA mycorrhizal fungus , *G. mosseae* (obtained from Plant Pathology Dept. , Moshtohr Fac., Zagzig Univ. Egypt)was produced on onion plants in a peat /vermiculite (1:1,w/w) mixture (Jenkines ,1964).

Peat and vermiculite (100g) of mycorrhizal treatment was mixed with 10 ml of the inoculum suspension of different biocontrol agents. The pH was adjusted to 7.0.

Pots (25 cm^3 in diam.) containing clay soil were mixed with 1% of peat and vermiculite of inocula source with 5 cuttings of geranium per pot were planted. Before sowing, soil was supplemented with 10 ml of either *F.solani* or *M. phaseolina*

cultures. Control plants were similar supplied with 10 ml of sterilized water. The plants were grown in a greenhouse and watered daily. Five replicates were used for each treatment. After 75 days of plant growth, the percentage of root-rot disease incidence and population counts of either biocontrol agents or pathogens by plate diluted technique were recorded .
The percentage of root infection with *G. mosseae* assayed by modification methods of Phillips and Hyaman (1970).

Field experiments: Two field experiments were established under the nature clay soil in El-Kalubia Governorate during 2000 and 2001 seasons. For inoculation the geranium cutting , a thin layer of peat and vermiculite of inocula source (10 g) and 0.1% caboxymethyl cellulose were placed below the surface of the soil before planting. Randomized complete block design was used for each treatment with 50 plants per replicate.

Disease assessment: The percentage of root-rot disease incidence was recorded during growth periods.

Population density : The percentage of root infection with *G. mosseae* were measured. Freshly rinsed roots were cut into 1-3 cm segments, from the root base. Ten root segments of each treatment were randomly selected for staining.
Population counts of either BCAs or pathogens were also calculated in soil rhizosphere as mentioned previous above.

Electrophoresis: Chitinase activity in plant roots was determined after 60 days of sowing. Roots were cut and macerated at 4C ^{0} in ice chilled mortar with liquid nitrogen. The resulting material was suspended in 100 mM MacIL vaine (Citric acid/ Na2 HPO4) extracting buffer, pH 6.8. Crude homogenates were centrifuged at 15000 g for 30 min at 4 ^{0}C. Extracts (10 ųg) per sample were analyzed by activity staining in 12% gel electrophoresis (PAGE) using N-acetyl glucoseamine as substrate for chitinase activities according to Kang *et al*. (1989).

Harvested: After three months of plant transplanting, the plant length and dry weight were measured.
The Phosphorus (P) concentration in the shoot was determined using a spectrophotometer at 660 nm (Olsen and Sommers , 1982).
Volatile oil in shoot was also collected and determined with hydrodistillation according to Guenther (1961).

Dual culture: Stimulation of biocontrol on VAM growth and its activity were studied by means of dual culture trials carried out in 9 cm petri dish VA mycorrhizae fungi were grown on the medium (Abdel-latif , Faten , 1999 and 2001) modified from Murashing and Ckoog (1962) medium. This medium contains of nutrient elements as mg/1L, $CaCl_2.2H_2O$ (440); $MgSo_4$,$7H_2O$ (370); KH_2PO_4 (170); Na_2 EDDA (33.6);$FeSO_4$.$7H_2O$ (278); NH_4NO_3 (1.65); KNO_3 (1.9); Mn $SO_4.4H_2O$ (22.3); $CuSO_4.5H_2O$ (0.025); $CaCL_2.6H_20$ (0.025); H_3BO_3 (6.2); KI (0.83); $NaMoO_4.2H_2O$ (0.25); Glycine (2.0); Thiamine (0.1); Pyridoxine. HCl (0.5); Nicotinic acid (0.5); Inositol (100); Sucrose (30.0) and 1% agar in /1L distilled water ; pH of the medium was adjusted to 5.5 before sterilize in -9 cm -Petri dishes Discs of mycorrhizae were cut with a 9 mm diam. Cork borer from the edge of young cultures growing on the same medium were placed on petri dish. The experiment consisted of a standard petri dish of single VAM as control treatment. Ten replicates for control and each containing biocontrol mat were used. The cultures were incubated at 35C ^{0} for 30 days in the dark. Stimulation or inhibition of mycorhizal growth were assessed at the end of inocubation by light microscopy. At the same time, chitinase produced by *G. mosseae* in the medium containing 1% agar was also measured as previous above. Data obtained were subjected to statistical analysis according to the procedures outlined by Snedecor and Cochran (1980).

