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A study toward the control of *Pythium* damping-off of cotton using arbuscular mycorrhizal fungi

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Abstract: In a trial to evaluate the efficacy of arbuscular mycorrhizal (AM) fungi to control *Pythium* damping-off of cotton, an increase of soil population of AM fungi was achieved by growing a mixture of trap crops for several months whereby a total count of 996 spores/100 g soil was achieved. As expected, plants grown in soil with increased mycorrhization showed a prominent positive change in all growth parameters by comparison with the non-mycorrhized-control soil. The results revealed also that AM fungi induced a significant reduction of disease incidence in infested soil from 68 % to 29% in pots and from 77.3% to 50.7% in the field. However, such values of reduction in disease incidence although mathematically significant, are not economically acceptable and make the reliability of AM alone, as a control mean of cotton damping-off by *Pythium*, questionable. Justifications of such limitation are given and suggestions of improvement of efficacy of AM fungi are presented. The systemic fungicide mefanoxam, by comparison with mycorrhiza, showed up a high degree of efficacy by reducing the disease incidence down to 12% in pots and 9.6% in field.

Key words: Arbuscular mycorrhizal (AM) fungi, *Pythium aphanidermatum*, damping-off, cotton, biocontrol.

Introduction

Cotton *"Gossypium barbadense* L.*"* is considered one of the most strategic crops in Egypt. Since 1960 it has been facing many problems and challenges that had led to an incredible decline in its annual production and the emergence of strong competitors in the world market like India and China (Hamza and Maldonado 2012). Pest infections and inadequate technologies used in crop collection and storage along with the incredible cut in acreage cultivated with cotton may be considered the major causes of such great drop in the annual cotton production. On the other hand, like many other crops, cotton is vulnerable to the attack by various pests of which fungal infections are considered the most devastating. Among these, damping-off could be the most serious.

Cotton seedling damping-off is a worldwide disease, caused by various fungal pathogens belonging to *Pythium*,*Phytophthora, Fusarium, Rhizoctonia, Verticillium, Macrophomina, Alternaria* and recently *Rhizopus* (Howell, 2002). A critical survey of previous studies carried out in Egypt on the subject indicated that out of the preceding pathogens only four are implicated with this disease, *Pythium* (Fletscher 1902, Esia 1983,Mazen *et al.* 1986, Omar *et al.* 2007), *Rhizoctonia* (Ashour 1957, Moubasher 1958, Tolba & Ali 1972, El-Mehalawy *et al.* 2007, Hassanin *et al.* 2007, Kasem 2009), *Fusarium* (El-Samawaty 2004, Abd-Elsalam *et al.* 2006) and *Macrophomina phaseolina* (Omar 2005).

Trials to reduce disease incidence in Egypt have started in the early 1950s by improvement of cultural practices, application of non-systemic fungicides (Ashour 1957, Moubasher 1958) or inducing resistance (El-Mehalawy *et al*. 2007, Hassanin *et al.* 2007) but the obtained results were not completely satisfactory. This stimulated investigators to look for alternative techniques. More recent trials concentrated mostly on two approaches, either application of systemic fungicides which usually add more cost effect or adoption of biological control techniques which could be achieved through various methods of which endomycorrhiza (AM fungi) might be the most widely used worldwide.

Endomycorrhizal fungi have become an important element in the last few decades in sustainable agriculture. Since recognized as useful technique of bicontrol, it has been receiving considerable attention worldwide to meet the growing public concern regarding the widespread and misuse of hazardous agrochemicals in best management (Mulder 1979). It has already found its way in application, as a safe approach, toward the control of root pathogens. The great importance of this approach might be due to its relatively low cost effect as well as its safety.

Two major factors must be fulfilled for AM fungi to confer its successful protection against root pathogens: a- soil endomycorrhizal population must be of high density and diversity, and b- high rate of colonization would be achieved by host-roots before infection takes place. The main objective of the present

investigation was to study the efficacy of AM fungi, through increasing soil mycorrhization before sowing seeds, to reduce cotton seedling damping-off in Ismailia Governorate.

