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Control of Fusarium Wilt of Cucumber Seedlings by Inoculation with an Arbuscular Mycorrhizal Fungus

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ABSTRACT

A glasshouse pot experiment was conducted to investigate the impact of inoculation of cucumber at the germination stage with *Glomus etunicatum* BEG168 on plant yield and incidence of *Fusarium oxysporum* f.sp. *cucumerinum* inoculated 28 days after the start of the experiment. Inoculation with the AM fungus decreased both disease incidence and disease index. Mycorrhizal inoculation also increased P concentrations in the cucumber seedlings. The mycorrhizal seedlings had higher concentrations of proline and polyphenol oxidase activity but lower malondialdehyde than non-mycorrhizal seedlings, indicating that AM inoculation may have protected membrane permeability and reduced the extent of the damage caused by *F. oxysporum*. The results indicate that the mycorrhizal fungus may influence plant secondary metabolites and increase resistance to wilt disease in cucumber seedlings and may therefore have some potential as a biological control agent.

Keywords: arbuscular mycorrhizal fungi, cucumber, wilt disease

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INTRODUCTION

Fusarium oxysporum f. sp. *cucumerinum* is an important soil-borne plant pathogen affecting cucumber plants (*Cucumis sativus* L.). Infected plantlets may develop root rot or stem canker, often leading to wilting and to plant death in severe cases (Rose et al., 2003). Fusarium wilt disease of cucumber is a worldwide problem. It is widespread in North China and is especially serious in glasshouse cropping systems (Huang et al., 1994).

Plant pathogen-root interactions and the biochemical activities that occur during root infection have been studied in some detail. There are numerous reports on the disease mechanisms and the most important factor seems to be disruption of the transport of water and nutrients in host plant vessels by the mycelium of *F. oxysporum* (de Boer et al., 2003). The resulting stress involves many physiological and biochemical changes (Magnoli et al., 1999), for example changes in enzyme activities and damage to membrane permeability.

Current cucumber disease management is based mainly on cultural practices and the use of fungicides, although chemical control is not always efficient or economically feasible for control of the disease. Biological control of the pathogen using mutualistic micro-organisms has been suggested as a potential alternative to chemical control (Park et al., 1988).

Arbuscular mycorrhizal (AM) fungi colonize species belonging to over 80% of all plant genera (Bonfante-Fasolo, 1987) and are considered to be the most widespread symbionts in plants. Arbuscular mycorrhizas are known to enhance plant uptake of phosphate (P) and other mineral nutrients under certain conditions (Abbott and Robson, 1984). Recently, there have been reports that arbuscular mycorrhiza can induce resistance or increased tolerance to some root pathogens (Cordier et al., 1996; Trotta et al., 1996; Lingua et al., 2002), and the use of AM fungi as potential biological control agents of soil-borne plant pathogens has been suggested. However, studies have indicated that sometimes arbuscular mycorrhiza can have harmful effects depending on the pathogens involved and other factors (Druge and Schonbeck, 1992; Shaul et al., 1999).

In the present investigation, a pot experiment was conducted to study the effects of inoculation with the AM fungus *Glomus etunicatum* BEG 168 on Fusarium wilt disease of cucumber seedlings under glasshouse conditions. In accordance with commercial horticultural practice, cucumber was inoculated with the AM fungus at germination and then supplied with a zoospore suspension of *F. oxysporum* 28 days later to determine any potential biological control effects of the AM fungus and changes in several secondary metabolites related to water stress and membrane permeability.

MATERIALS AND METHODS

Seeds of cucumber (*Cucumis sativus* L. cv. Jinlv 3) obtained from the Cucumber Research Institute, Tianjin, China, were used in experiment. The seeds were surface sterilized in 10% w/w H₂O₂ for 10 min, washed thoroughly in sterile distilled water, and then pre-germinated for two days on moist filter paper at 28°C in the dark. Seedlings were selected for uniformity of size prior to planting.

The plant growth medium consisted of a 2:1 w/w mixture of peat and vermiculite with the following properties: pH (soil:water ratio 1:2.5) 7.2, organic matter 27.1 g kg⁻¹, Olsen-P 67.2 mg kg⁻¹, NaOH-extractable N 23.3 mg kg⁻¹ and NH₄Ac-exchangeable K 103.1 mg kg⁻¹. The medium was steam-sterilized at 121°C for 30 min and air-dried. The plants were grown in 0.8-L plastic pots sterilized with 70% v/v ethanol.

