EFFECTS OF THREE *GLOMUS SPECIES* AS BIOCONTROL AGENTS AGAINST VERTICILLIUM-INDUCED WILT IN COTTON

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Abstract: The objective of this investigation was to study the effects of three *Glomus* species: *G. etunicatum*, *G. intraradices* and *G. versiforme* on the development of verticillium wilt in cotton plants. Results indicated that the influence of arbuscular mycorrhizal fungi as a biocontrol agents were different among three *Glomus* species. In diseased cotton plants colonized by *G. etunicatum*, the disease index was lower than others and also, higher colonization percentage was relevant to these plants. On the other hand, the establishment of mycorrhizal symbiosis and development of structure of AMF were reduced when both symbiotic and pathogenic fungi infected the same root. In addition, the symptoms of veticillium wilt were diminished too. These results revealed that the beneficial effects of mycorrhiza could alleviate the pathogenic effects of *V. dahliae* and also a competitive interaction existed between these pathogenic and symbiont fungi.

Key words: arbuscular mycorrhizal fungi, biocontrol, cotton, verticillium wilt, resistance

INTRODUCTION

Verticillium wilt of cotton, caused by the soil-borne fungal pathogen, *Verticillium dahliae* Kleb., is one of the most important diseases in cotton production areas of the world (Eldon and Hillocks 1996) with drastic reductions (Schnathorst and Mathre 1966). The fungus enters the root and hyphae then move to vascular tissues (Schnathorst 1981). Xylem colonization by the fungus increases the resistance to water flow within the plant thus resulting in leaf water deficits and reduced photosynthetic and transpiration rates (Adams *et al*. 1987). Recent studies suggested that some arbuscular mycorrhizal fungi (AMF) can alleviate the deleterious effects of *V. dahliae* on pepper yield and growth (Garmendia *et al*. 2004b). The use of biocontrol agents to control wilt would allow to reduce the use of chemical plant protection products with the subsequent enhancement in global sustainability in agriculture (Azcón-Aguilar and Barea 1997). Several mechanisms are involved in bioprotection by AMF against plant pathogens (Azcón-Aguilar *et al*. 2002). In fact, the mutualistic symbiosis between AMF and plant roots plays an important role in nutrient cycling in the ecosystem and also can protect plants against environmental and cultural stress (Garmendia *et al*. 2004b).

The objective of this study was to evaluate the effects of three *Glomus* species; *G. etunicatum*, *G. intraradices* and *G. versiforme* on cotton verticillium wilt under controlled condition.

MATERIALS AND METHODS

Biological material, growth condition and experimental design

A cotton cultivar susceptible to *V. dahliae*, (cv. Mehr) was kindly provided by the Moghan Agricultural Research Center–Cotton seeds were delinted by sulphuric acid for 5 min and neutralized by ammonia and then washed with sterile distilled water. Delinted seeds germinated at 23–27°C and germinating seeds with approximately equal radicle length were selected for sowing in 1.5 liters pots filled with an autoclaved mixture of sand-soil (3 : 1 v/v).

Plants were initially divided into four groups: (a) non mycorrhizal plants, (b) plants inoculated with *G. etunicatum* (Ge), (c) plants inoculated with *G. intraradices* (Gi) and (d) plants inoculated with *G. versiforme* (Gv). Mycorrhizal inoculum was supplied by Agrology Department of Tabriz University. The AMF was used as a soil based inoculum (70 g per pot) including spores and hyphae and heavily colonized roots from three-month-old culture of corn roots cut into small pieces (Garmendia *et al*. 2006).

The isolate of *V. dahliae* used in this experiment was isolated from naturally infested cotton growing areas at Moghan. The pathogenic fungus was grown in plates on potato dextrose agar (PDA) medium at 25°C in the dark. Then, ten colonized plugs of agar from primary plates were cut out and placed in 500 ml glasses flask (closed by cotton wool) containing autoclaved 190 g sand, 10 g barley meal, 15 ml distilled water. The flasks were incubated at 25°C for 1 month (Huang *et al*. 2006). Content of half

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of plants from each group were inoculated with *V. dahliae* at a concentration of 4% (w/w). Autoclaved inocula of three *Glomus* species and *V. dahliae* were used as the control. Therefore, eight treatments were compared: non mycorrhizal plants inoculated (NMV) or not (NMNV) with *V. dahliae*, plants associated with *G. etunicatum* and inoculated (GeV) or not (GeNV) with *V. dahliae*, plants associated with *G. intraradices* and inoculated (GiV) or not (GiNV) with *V. dahliae* and plants associated with *G. versiforme* and inoculated (GvV) or not (GvNV) with *V. dahliae.*

Treated plants were grown in a greenhouse at 25/31°C with a day/night regime and 14 h photoperiod. Cotton plants were given every day alternatively water and half-strength Hoagland nutrient solution lacking phosphorus. The plants were harvested after 2 months.