Materials and Methods

Pathogen isolation and pathogenicity

A screening for pathogenic *Pythium* spp. was carried out using rhizospheric soil samples collected from diseased crops in Ismailia Governorate by adopting baiting technique using the following baits: cucumber seeds & cubes, potato cubes, grass cuttings. Grass cuttings proved to be the most suitable for isolation due to the development of less competitors like *Fusarium* and *Acremonium.* Purified isolates were grown on PDA (Potato Dextrose Agar), and tested for their pathogenic potentials against several hosts hopefully to find one or more able to attack cotton. Only one of the tested isolates showed strong ability to induce cotton damping-off. Based on microscopic characters and according to the scheme of van der Plaats-Niterink (1981) and Dick (1990), it was identified as *Pythium aphanidermatum*. Furthermore, identification was confirmed by molecular analysis at SOLGENT Co. ltd, South Korea by DNA sequencing using the 18S rDNA.

Soil mycorrhization

The increase in the diversity of soil endomycorrhiza, was carried out using rhizospheric soils of ten plant species to determine among them those to be used for increasing spore count. Ability of spore production came in the following order: wheat followed by garlic, corn and onion. Rhizospheric soils of these plants were collected and thoroughly mixed to form a composite soil. This mixed soil was dispensed in pots thereafter cultivated once again with the same trap crops and left for eight months to achieve good AM diversity and bring spore count to maximum as recommended by Oehl *et al.* (2003), and Shirmohammadi and Aliasgharzad (2013). Soils of the abovementioned trap crops were lumbed and very well mixed to show a spore count of 996 spores/100 g soil. For spore extraction, the wet sieving and decantation technique by Gerdeman and Nicolson (1963) was used. The microscopic identification (according to Trappe 1982, Sharma *et al.* 2009, Khade 2011) of this mixed soil showed clearly that *Glomus* was the most dominant accounting for 88.60% of examined spores, followed by *Gigaspora margarita* (8.52%), *Acaulospora spp.* (3.38%) in addition to unknown taxon by 0.5%.

Root colonization

The technique described by Brundrett *et al.* (1984) was adopted where colonization percentage of root was measured after 4 weeks of plant growth. Root clearing was carried out using 10% KOH then stained with acidic glycerol containing 0.05% trypan blue for 24 h at room temperature. Root samples were then de-stained, at room temperature, in acidic glycerol (Koske & Gemma 1989) and then randomly selected segments were mounted on microscope slides to detect the presence of hyphae, vesicles, arbuscules and any unusual structures. The percentage of colonization rate by AM fungi was calculated using the following formula:

$$\% \text{ Colonization} = \frac{\text{No. of colonized root segments}}{\text{Total no. of root segments examined}} \times 100$$

Soil infestation

Soil infestation was carried out using the pathogen (*P. aphanidermatum*) grown on sand-corn medium (1:1 w/w) using the technique described by Koffi *et al.* (2010). The appropriate concentration of the pathogen for the induction of high rate of infestation was determined according to the following procedures: equal soil samples, in two sets of pots, were inoculated at the following ratios: 2.5%, 5%. 7.5%, 10% and 12.5% (w/w) and thoroughly mixed. Inoculated pots were incubated for sometimes to allow good colonization by the pathogen before sowing seeds. One set was incubated for one week while the other for two weeks.

Evaluation of AM efficacy to control cotton damping-off

The ability of AM fungi to reduce the incidence of cotton damping-off was achieved using a set of five treatments at both small scale level (in pots) and at a large scale in the field (see Fig. 1). Evaluation of the biocontrol efficacy of AM was done by comparison with the fungicide Maxim XL (at a concentration of 3.5% mefanoxam). The five treatments designed were:

a- Control (natural soil, i.e. non-infested, non mycorrhized),
b- Infested soil (with a pathogen concentration at 5% w/w),
c- Mycorrhized infested soil,
d- Mycorrhized soil (alone), using 150 g aliquots as seed beds
e- Seeds treated with the fungicide Maxim XL (at the rate of 2 ml/ kg seeds) in infested soil.