The AM fungus *Glomus etunicatum* BEG168 was propagated in pot culture on maize (*Zea mays* L. cv. ND108) plants in a sandy soil for 10 weeks. Inoculum from the pot culture was a mixture of spores, mycelium, sandy soil and maize root fragments. Inoculum (15 g) was mixed with the rooting medium (185 g) to produce mycorrhizal treatments, and 15 g sterilized inoculum plus 10 mL mycorrhizal fungal-free filtrate from the inoculum suspension were added to produce the non-mycorrhizal treatments in order to provide a similar microflora except for the absence of the mycorrhizal fungus.

Fusarium oxysporum f.sp. *cucumerinum* was grown on Potato Dextrose Agar. Synthetic medium for *F. oxysporum* consisted of liquid PDA without agar. Two pieces of 5-day-old *F. oxysporum* cultured on PDA were used as the inoculum for a 100 mL flask containing 50 mL of synthetic medium. The flask was shaken at 50 rpm at 25°C to allow spore germination. Fourteen days later the mycelial inoculum was separated from the growth medium by centrifugation at 9000 rpm and 4°C, and then washed three times in 100 mL of sterile 10 mmol L⁻¹ MgSO₄·7H₂O solution.

After growth of the seedlings for 24 days, 5 mL of the mycelial inoculum was added aseptically to the pots at a final concentration of 10⁶ germinated spores mL⁻¹ (as counted with a haemocytometer). Control plants were treated with sterile distilled water instead of the *Fusarium* spore suspension.

The experiment was conducted from April to July 2004 under glasshouse conditions. Deionized water was used daily to adjust the pots to field capacity. All treatments received application of 50% Hoagland nutrient solution at a rate of 40 mL per week.

The experiment consisted of two factors, a mycorrhizal treatment (non-mycorrhizal and inoculated with the AM fungus) and a pathogen treatment (control and inoculation with *F. oxysporum*) to give four treatments: control (no AM fungus and no *F. oxysporum*), inoculation with *Glomus etunicatum* only, inoculation with *Fusarium oxysporum* only, and inoculation with both *Glomus etunicatum* and *Fusarium oxysporum*. The plant pots were arranged in

a completely randomized design with 16 replicates of each treatment, giving a total of 64 pots.

Harvest and Chemical Analysis

Final observations on disease development were made 14 days after inoculation as described by Vakalounakis et al. (2004). Eight replicate pots of each treatment were used. Tests were repeated once. Disease severity was assessed using a visual scale of 0 to 3, where 0 = no symptoms, 1 = light or moderate wilt with light vascular discoloration in the stem, 2 = severe wilt with vascular discoloration in the stem, and 3 = dead seedlings.

Six repeats of each treatment were harvested before and 14 days after inoculation with *F. oxysporum*. Samples were carefully washed with tap water and then deionized water. Three were oven dried at 70°C for 48 hours to determine the biomass and P concentration. The others were kept at -80°C prior to further processing of determination of proline and malondialdehyde (MDA) contents and polyphenol oxidase activity. Subsamples of fresh roots were collected for determination of the proportion of root length colonized by the AM fungus.

Mycorrhizal colonization was estimated using the root fragment method of Brundrett et al. (1996). Root samples were cut into 1-cm-long segments, cleared in 10% (w/v) KOH at 90°C for 12–15 min in a water bath, acidified in 2% (v/v) HCl and then stained with Trypan blue at 90°C for 15 min. Thirty root segments from each sub-sample were observed under the microscope for colonization.

Plant samples were digested using H₂SO₄-H₂O₂, and phosphorus (P) was analyzed using a Model FIA-2400 flow injection analyzer.

Lipid peroxidation was estimated by measuring the formation of MDA with TBA (2-thiobarbituric acid) according to de Vos et al. (1989). Samples were harvested and transferred to 4 ml of 0.25% (w/v) TBA in 10% (w/v) trichloroacetic acid (TCA) and heated at 95°C for 30 min. After cooling in ice, the mixture was centrifuged at 5,000 rpm for 10 min. MDA was determined by subtracting the absorbance of the supernatant at 600 nm from that at 532 nm (Kwon et al., 1965).