Plant growth parameters

The aerial plant parts were cut at the level of cotyledons. Length of shoots and roots were measured separately and fresh weight (FW) of shoot and root recorded. Total leaf area was measured by Flachenberechnung einer Sw-Grafik software (Samadi 2007).

Disease assessment and estimation of AMF colonization

The disease severity was non-destructively estimated by calculating a disease index as the sum of wilted, chlorotic and necrotic leaves related to total leaves per plant, expressed as a percentage (Garmendia *et al*. 2004b)**.**

Fine root samples (< 1mm diameter) were cleared and stained in trypan blue according to the method of Philips and Hayman (1970) and the percentage of AMF root colonization was assessed by examining a minimum of 80–90 1cm root fragments for each treatment (Hayman *et al*. 1976). In addition, mycorrhizal colonization was characterized by assessing the presence or absence of arbuscules and vesicles.

Statistics

Plant growth parameters (Table 1) were analyzed with two-way ANOVA, with AMF colonization and *V. dahliae* infection as the main effects. Mean ±SE were calculated and when the F-ratio was significant, least significant differences were evaluated by the tukey-b test. Percentage of Disease index (Fig. 1), percentages of mycorrhizal colonization, arbuscules and vesicles (Fig. 2) were analyzed with one-way ANOVA. Mean ±SE were evaluated and when the F-ratio was significant, least significant differences were evaluated by the Tukey-b test. Significant levels were always set at 5% level.

RESULTS

Colonization with mycorrhiza increased total leaf area (Table 1). Although all mycorrhizal plants received identical nutritions, those associated with *G. etunicatum* had higher total leaf area than plants colonized by *G. intraradices* and *G. versiform.* ANOVA results corroborated the significant interaction ($p < 0.001$) between AMF and *V. dahliae* on total leaf area. There was no significant effect of mycorrhiza and *V. dahliae* on shoot length (Table 1). In addition, when comparing healthy plants, no significant difference was observed in root length between mycorrhizal and non-mycorrhizal treatments (Table 1).

Table 1. Total leaf area, shoot length, root length, shoot fresh weigh (FW) and root fresh weigh (FW) in non-mycorrhizal (NM) plants and plants associated with *G. etunicatum* (Ge), *G.intraradices* (Gi), *G. versiforme* (Gv), inoculated (V) or not (NV) with *V. dahliae*

Root FW [gr]	Shoot FW [gr]	Root length [cm]	Shoot length [cm]	Total leaf area [cm^2]	Treatment
.464 ab	2.01 a	24.00 a	31.00 a	73.43 a	NMNV
1.142 d	3.84 c	30.25 abc	34.00 a	263.04 c	GeNV
.743 c	3.24 bc	32.50 c	33.87 a	256.17 c	GiNV
.971 d	4.04 c	29.75 abc	34.25 a	160.63 b	GvNV
.354 a	1.40 a	23.00 a	30.75 a	51.27 a	NMV
.662 bc	3.30 bc	29.50 abc	32.50 a	187.03 b	GeV
.699 c	2.51 ab	30.75 bc	30.90 a	84.81a	GiV
.393 a	2.01 a	24.75 ab	33.75 a	93.14 a	GvV
***	***	***	*	***	AMF
***	***	ns	ns	***	*V. dahliae*
***	*	ns	ns	***	interaction

*** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, ns non-significant

Data were analyzed with two-way ANOVA with AMF and *V. dahliae* as the main effects. Mean ± SE (n = 3 plants) were calculated and, when the F-ratio was significant, least significant differences were evaluated by the tukey-b test. Within each column values followed by a common letter are not significantly different ($p < 0.05$)

V. dahliae reduced root FW in plants colonized with three *Glomus* species and the highest reductions were found in plants associated with *G. versiforme* (Table 1). AMF increased shoot FW in plants non-inoculated with *V. dahliae.* Furthermore, ANOVA results showed a significant interaction ($p < 0.05$) between symbiotic and pathogenic fungi on shoot FW (Table 1).