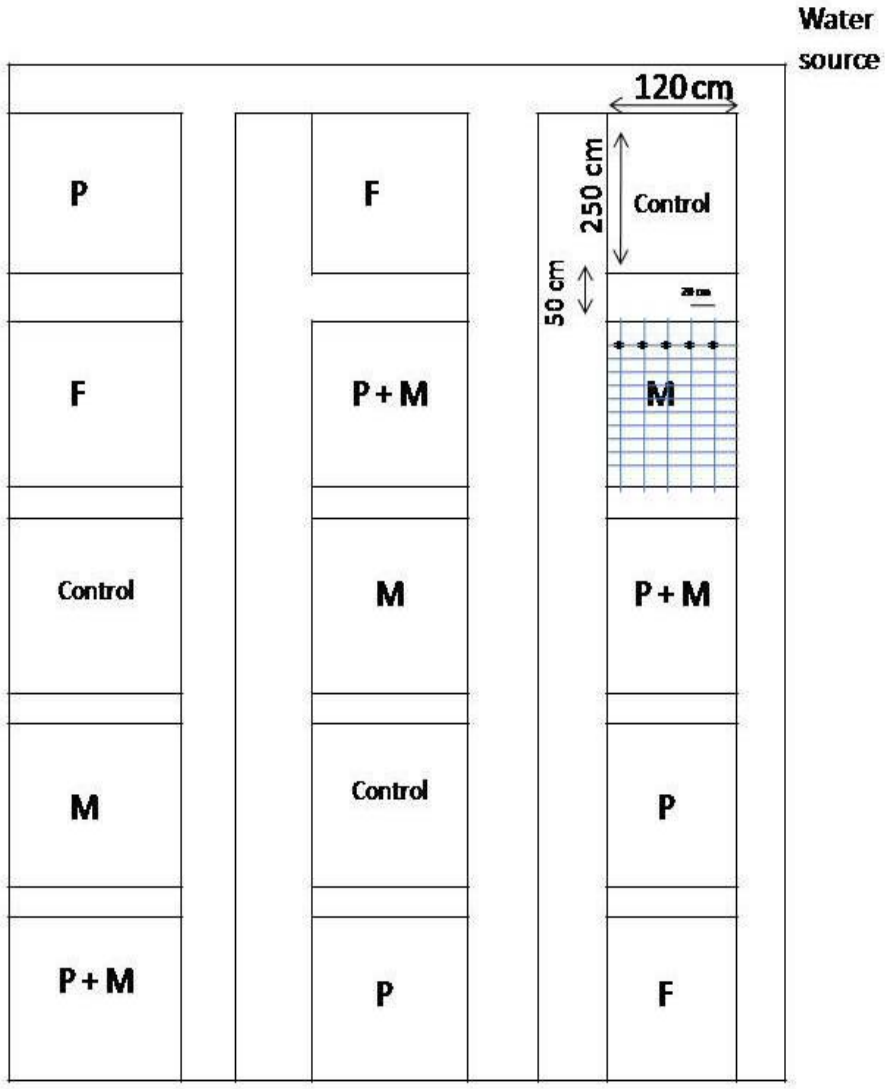

Fig.1: Layout of field experiment. **Abbreviations: C**, Control soil; **F**, Fungicide; **M**, Mycorrhizal soil; ***P,*** *Pythium*-infested soil. The field was divided into plots (250cm X 120cm), each represented one treatment. In each plot, cotton seeds were planted in wells (at 20 cm intervals) at the rate of two seeds per each well. Wells were arranged in five columns and ten rows.

Pre-emergence damping-off was determined after two weeks of sowing and calculated as percentage of the developing plants in relation to the total number of seeds used, while post-emergence was recorded after four weeks and calculated as percentage of the plants remaining after emergence but showing symptoms of infection. Assessment of disease was measured after 4 weeks by using the scale developed by Akköpru & Demir (2005) to rank disease severity of damping-off symptoms (Table 1).

Table 1: Rank of disease severity of damping-off disease

Rank	Mean
0	Plant showing no symptoms
1	Slight damage
2	Moderate damage
3	Dead plant

The Disease Index (DI) was calculated using the following formula:

$$DI\ (\%) = \frac{\Sigma\ (R_i\ X\ N_i)}{R_t\ X\ N_t}\ X\ 100$$

Where R_i is the disease rating, N_i is the number of plants with a particular disease rating, R_t is the highest rated diseased plant and N_t is the total number of plants rated. Effect of AM fungi on host growth was evaluated according to the change in plant height (cm), fresh and dry weight of shoots and roots (g).