Results

Under artificial infested soil with *F. solani* or *M. phaseolina* pathogens, geranium plants had significant lower root- rot disease levels when soil amended with mycorrhizal /or BCAs compared with un-inoculated plants (Table 1). Plants inoculated with mycorrhizal combined with BCAs displayed the highest effect on reducing disease incidence . The treatment of *G. mosseae* with *P. oxalicum* depressed root infection with *F. solani* , but not much as the soil inoculated with *M. phaseolina* . Wheares, soil amended with *G. mosseae* and *T. harzianum* , significantly depressed infection of root –rot disease incidence caused by *M. phaseolina.*
After 75 days of transplanting, the counts of either *F. solani* or *M. phaseolina* in soil rhizosphere were significantly affected and depressed by amending the soil with *G. mosseae* or BCAs. Also, population of the pathogens become lower with the application of mycorrhizal combined with biocontrols . The lowest counts of *F. solani* was recorded in *G. mosseae* with *P. oxalicum* combination treatment. *M. phaseolina* had adversed effect by amended *G. mosseae* with *T. harzianum*.
The biocontrol agents in the soil rhizosphere indicated that, population counts were generally higher in plants inoculated with pathogens. Also, Where they increased under treatments of *G. mosseae* inoculation.
Geranium plants had higher infection levels of VAM when soil amended with the BCAs. The presence of *T. harzianum* had the higher effect on infection levels, and increase of VAM counts.Under naturally infested soil with pathogens, the root-rot disease incidence of geranium plants subjected to the combination of *G. mosseae* and BCAs inoculated treatments is illustrated in Fig. (1). After 15 days of planting, root-rot disease showed an increase in un-inoculated by 36.4% and 32.6% and reach to 73.3 and 69.6% after 90 days in 2000 and 2001 seasons, respectively. The percentage of root-rot disease was decreased under VAM or BCAs treatments compared with un-inoculated one .
However, plants mycorrhizal with *G. mosseae* displayed the highest effect on reducing root- rot disease when combined with BCAs. The best treatments were those involving *G. mosseae* combined with either *T. harzianum* with 0.6 and 0.3% or *P. oxalicum* with 1.3 and 0.6%, in both seasons, respectively. Still, *G. mosseae* combined with *B. subtilis* was effective on controlling root-rot disease with 3.6 and 1.3% in different seasons, respectively.
One month after inoculation, mycorrhiza was formed, rapidly and increased during growth period containing vasicular, arbuscular mycorrhizae (VAM) spores and mycelium (Fig. 2). Overall, roots colonized by VAM was affected by the different BCAs. Since, inoculation with biocontrol agents led to increase in mycorrhizal colonization three months after sowing. The highest VAM counts were found in treatment with *T. harzianum* followed by *P. oxalicum* .
Growth curves and survival of different BCAs and pathogens in geranium rhizosphere during the growth period under natural field conditions were apparent in Fig. (3).In non-inoculated mycorrhiza, population density of different biocontrol agents were increased up to 60 days of growth period for *T. harzianum* and *P. oxalicum* and 45 days for *B. subtilis*, thereafter the counts slightly declined. Meanwhile, in plants inoculated with *G. mosseae* , counts were increased in population and survival over 90 days for all test biocontrol. The highest spores yield was gained by *P. oxalicum* followed by *T. harzianum* in plants inoculated with *G. mosseae.*
At the same time, population counts of both pathogens conferred a visible increase over 90 days in untreated plants

Table 1: Influence of geranium cuttings inoculated with combined of mycorrhizae with biocontrol microorganisms on root- rot disease incidence under artificial soil with *Fusarium solani* and *Machrophomina phaseolina* after 75 days of transplanting.