Proline content was estimated by the method of Bates et al. (1973) with slight modification. Samples (1 g fresh weight) were each extracted with 5 mL of 5% (w/v) sulfosalicylic acid at 100°C for 10 min. After cooling, the sample solution was filtered. Following this, 2 mL of the resulting supernatant, 2 mL of acid ninhydrin reagent (1.25 g ninhydrin + 30 mL glacial acetic acid + 8 mL orthophosphoric acid + 12 mL dH₂O) and 2 mL of glacial acetic acid were added, and the mixture was boiled for 1 h in a water bath. The reaction was terminated by dipping the test tubes in an ice bath. Toluene (4 mL) was then added and the reaction mixture vigorously mixed on a Cyclomixer for 30 s. The resultant chromophore extracted into the toluene phase was then aspirated

from the aqueous phase and its absorbance was read at 520 nm. A standard plot was also calibrated by using L-proline.

Polyphenol oxidase activity was determined by the method of Lobo and Cano (1998). Lyophilized homogenized samples (0.5 g) were mixed in a mortar and pestle with 0.2 mol L⁻¹ sodium phosphate buffer (pH 6.5, 25 mL). Homogenates were centrifuged in a Himac model RC-21F refrigerated superspeed centrifuge for 15 min at 10000 rpm and 4°C until assayed for polyphenol oxidase (PPO). PPO activity was determined at 25°C by measuring the initial rate of increase in absorbance at 420 nm. Unless otherwise specified, the assays of activity were carried out on 3 mL of reaction mixture, consisting of 0.05 mol L⁻¹ catechol in 0.05 mol L⁻¹ sodium phosphate buffer (2.75 mL, pH 6.5), and on aliquots of enzyme extract (0.25 mL). The enzyme activity unit was defined as the change in absorbance per minute per milligram of protein.

Statistical analysis of the data was carried out using the SPSS 11.0 package. Significant differences between means were established by calculation of LSD at the 5% level.

RESULTS

Mycorrhizal Colonization

No mycorrhizal colonization was observed in the roots of plants that were not inoculated with the AM fungus (Table 1). Both during the period before the plants were inoculated with *F. oxysporum* (28 days after seed germination) and 14 days after inoculation with *F. oxysporum* (42 days after seed germination), the inoculated plants had over 35% of their root length colonized. Thus, *G. etunicatum* produced substantial root colonization before the plants were

Table 1

Proportion of cucumber root length colonized by the AM fungus. Each value is the mean of 3 replicates. Within columns, treatments with the same letter are not significantly different (LSD α = 0.05)

Treatment	Mycorrhizal colonization (%)	
	Immediately after inoculation with <i>F. oxysporum</i>	14 days after inoculation with <i>F. oxysporum</i>
Uninoculated control	0b	0c
<i>G. etunicatum</i>	36a	37a
<i>F. oxysporum</i>	0b	0c
<i>G. etunicatum</i> + <i>F.</i> <i>oxysporum</i>	36a	32b

Table 2
Fusarium wilt disease incidence and severity index of cucumber inoculated with a zoospore suspension of *Fusarium oxysporum*. The results were determined 14 days after inoculation with Fusarium

Treatment	Incidence(%)	Disease index(%)
Uninoculated control	0.0	0.0
<i>G. etunicatum</i>	0.0	0.0
<i>F. oxysporum</i>	12.5	4.1
<i>G. etunicatum</i> + <i>F. oxysporum</i>	0.0	0.0

inoculated with *F. oxysporum*. Inoculation with *F. oxysporum* led to a decrease in the proportion of root length colonized by *G. etunicatum* in mycorrhizal plants, but mycorrhizal colonization remained at about 30% of root length with a decrease of about 5%.

Disease Development

The incidence and disease severity index of mycorrhization with BEG168 were both decreased compared with non-mycorrhizal plants by inoculation with a zoospore suspension of *F. oxysporum*, and there was no disease incidence in the plants not inoculated with *F. oxysporum* (Table 2). Compared with the untreated control plants, only those inoculated with *F. oxysporum* showed typical Fusarium wilt and yellowing of the leaves with root and stem rot symptoms. In contrast, plants inoculated with *G. etunicatum* at germination and then inoculated *F. oxysporum* 28 days later showed no disease symptoms.

Growth Parameters

Leaf area showed no significant difference between mycorrhizal and non-mycorrhizal plants before inoculation with *F. oxysporum* but a clear effect was apparent 14 days after *F. oxysporum* inoculation (Table 3). Compared with the control, the plants inoculated with *F. oxysporum* had a smaller leaf area, while there was no significant difference between the controls and the plants inoculated with both the AM fungus and the Fusarium treatment 14 days after *F. oxysporum* inoculation.