Statistical analysis

Data of pot and field experiments were subjected to the analysis of variance (ANOVA) while Duncan`s multiple range tests (Duncan 1958) was used for comparison among means.

Results

Effect of inoculum level of the pathogen on disease incidence

The test of infestation to determine the suitable amount of pathogen to be used to induce high rate of infestation is given in Table (2) where the results indicated that the increase of inocula ratio was always associated with a prominent increase in the number of infected plant. Data of these tests revealed that a high rate of damping-off (94.3%) could be achieved by adding the pathogen inoculum to the soil either at the ratio (w/w) of 5% and incubated for a period of two weeks before sowing seeds, or at the ratio of 12.5% with an incubation period of only one week.

Interaction between AM fungi & *Pythium* on host growth & disease incidence

The role of AM fungi alone and together with the pathogen (*P. aphanidermatum*) on host growth and disease incidence is given in Tables (3 & 4). The data also showed the effect of the systemic fungicide mefanoxam on disease incidence. A glimpse to the data of the two tables clearly showed a positive effect of increasing soil mycorrhization on the host growth as indicated by the effects on all growth parameters of treated hosts as compared with control.

a- Pot experiments

The data of pot experiments (Table 3) clearly show the significant role of soil mycorrhization in reducing the disease incidence in infested soil from 68% down to 29%. The same trend of disease reduction was also noticed in pre- and post-emergence where the former scored a sharp decline from 37% down to 9% and the latter from 31% to 20%. All growth parameters considered, revealed also positive changes highly significant particularly in root fresh and dry weights. Application of the systemic fungicide mefanoxam showed greater reduction in disease incidence than AM fungi by scoring a total incidence of only 12% compared to 29% by AM fungi.

b- Field experiments

The data of Table (4) revealed also the same effect of soil mycorrhization on disease incidence and host growth parameters where a significant decrease of disease incidence from 77.3% in infested soil to 50.66% in infested-mycorrhized soil was noticed. The same trend of reduction was also reported in pre- and post-emergence damping-off.

As reported in pot experiments, application of the systemic fungicide mefanoxam also showed a much greater reduction in disease incidence than AM fungi by scoring a total incidence of only 6.9% compared to 50.7% by AM fungi.

The severity of cotton damping-off was assessed using the disease index. The results showed that using AM as a biocontrol agent reduced the disease index in infested soil from 89.2% to 63.2%. In case of mefanoxam the disease index showed a sharp drop to reach 11.922%. The negative effect of soil infestation by *P. aphanidermatum* on root mycorrhizal colonization rate was also significant where a reduction from 71%, in mycorrhized soil, down to 60% in mycorrhized-infested soil was observed.

Table 2: Effect of inoculum density (or ratio) on total disease incidence and pre- and post-emergence damping-off.

Inoculum left for one week				
Inoculum mass (or ratio)	Disease incidence (%)		Infected plants (%)	Healthy plants (%)
	Pre-emergence	Post-emergence		
2.5.%	40	30.7	70.7	29.3
5.0%	55.5	27.9	83.4	16.6
7.5%	60.7	26	86.7	13.3
10.0%	77.4	13.3	90.7	9.3
12.5%	75	18.4	93.4	6.6
Inoculum left for two weeks				
Inoculum mass (or ratio)	Disease incidence (%)		Infected plants (%)	Healthy plants (%)
	Pre-emergence	Post-emergence		
2.5.%	45	28	73	27
5%	48.5	44.9	93.4	6.6
7.5%	52.4	30.6	83	17
10%	43.5	44.5	88	12
12.5%	50	40	90	10

Table 3: Effect of AM fungi on the disease incidence and host growth parameters (in pots)