Treatment	*Fusarium solani*						*Machrophomina pheseolina*					
	% infection	*Fusarium** counts	Biocontrol counts	Mycorrhizal counts[y] V	A	M	% infection	*Macrophomina* counts	Biocontrol counts	Mycorrhizal counts V	A	M
Untreated soil	74.6 a	35.6 a	0.0f	0.0	0.0	0.0	58.7 a	18.6 a	0.0f	0.0	0.0	0.0
Glomus mosseae	33.6 b	17.5 b	0.0f	3.0	5.2	2.3	25.6 b	9.6 b	0.0f	3.0	2.5	1.0
Trichoderma harzianum	16.6 cd	13.3 bc	86.4 c	0.0	0.0	0.0	12.6 cd	7.6 c	84.3 c	0.0	0.0	0.0
Penicllium oxalicum	13.3 d	10.5 c	96.2 b	0.0	0.0	0.0	10.3 d	6.6 cd	86.7 c	0.0	0.0	0.0
Bacillus subtilis	20.0 c	14.6 bc	13.6 e	0.0	0.0	0.0	16.3 c	9.6 bc	12.6 e	0.0	0.0	0.0
G. mosseae+ T. harzianum	3.6 ef	4.6 d	109.4 a	39.0	12.2	13.0	0.0 e	0.3 d	114.3 a	35.0	23.0	15.1
G. mosseae+ P. oxalicum	0.0 f	0.6 e	113.4 a	16.0	10.1	6.3	2.6 e	2.3 d	105.6 b	32.1	16.6	11.0
G. mosseae+ B. subtilis	6.6 e	9.8 c	29.4 d	3.3	5.6	2.6	4.6 e	7.8 c	26.4 d	3.3	3.2	2.0

* Values within a column followed by the same letter are not significantly different.
[y]V= Vesicular (small spoes), A= Arbuscular (Large spores) and M= Mycelium.

Table 2: Growth parameters of geranium plants as influenced by combined of mycorrhizae with biocontrol microorganisms

Treatment	2000 season		2001 season	
	Plant length (cm)	Plant dry weight (g)	Plant length (cm)	Plant dry weight (g)
Untreated control	39.6 f*	8.9 d	40.7 g	9.5 e
Glomus mosseae	59.6d	13.4 bc	63.2 d	14.9 bc
Trichoderma harzianum	55.5 d	12.0 c	45.2 f	12.6 cd
Penicllium oxalicum	52.0 de	10.9 cd	54.6 e	11.9 cd
Bacillus subtilis	48.8 e	10.2 cd	42.6 f	9.8 d
G. mosseae+ T. harzianum	95.6 a	20.2 a	90.8 a	19.6 a
G. mosseae+ P. oxalicum	86.4 b	16.9 ab	80.3 b	16.4 ab
G. mosseae+ B. subtilis	72.6 c	14.0 bc	70.7 c	13.4 bc

* Values within a column followed by the same letter are not significantly different.

Table 3: Phosphorus concentration and oil percentage of geranium plants as influenced by combined of mycorrhizae with biocontrol microorganisms

Treatment	2000 season		2001 season	
	P conc. in dry tissue (mg/plant)	Oil % (v/w)	P conc. in dry tissue (mg/plant)	Oil % (v/w)
Untreated contol	3.31	0.108	3.21	0.10
Glomus mosseae	4.62	0.150	4.71	0.149
Trichoderma harzianum	3.62	0.144	3.71	0.140
Penicllium oxalicum	3.42	0.134	3.62	0.129
Bacillus subtilis	3.40	0.132	3.54	0.126
G. mosseae+ T. harzianum	5.02	0.182	5.82	0.171
G. mosseae+ P. oxalicum	4.98	0.173	5.02	0.169
G. mosseae+ B. subtilis	4.90	0.151	4.94	0.150