Mycorrhiza reduced generally visible symptoms of pathogen's infection. However, only the disease index of plants associated with *G. etunicatum* showed a significant difference, compared to non mycorrhiza ones (Fig. 1).

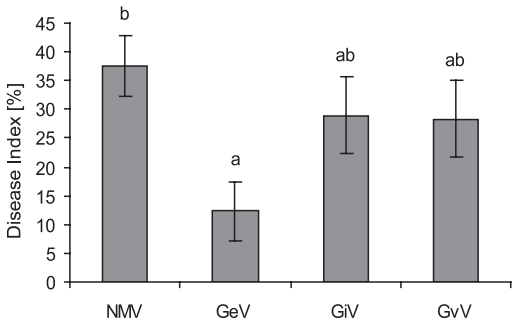

Fig. 1. Percentage of disease index for plants associated with *G. etunicatum* (Ge), *G. intraradices* (Gi) or *G. versiforme* (Gv), inoculated with *V. dahliae* (V). Values are means ±SD (n = 3 plants). Means compared with Tukey-b test. Different letters indicate significantly different ($p < 0.05$).

Percentages of mycorrhizal colonization varied among plants colonized with three *Glomus* species. However, when compared to only AMF inoculated plants, root colonization in mycorrhizal plants reduced significantly following double inoculation (mycorrhiza and verticillium) and difference of values beetwen healthy and diseased mycorrhizal plants amounted to 19.71, 25.02 and 32.2% in cotton colonized by *G. etunicatum*, *G. intraradices* and *G. versiforme*, respectively (Fig. 2A). Healthy mycorrhizal plants showed higher percentage of arbuscules (Fig. 2B) and vesicles (Fig. 2C), compared to diseased ones and presence of *V. dahliae* declined development of number of arbuscules and vesicles in cortex. Percentage of roots with arbuscules and vesicles was greater in plants colonized with *G. intraradices* independently of *V. dahliae* infection.

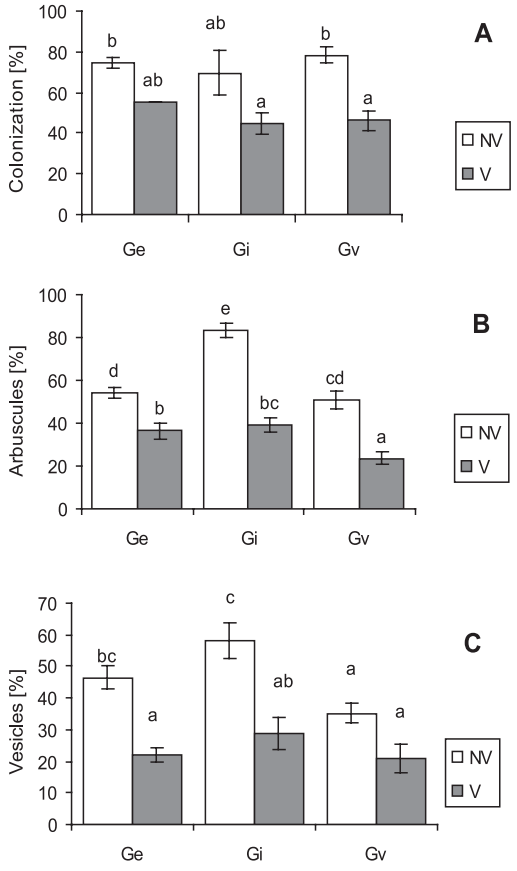

Fig. 2. Percentages of mycorrhizal colonization [%] (A), arbuscules [%] (B) and vesicles [%] (C) for plants associated with *Glomus etunicatum* (Ge), *G. intraradices* (Gi) or *G. versiforme* (Gv), inoculated (V) or not (NV) with *Verticillium dahliae*. Values are means ± SD (n = 3 plants). Means compared with Tukey-b test. Within each graph, different letters indicate significantly different ($p < 0.05$)

DISCUSSION

Interactions of the AMF with pathogenic soil fungi have been studied repeatedly e.g. *Phytophthora fragariae* (Norman *et al*. 1996), the wilt pathogen *Fusarium oxysporum* and the shoot pathogen *Oidium lini* (Dugassa *et al*. 1996), with the results showing that AMF increases tolerance to the disease.