Inoculation with *G. etunicatum* increased plant biomass significantly during the period before *F. oxysporum* inoculation (Table 3). Fourteen days after *F. oxysporum* inoculation there were no differences between other treatments and the control, but AM fungal inoculation produced greater plant dry weight was more than did *F. oxysporum* inoculation.

Table 3

Leaf area and dry weight of the cucumber plants. Each value is the mean of three replicates. Within each column, treatments with the same letter are not significantly different (LSD $\alpha = 0.05$)

Treatment	Leaf area (cm ²)		Dry weight (g)	
	Immediately after inoculation with <i>F. oxysporum</i>	14 days after inoculation with <i>F. oxysporum</i>	Immediately after inoculation with <i>F. oxysporum</i>	14 days after inoculation with <i>F. oxysporum</i>
Uninoculated control	360a	583b	1.11a	3.71ab
<i>G. etunicatum</i>	366a	764a	1.22b	4.44a
<i>F. oxysporum</i>	360a	481c	1.11a	3.11b
<i>G. etunicatum</i> + <i>F. oxysporum</i>	366a	564b	1.22b	3.92ab

Phosphate (P) Concentration

Shoot P concentration was increased by application of *G. etunicatum* both before inoculation with Fusarium and 14 days afterwards irrespective of whether *F. oxysporum* inoculation was done (Table 4). Inoculation with *F. oxysporum* had no influence on shoot P concentration.

Malondialdehyde (MDA) Production

Malondialdehyde (MDA) production of controls and plants inoculated with the AM fungus only showed no significant difference at whether before or 14 days

Table 4

Phosphorus (P) concentrations in cucumber plants. Each value is the mean of three replicates. Within each column, treatments with the same letter are not significantly different (LSD $\alpha = 0.05$)

Treatment	P(g/kg)	
	Immediately after inoculation with <i>F. oxysporum</i>	14 days after inoculation with <i>F. oxysporum</i>
Uninoculated control	1.5b	1.4b
<i>G. etunicatum</i>	2.5a	2.5a
<i>F. oxysporum</i>	1.5b	1.7b
<i>G. etunicatum</i> + <i>F. oxysporum</i>	2.5a	2.7a

Figure 1. Malondialdehyde (MDA) concentrations in cucumber plants. Each value is the mean of three replicate. At each time, treatments with the same letter are not significantly different (LSD $\alpha = 0.05$).

after *F. oxysporum* inoculation (Figure 1). *Fusarium oxysporum* increased MDA production markedly. In contrast, there was no significant difference between the controls and the plants inoculated with both the AM and wilt fungi 14 days after *F. oxysporum* inoculation, indicating that inoculation with the AM fungus reduced the MDA concentrations under conditions of disease stress.

Proline Content

Proline concentrations were not influenced by the AM fungus during the period before *F. oxysporum* inoculation, but 14 days after *F. oxysporum* inoculation the mycorrhizal plants had higher proline concentrations than non-mycorrhizal plants (Figure 2). Inoculation with *F. oxysporum* only increased proline concentrations compared with the control, but less than inoculation with both fungi, the treatment which gave the highest proline concentrations.

Polyphenol Oxidase (PPO) Activity

Before *F. oxysporum* inoculation, there were no significant effects on PPO activity (Figure 3). Fourteen days after *F. oxysporum* inoculation the PPO activity of mycorrhizal plants was higher than of non-mycorrhizal plants. However, there was no difference between plants inoculated with *F. oxysporum* only and controls, and plants inoculated with both fungi showed the highest PPO activity.

Figure 2. Proline concentrations in cucumber plants. Each value is the mean of three replicates. At each time, treatments with the same letter are not significantly different (LSD $\alpha = 0.05$).

DISCUSSION

Although cucumber wilt disease does not reach its peak until the plants are producing fruit, the present study showed that the nonmycorrhizal plantlets inoculated *F. oxysporum* showed symptoms of severe root rot with dark, softened

Figure 3. Polyphenol oxidase (PPO) activity in cucumber plants. Each value is the mean of three replicates. At each time, treatments with the same letter are not significantly different (LSD $\alpha = 0.05$).

roots and wilting of the shoots. This was in contrast to the mycorrhizal plants, which were not damaged to the same degree. The experiment confirms that the AM fungus can reduce the damage to the plants caused by the root pathogen.