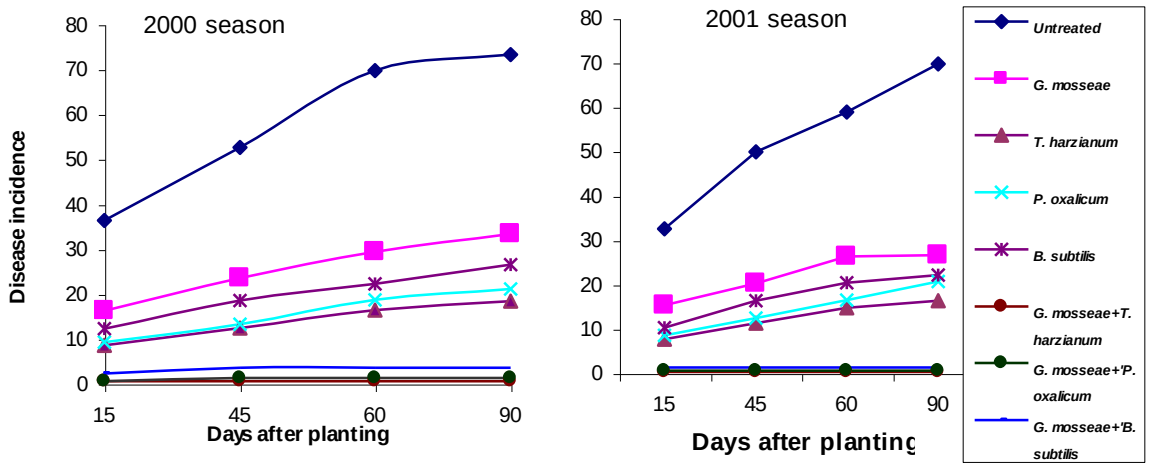

Fig. 1: Influence of geranium cuttings inoculated with combined of mycorrhizae and biocontrol agents microorganisms on the percentage of root- rot disease incidence under natural soil.