Treatment	Plant height (cm)	Shoot fresh weight (g)	Shoot dry weight (g)	Root fresh weight (g)	Root dry weight (g)	Healthy plants (%)	Percentage of plants showing damping-off		Total disease incidence (%)
							Pre-emergence	Post-emergence	
Control	18.8 ± 0.714 [c]	1.32 ±0.132 [c]	0.18 ± 0.009 [c]	0.08 ±0.002 [c]	0.0058 ± 0.00007 [d]	91±0.27 [a]	0±0[c]	0±0[d]	0±0[d]
AM fungi	22.10± 0.387 [b]	1.97 ±0.159 [b]	0.28 ± 0.0061 [b]	0.14 ±0.013 [a]	0.0253 ± 0.0003 [a]	90±0.298 [a]	0±0[c]	0±0[d]	0±0[d]
Pathogen	14.70 ±0.464 [d]	0.94 ±0.098 [d]	0.13 ± 0.004 [d]	0.04 ±0.0052 [d]	0.0044 ± 0.0001 [e]	23±5.38[d]	37±0.339 [a]	31±3.14[a]	68±0.538 [a]
Pathogen +AM fungi	15.06 ±0.270 [d]	1.13 ±0.106 [cd]	0.16 ± 0.0026 [d]	0.09 ±0.0041 [bc]	0.0092 ± 0.0004 [c]	62± 4.66 [c]	9±0.249[b]	20±0.33 [b]	29±0.466 [b]
Pathogen +Fungicide	23.90 ±0.388 [a]	2.63 ±0.173 [a]	0.37 ± 0.0126 [a]	0.11 ±0.0048 [b]	0.0218 ± 0.0005 [b]	79 ±4.58 [b]	3± 3.2 [b]	9± 3.48 [c]	12±0.458 [c]

- All values represent the mean of 10 plants / treatment.
- Data expressed as mean ± standard error.
- At the same column, values followed by the same letter(s) do not show significant difference at $p < 0.05$.
- Percentage of seed germination in the control treatment = 91 (9% of seeds did not germinate due to internal factors).

Table 4: Effect of AM fungi on the disease incidence, host growth parameters and root colonization (in the field).

Treatment	Plant height (cm)	Shoot fresh weight (g)	Root fresh weight (g)	Shoot dry weight (g)	Root dry weight (g)	Healthy plants (%)	Percentage of plants showing damping-off		Total disease incidence (%)	Total disease index (%)	Mycorrhizal colonization (%)
							Pre-emergence	Post-emergence			
Control	21.3± 0.606[a]	2.08± 0.012 [b]	0.158± 0.003[b]	0.3837± 0.0007[b]	0.0332± 0.00008[b]	91± 0.27 [a]	0±0[c]	0±0[d]	0.00[c]	0[c]	-
AM fungi	22.8± 0.208[a]	2.262± 0.208[a]	0.188± 0.025[a]	0.3928 ± 0.00008[a]	0.0361± 0.00028[a]	90± 0.298 [a]	0±0[c]	0±0[d]	0.00[c]	0[c]	71 ±3.96[a]
Pathogen	17.78± 0.431[c]	1.63± 0.060[c]	0.094± 0.008[c]	0.3124± 0.00008[e]	0.01055± 0.00011[e]	23 ±5.38[d]	37± 0.339 [a]	31± 3.14[a]	89.2[a]	77.32 ±1.89 [a]	-
Pathogen +AM fungi	19.51± 0.529[b]	1.73± 0.041[c]	0.128± 0.0020[b]	0.3806± 0.00007 [c]	0.0264± 0.00007 [c]	62± 4.66 [c]	9± 0.249[b]	20± 0.33 [b]	63.2[b]	50.66 ±2.027 [b]	60 ±2.991[b]
Pathogen +Fungicide	19.82± 0.264[b]	1.68± 0.0264[c]	0.124± 0.001[bc]	0.3294 ± 0.00008 [d]	0.02576± 0.0006[d]	79 ± 4.58 [b]	3± 3.2 [b]	9± 3.48 [c]	11.922[c]	6.93 ±0.521 [c]	-

- All values represent the mean of 20 plants / treatment
- Data expressed as mean ± standard error.
- At the same column, values followed by the same letter(s) do not show significant difference at $p < 0.05$.
- Percentage of seed germination in the control treatment = 91.33

Discussion

It is known that the efficacy of AM fungi as a type of biocontrol varies markedly from one case to another depending on several factors among which: i- density and diversity of AM population, a factor that is greatly affected by the soil physicochemical characters, ii- the ability of host plant roots to allow high colonization and to develop spores in high count, iii- the type of pathogen and degree of infestation. Earlier studies indicated also that the protection ability of AM fungi, against root pathogens, vary considerably from species to another. Thereafter, it has been recommended that an assemblage of AM fungi derived from a multiple species would exhibit greater potential than a single species (Habte *et al.* 1999, Pozo *et al.* 2002).