Mycorrhizal colonization increased the leaf area and biomass 14 days after inoculation with *F. oxysporum*, indicating that the growth-promoting effect of the mycorrhiza required time to develop. The effect of mycorrhiza on growth may thus be related to P nutrition. Smith and Gianinazzi-Pearson (1988) pointed out that inoculation with AM fungi enhanced plant growth mainly due to P absorption, which might account for the higher tolerance of mycorrhizal plants to pathogens (Hooker et al., 1994; Linderman, 1994). Plant growth promotion due to AM colonization of the root systems was shown by Cordier et al. (1996) to be responsible for increased disease resistance. In contrast, inoculation with *F. oxysporum* decreased plant growth as a result of the damage to the plantlets from metabolic changes and water stress. The mycorrhizal plants infected with *F. oxysporum* were not significantly different from the controls, indicating that promotion of plant growth can ameliorate wilt disease. This is in agreement with Cordier et al. (1996) and may represent an indirect contribution to biological control of the root rot.

AM fungal colonization of plant roots induces changes in secondary metabolites that are known to alter root physiology (Linderman, 1994) and induce local and systemic resistance to fungal pathogens (Cooper and Grandison, 1986). The activation of specific plant defense mechanisms as a response to AM colonization is an obvious basis for the protective capacity of AM fungi. The elicitation by an AM symbiosis of specific plant defense reactions could predispose the plant to an early response to attack by a root pathogen (Gianinazzi-Pearson et al., 1994).

Induced premature senescence is often quantified by increases in membrane permeability and lipid peroxidation. Lipid peroxidation is usually determined by MDA content. As a rule, the cellular level of MDA is relatively low, representing a balance between the rate of univalent oxidation and the combined rates of reduction and formation of MDA. When cells suffer from oxidative stress, production of MDA is inevitable because oxidation radicals are commonly trapped, thereby generating MDA (Heber et al., 1996). There were no significant differences in MDA production between unstressed and *F. oxysporum*-inoculated mycorrhizal plants during our experiment in contrast to plants inoculated with *F. oxysporum* only and the controls. This data supports the hypothesis that the inoculation with AM fungi can reduce the production of MDA compared with non-mycorrhizal plants under *F. oxysporum* stress.

As expected, inoculation with *F. oxysporum* impeded water transport in the plants. Higher plants accumulate some metabolites in response to osmotic stress such as water stress (Delauney and Verma, 1993; Hasegawa et al., 2000). Proline appears to be the most widely distributed compatible solute in organisms ranging from bacteria to plants (Flowers et al., 1977). Some investigators have reported a positive correlation between proline accumulation and adaptation

to water stress (Voetberg and Sharp, 1991; Kishor et al., 1995). The results of this study indicate that more exogenous proline was produced to protect mycorrhizal plants under pathogen stress. Thus, the tolerance conferred by mycorrhizal colonization may have been further promoted by increasing the proline concentration in the plants.

Because of their conspicuous reaction products and their wound and pathogen inducibilities, PPOs have frequently been suggested to participate in plant defense against pests and pathogens (Thipyapong et al., 1995, 1997). There are reports pointing out that PPO is prominent amongst the defense proteins induced in crop plants (Constabel et al., 1995; Constabel and Ryan, 1998). Inoculation with AM fungi may induce an increase in PPO activity and accumulation of defense proteins in view of reports that active quinones produced by PPOs may possess direct antibiotic and cytotoxic activities to pathogens (Peter, 1989). In the present study, the mycorrhizal plants under disease stress showed higher PPO activity than non-mycorrhizal plants, suggesting an important role of AM fungi in limiting plant disease development, and providing some evidence for the involvement of PPO in disease resistance.

The data indicate that inoculation with the AM fungus may release water stress, protect membrane permeability, promote oxidation of hydroxybenzene (which can restrain the activity of *Fusarium*), and reduce the extent of the damage to cucumber seedlings caused by *F. oxysporum*. Thus, the mycorrhizal fungus may improve the growth and the resistance to wilt disease of the cucumber seedlings.

Furthermore, *F. oxysporum* and its metabolites may suppress the growth of AM fungi and inhibit the development of the mycorrhizal association symbiosis, effects similar to those reported by Liu (1995). This may be very important since such effects may hinder the beneficial effects of AM fungi because of inhibition sporangium production, zoospore germination (Catska, 1994), and competition for host photosynthates and colonization sites (Linderman, 1994; Jalali and Jalali, 1991).

This preliminary study has indicated that inoculation with an AM fungus reduced the development of *F. oxysporum* disease on cucumber plantlets. Though the results showed beneficial effect of the AM fungus on the growth and disease resistance of the plantlets under glasshouse conditions, further studies are required to clarify whether these effects can persist over the long term and under field conditions.

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