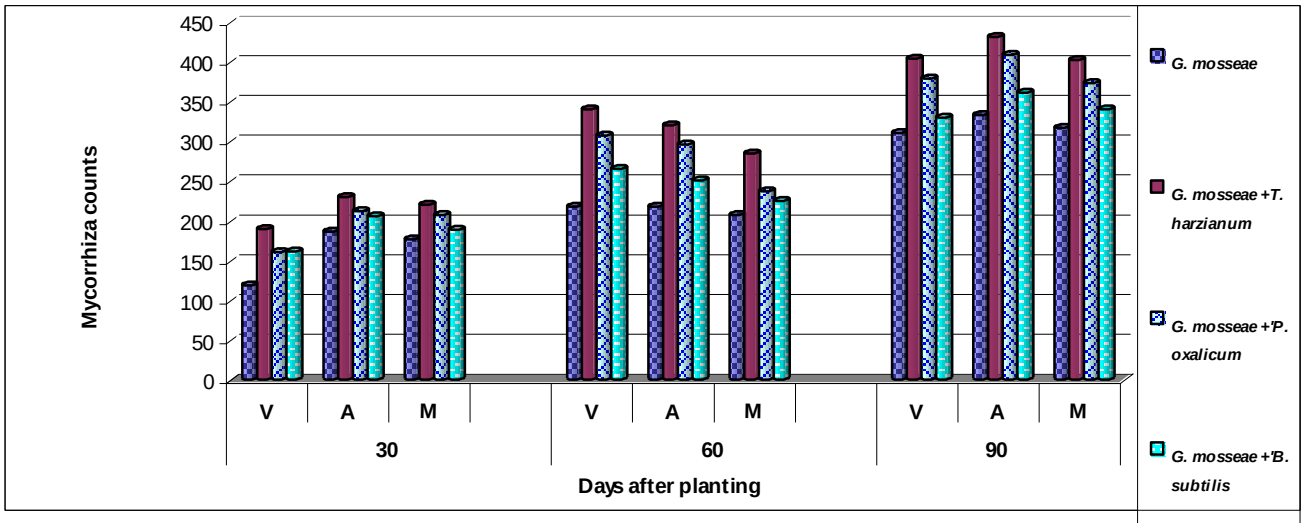

Fig. 2: Influence of soil amended with biocontrol microorganisms on vescular arbscular mycorrhizae fungi forming, infection, colonization in geranium plants under natural soil. V= Vesicular (small spoes) A= Arbuscular (Large spores) M= Mycelium

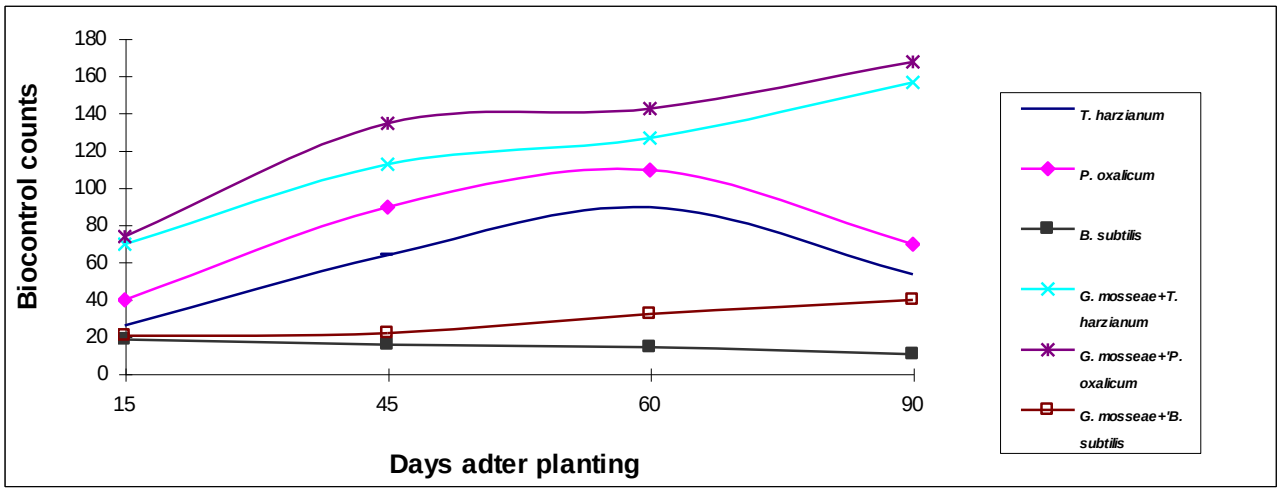

Fig. 3: Population counts of biocontrol microorganisms in the soil rhizosphere of geranium plants as influenced by adding *Glomus mosseae*. Population counts were estimated by diluted plate technique and expressed as cfux 10^4 of fungi and 10^7 of bacteria

Fig. (4). In the treatment with either mycorrhizae or biocontrol agents during transplanting, there was a decrease of the population counts of both pathogens. Moreover, population become lower up to 90 days with the application of *G. mosseae* combined with biocontrol agents. The lowest population of *F. solani* was recorded in *G. mosseae* combined with *P. oxalicum* , whereas *M. phaseolina* was reduced by *G. mosseae* combined with *T. harzianum* , which led to slight increase up to 60 days before began to decline where no propagules were detected up to 90 days.

Mycorrhiza or BCAs stimulated the chitinase induction in plant roots compared with untreated plants, corresponding to constitutively expressed isoenzymes on gel electrophoresis (Fig. 5). Activity was detected maximum at combined mycorrhiza with BCAs. A general increase in chitinase activity induction and a high band corresponding to isozymes was observed in G. *mosseae* combined with *T. harzianum* .