Data of the pot and field experiments showed some minor differences in values. Both data revealed clearly the positive effect of increasing soil mycorrhization on plant vigour where all growth parameters showed significant increase by comparison with the non-mycorrhized, control soil. Nevertheless, the ability of mycorrhiza to reduce the incidence of cotton damping-off was rather limited where a reduction from 68% to 29% was reported in pots and from 77% to 50.7% in field. These amounts of reduction in disease incidence although mathematically significant, it is not economically acceptable by comparison with the systemic fungicide mefanoxam which revealed greater values of reduction, than AM fungi, by showing an incidence of only 12% in pots and 9.6% in field.

The limited reduction of disease incidence, obtained in the present study, might be attributed to two reasons. The first, is related to the density of endomycorrhizal population which showed after eight months of mycorrhization a mean count of 996 spores/ 100 g soil. Such amount of spore density could not be high enough to afford the required protection of host roots against infection because the soil used for experimentation was sandy (El-Bramawy and Osman 2012) with relatively poor organic matter content of 0.4%. Such spore count is considered low if compared to other Egyptian cultivated soils which accommodate greater counts reaching as many as 2373 spores/100 g soil as reported by Abdel-Azeem *et al.* (2007). The second reason is related to the type of pathogen and degree of infestation where infection by *Pythium* as a pathogen takes place through vast numbers of zoospores which represent a strong inoculum potential able to penetrate through large areas of the root surface at one time. In addition, incubating the pathogen in the test soil for two weeks before sowing seeds greatly increased the inoculum potential to score an infestation rate of 93% which could be too high to mycorrhiza alone to bring down of disease incidence to an economically acceptable rate.

Our observation on the limited efficacy of AM fungi to protect cotton against damping-off by *Pythium* was also reported by many investigators dealing with cotton or some other crops as reported by Haggag *et al.* (2001) on geranium root rot caused by *Fusarium solani* and *Macrophomina phaseolina*, Elwan *et al.* (2002) on cotton damping-off by *Rhizoctonia solani*, El-Khallal (2007) on tomato wilt by *Fusarium oxysporum*, Abdel-Fattah *et al.* (2011) on the root rot of common bean by *Rhizoctonia* sp., and Zhang *et al.* (2012) on cotton wilt by *Verticillium dahliae.*

Like growth parameters, root-colonization rate was also negatively affected by soil infestation with *P. aphanidermatum* where a drop in the colonization rate from 71% to 60% was reported. The same observation was also noticed by several investigators who attributed the decrease in root colonization to the presence of competition between the antagonist (AM fungus) and the pathogen for common resources within the root such as infection sites and place (St-Arnaud *et al.* 1994, Bodker *et al.* 2002, Filion *et al*. 2003).

It is evident that AM fungi alone as a biocontrol mean of root infections cannot afford economically acceptable rates of disease reduction. Therefore, improvement of AM efficacy is likely a must and various approaches are available in which AM fungi would act as a part of an integrated control of a bipartite or tripartite type. In the bipartite, endomycorrhizae are usually mixed with any of the following candidates as antagonists: soil fungi such as *Trichoderma*, *Gliocladium*, *Penicillium*, *Epicoccum*, *Paecilomyces* or bacteria such as *Pseudomonas*, *Rhizobium*, *Bacillus*, *Paenibacillu*s and *Burkholderia*. However, in the tripartite, AM fungi are mixed with dual organisms, either a soil fungus plus a bacterium such as *Pseudomonas simplicissimum* and *T. harzianum* (Chandanie *et al*. 2009), *P. flurescens* and *T. harzianum* (Sukhada *et al.* 2002), *Bacillus subtilis* and *T. viride* (Zeidan *et al.* 2011), or two bacteria like *Rhizobium* and *Pseudomonas* (Akhtar and Siddiqui 2009).

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