Results revealed that among mycorrhizal plants, infection by pathogen reduced the number of root segments colonized by AMF. Rate of root exudation influences strongly the extent of AMF infection in the root (Baath and Hayman 1983). Therefore, verticillium might decrease the rate of root exudation and thus induces a decline in mycorrhizal infection. Statistics analysis showed that *V. dahliae* decreased total biomass in cotton plants. Definitely it is possible, verticillium wilt diminishes the photosynthesis of the plant (Mathre 1968), that less photosynthate is transported to the roots and exuded, therefore colonization of AMF decreases. On the other hand, the significant effect of *V. dahliae* on the percentage of root cortex colonization by AMF in roots suggests existence of competition between pathogen and AMF for space and/or host resources (Garmendia *et al*. 2005). As a consequence of the competition between pathogenic and symbiotic fungi, the efficiency of AMF as a biocontrol agent could have been decreased. In this study, the production of vesicles and arbuscules diminished in double-inoculated cotton plants. Thus, the low biomass of such plants could be in part due to a relevant transport of carbohydrates from shoot to mycorrhiza in roots similarly to previous studies (Goicoechea *et al*. 2004)

AMF can effectively reduce root disease caused by a number of soil-borne pathogens (Dumas-Gaudot *et al*. 2000). The lower disease index and increasing growth of plants when coinfected by mycorrhizae and pathogen indicated that cotton colonized by mycorrhizal fungi is resistant to verticillium wilt. The lowest disease index was observed in plants colonized by *G. etunicatum* that could be vindicated by maximum colonization by AMF. However, results showed that percentage of arbuscules and vesicles did not affect disease index. *Lorenzini et al. (*1997) found that verticillium accelerated senescence of plants inoculated with the pathogen due to greater leaf abscission and lower photosynthetic rates comparing to their healthy control. However, in general, mycorrhizal plants are more vigorous due to alteration of leaf hydration, leaf osmotic potential, stomatal conductance, reproduction, photosynthesis and transpiration (Smith and Read 1997; Auge 2001)

Mechanisms of plant protection by AMF are nutritional effects, morphological changes in roots and root tissues, changes in chemical constituents in plant tissue and microbial changes in the mycorrhizosphere. The increased capacity for nutrient uptake by the mycorrhizal association may allow host plants to be more vigorous and consequently, more resistant or tolerant to pathogen attack (Azcón-Aguilar *et al*. 2002).

In the interaction of mycorrhizal roots with pathogen, implication for plant bioprotection could be drown from the accumulation of phenolic compounds in plant cell wall reflecting increased lignification (Cordier *et al*. 1998). Lignification, as an essential mechanism for disease resistance (Morandi *et al*. 1984), may contribute to reduced pathogen proliferation in mycorrhizal roots.

In summary, the effect of myorrhiza as biocontrol agent against verticillium varied among different *Glomus* species, which was similar to the results of another study concerning nematode damage (Habte *et al*. 1999).

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POLSH SUMMARY

SKUTECZNOŚĆ TRZECH GATUNKÓW *GLOMUS* JAKO CZYNNIKÓW BIOLOGICZNEGO ZWALCZANIA WERTICILLIOZY NA BAWEŁNIE

Celem prezentowanych badań była ocena wpływu trzech gatunków *Glomus*: *G. etunicatum*, *G. intraradices*, *G. versiforme* na rozwój werticilliozy na roślinach bawełny. Wyniki badań wykazały, że skuteczność arbuskularnych grzybów mikoryzowych jako czynników biologicznego zwalczania była różna dla poszczególnych gatunków *Glomus*. Rośliny bawełny porażone werticilliozą i jed-

nocześnie zasiedlone przez grzyb *G. etunicatum*, charakteryzowały się niższym wskaźnikiem choroby, a także wyższym procentem zasiedlenia przez ten gatunek w porównaniu do pozostałych obydwóch gatunków *Glomus*. Zapoczątkowanie symbiozy mikoryzowej i dalszy rozwój grzybni arbuskularnych grzybów mikoryzowych (AMF) były ograniczone, gdy zarówno symbiotyczne jak i patogeniczne grzyby infekowały ten sam korzeń. Ponadto stwierdzono, że pod wpływem grzybów mikoryzowych nasilenie objawów werticilliozy ulegało zmniejszeniu. Wyniki badań sugerowały korzystny wpływ mikoryzy polegający na łagodzeniu skutków patogeniczności grzyba *V. dahliae* oraz występowania konkurencyjnej interakcji pomiędzy patogenicznymi i symbiotycznymi grzybami.