Geranium growth was affected by either BCAs or VAM development treatment if compared with un-inoculated plants in both seasons (Table 2). *G. mosseae* treatment promoted the growth of geranium plants but these had a significant increase when combined with BCAs in both seasons. Similar results have been achieved by BCAs either in single or combined with mycorrhizae. The highest plant length and dry weight were recorded in *G. mosseae* combined with *T. harzianum* followed by *P. oxalicum* in both seasons .

The P concentration and oil percentage in geranium plants showed similar trends to those recorded in plant growth in both seasons (Table 3). Mycorrhizae combined with BCAs

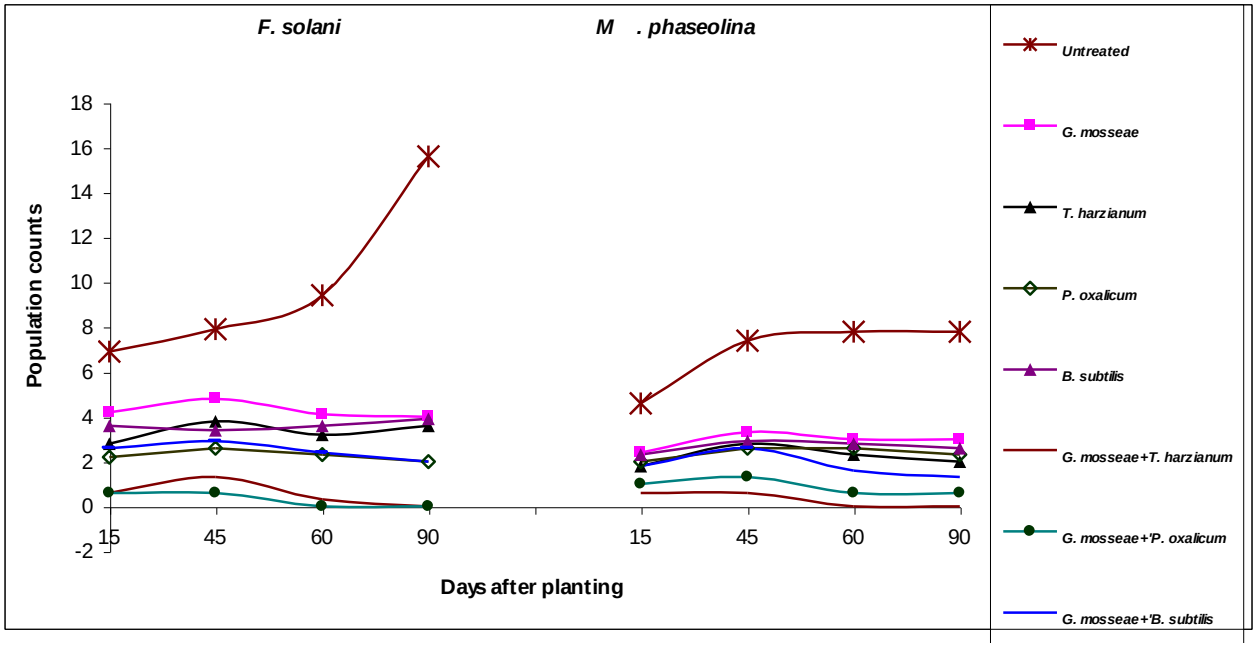

Fig.4: Population counts of the pathogens in the soil rhizosphere of geranium plants as influenced by adding *Glomus mosseae* and biocontrol microorganisms. Population counts were estimated by diluted plate technique and expressed as cfux 10^3

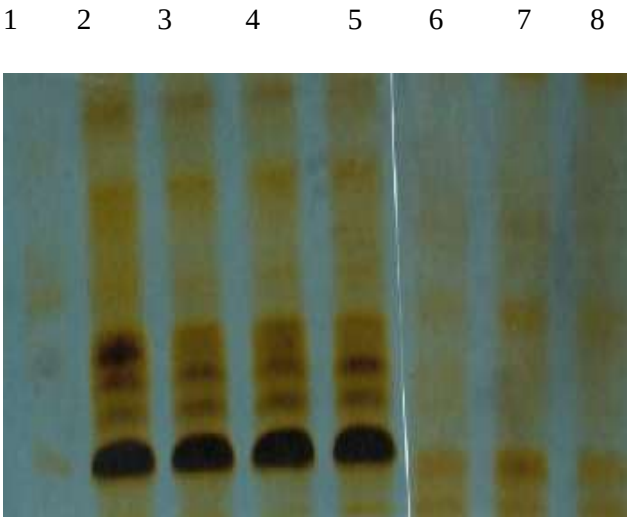

Fig .5:Isozyme bands displayed in geranium roots as influenced by combined of *Glomus mosseae* with biocontrol microorganisms after separation by poly acrylamide gel electrophoresis. Lane1 : untreated control , Lane 2: *G. mosseae* + *Trichoderma harzianum*, Lane3: *G. mosseae* + *Penicillium oxalicum*, Lane4: *G. mosseae* + *Bacillus subtilis*, Lane5: *G. mosseae*, Lane 6: *Trichoderma harzianum*, Lane7: *Penicillium oxalicum* and Lane 8: *Bacillus subtilis*

increased P concentration and oil percentage in geranium plants than in each one or in control treatments. Also, the contents were further enhanced with the application of *G. mosseae* combined with *T. harzianum* and un-amended treatment . Still, *B. subtilis* had a stimulatory effect on VAM contents.

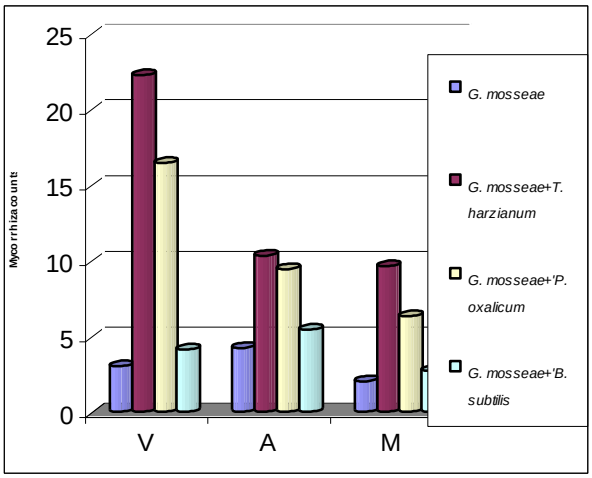

Fig. 6: Influenced of dual culture amended with biocontrol microorganisms mat on *Glomus mosseae* growth**.** V= Vesicular (small spoes), A= Arbuscular (Large spores), M= Mycelium

The mat of biocontrol agents stimulation VAM developed by *G. mosseae* in dual culture (Fig. 6). *T. harzianum* and *P. oxalicum* had a stimulate effect on mycorrhizal growth in comparison with un-amended control.

Also, data insinuated that mycorrhizae produced chitinase isozyme bands in dual culture (Fig.7). The activity of these isozymes appears to increase with biocontrol mat amended. A much higher levels of chitinase bands were detected in *T. harzianum* mat amended followed by *B. subilis* and *P.oxalicum.*

Discussion

The results demonstrated that vasicular -arbscular mycorrhizae ,*G. mosseae* fungi are compatible with BCAs. The

importance of arbuscular fungi for plant development and health is now widely demonstrated. Because of their role as bioregulators (Gianinazzi-Pearson *et al*., 1996), biofertilizers (Ravnskov and Jakobsen ,1999) and biocontrol agents (Barea *et al*., 1998), they represent potentially important tools for plant management techniques that prevent dependent on chemical inputs.

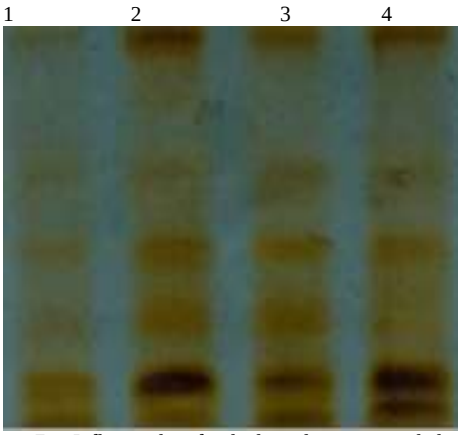

Fig. 7: Influenced of dual culture amended with biocontrol microorganisms mat on *Glomus mosseae* chitinase production. Lane 1: *Glomus mosseae*, Lane2: *G. mosseae* + *Trichoderma harzianum*, Lane3: *G. mosseae* + *Penicillium oxalicum*, Lane4: *G. mosseae* + *Bacillus subtilis*.

Glomus mosseae used in these studies has been demonstrated to have biocontrol potential as well as plant biofertilizer (Gianinazzi *et al*., 1996, Pozo *et al*., 1996, 1998 and Slezack *et al*., 2000).

The addition of BCAs in combination with VAM to plant media increases the possibility of obtaining disease control in the greenhouse stage of production or conferring resistance to the plant after it is out planted in the field. Generally, other soil microorganisms can influence the behaviour of mycorrhizal fungi. Most research reported that soil microflora and plant growth promoting rhizobacteria organisms present in soil or added with vasicular-arbscular mycorrhizae fungi to pott media or as seed treatments do not inhibit mycorrhizal fungi colonization of roots (Bianciotto *et al*., 1996, Kim *et al*., 1998 and Ravnskov and Jakobsen ,1999). Dhillion (1994) noted synergistic effect in different crop species included millet, sorghum, maize and wheat to inoculated with mycorrhizae and phosphate solubilizing fungi. In the treatment in which *G. mosseae* and BCAs were inoculated at the same time, disease incidence was lower during growth period in artificial or natural soil with pathogens.

Also, data indicated that certain mycorrhiza ,*G. mosseae* may have the potential to enhance the biocontrol counts in soil via this stimulation of soil microflora. Whereas, different biocontrol agents have been recorded at high level from soil rhizosphere of plants over 90 days of growth period by the presence of *G. mosseae* . Stable and increase in population of different biocontrol reflect their root and rhizosphere colonizing ability. At the same time, mycorrhizal infection was clearly increased by different BCAs addition. Root colonization did not prevent pathogenic infection only, but reduced the downward growth of the pathogens in the root rhizosphere. This reduction could be caused by competition between the two groups for physical space in the root, but may also be the result of antifungal compounds produced in the soil. Enhanced VAM hyphal growth was observed either in dual culture inoculated with biomass mat of BCAs. Also, stimulated VAM hyphal growth enhanced the chitinase production in dual culture. The reasons for this synergistic interaction between VAM and antagonistic microorganisms may be mediated by soluble factors or physical content. Similarly, Bianciotto *et al*.(1996) mentioned that *Pseudomonas* and rhizobacteria adhere to spores and hyphal structures formed by VAM fungi, showed higher density of attached bacteria dependent on their strain.

The combination of VAM fungi with BCAs increased chitinase activity in plant over to those obtained in non- mycorrhizae or mycorrhizae alone, corresponded to more disease control. Transient activation of chitinase has been reported in several VAM symbioses (Pozo *et al*., 1996 and 1998).The two groups of VAM and BCAs, when inoculated together on geranium plants increased plant length, dry weight , P content and oil percentage substantially over control and single treatments. Benefits in plant growth may be related to percent root colonization and spores levels in the root, since VAM higher in the presence of *T. harzianum* and *P. oxalicum* were those in which the incidence of disease was lowest and plant production was higher.

These results indicate that, combination of vasicular-arbscular mycorrhizae with antagonistic biocontrol agents, there is a possibility of improve disease control and plant yield.

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