Plant Pathology Journal

ISSN 1812-5387

Plant Pathology Journal 10 (3): 108-114, 2011
ISSN 1812-5387 / DOI: 10.3923/ppj.2011.108.114

Effects of Arbuscular Mycorrhizal Fungus and Different Phosphorus Doses Against Cotton Wilt Caused *Verticillium dahliae* Kleb.

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Abstract: The objective of this work was to study the influence of Arbuscular Mycorrhizal Fungus (AMF) and different phosphorus dosages on the development of *Verticillium dahliae* Kleb. induced wilt in cotton. Sayar-314 cotton variety which is sensitive to *Verticillium* wilt, AMF *G. intraradices* and phosphorus dosages of 0, 40 and 80 kg ha^{-1} were used in the experiments implemented under naturally *V. dahliae*-contaminated field conditions during the years of 2007 and 2008. According to the obtained results, it was found that there occurred a reduction in the disease chart. Also, it was observed that the application reduced the severity of the disease by 22-29.22% in green portions of cotton plants and their stem sections in field divisions particularly to which *G. intraradices* and phosphorus of 40 kg ha^{-1} had been applied together in both years. On the other hand, phosphorus dosage of 80 kg ha^{-1} had a negative effect in suppressing the infection. Phosphorus (P) content of cotton plant's leaves increased in mycorrhizal fungus treated divisions compared with those untreated. Besides, due to the infection, cotton yield decreased by 14-21% in the divisions without AMF application. It was also concluded that if AM fungus was applied along with lower dosages of phosphorus, it would mitigate the severity of *V. dahliae*-induced infection in cotton, increase the yield despite the infection and induce phosphorus uptake in the plant.

Key words: Cotton, arbuscular mycorrhizal fungi, phosphorus (P), *Verticillium dahliae* Kleb., disease severity, biological control

INTRODUCTION

Cotton is an important industrial raw material for the world's agriculture and commerce because it is used in many areas. Therefore, it is grown in many geographical regions across the world. Turkey ranks seventh according to the size and production of its cotton fields sand its share in the total production ranges between approximately 3.5 and 4.5% (Ozudogru, 2006).

Verticillium wilt, caused by the soil-borne fungus *Verticillium dahliae* Kleb. is one of the most important cotton diseases and causes great economic losses (Naraghi *et al*., 2008). It is difficult to control due to the broad host range of the fungal pathogen, long viability of the resting structure and the insufficiency of fungicides to affect the pathogene once it enters the xylem (Fradin and Thomma, 2006). Therefore, the most effective control is achieved by growing adapted resistant cultivars and management practices known to reduce disease severity (Azaddisfani and Zangi, 2007). On the contrary, breeding for resistance can be very difficult when no dominant gene is known. In addition difficulty in large-scale application of soil solarization and fumigation to reduce microsclerotia in soil make it necessary to consider other control methods (Erdogan and Benlioglu, 2010).

The difficulty in controlling, based on the above statements, *Verticillium* wilt has stimulated the research in biological control independently from the recent concern for environmental protection. One of the recent approaches used to control *Verticillium* wilt disease is Arbuscular Mycorrhizal Fungi (AMF) based biological control (Karagiannidis *et al*., 2002; Garmendia *et al*., 2005).

The symbiotic association between mycorrhizal fungi and the roots of plants is widespread in the natural environment (Gosling *et al*., 2006 Mahmood and Rizvi, 2010). There are a number of different types of fungi that form these associations but as for agriculture, it is the Arbuscular Mycorrhizal Fungi (AMF) of the phylum Glomeromycota (Schuller *et al*., 2001) that is the most important (Gosling *et al*., 2006). It is well known that AM fungi can enhance uptake several nutrients, especially phosphorus and improve the nutrient status of their host plants (Turkmen *et al*., 2005; Smith and Read, 2008; Wehner *et al*., 2010). Though nutrient uptake has been the focus of much research on the Arbuscular Mycorrhizae (AM) association there is evidence that

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AMF also a play a role in the suppression of crop pests and diseases, particularly soil-borne fungal diseases (Bharadwaj and Sharma, 2006; Demir and Akkopru, 2007; Aysan and Demir, 2009; Al-Askar and Rashad, 2010; Tahat *et al.*, 2010). Previous studies have shown that arbuscular mycorrhizal fungi can be used successfully to control *V. dahliae* in plants (Davis *et al.*, 1979; Liu, 1995; Matsubara *et al.*, 1995; Demir and Onogur, 1999; Karagiannidis *et al.*, 2002).

Phosphorus is one of the major elements for cotton plants and it has got an important role in the formation of seed and fiber. Also it allows early harvest cotton by encouraging early maturation (Albers *et al.*, 1993). Therefore, the optimum yield and high quality cotton, as well as a balanced fertilizer are necessary for an economic production. Cotton is highly dependent on mycorrhizae for phosphorus uptake (Albers *et al.*, 1993). Also several studies have shown that the association of root system of cotton with AM fungi can increase the ability of plants to absorb water and nutrients and can result in increased biomass production and yields (Zhengjia and Xiangdong, 1991; Zak *et al.*, 1998).

In this study, the effects of singular and double combinations of Arbuscular Mycorrhizal Fungi (AMF) and different phosphorus doses were aimed to investigate on the control strategy of *Verticillium* wilt disease.

MATERIALS AND METHODS

Materials: The cotton cultivar (*Gossypium hirsutum* L.) Sayar-314 which is known as susceptible cultivar against *Verticillium* wilt and AMF fungus (*Glomus intraradices*) were used as materials (Demir *et al.*, 2010). Phosphorus was applied in doses of 0, 40 and 80 kg ha^{-1}.

Field conditions and experimental design: During 2007 and 2008, the experiments were carried out in different previously cotton grown fields on the banks of the Dicle River that was naturally infested with *V. dahliae* and absence of autochthonous AMF inoculum. The natural population of microsclerotia of *V. dahliae* was determined from soil samples according to a standardized methodology from soil depths of 20-25 cm in an experimental field before planting the seeds of cotton. Soil samples were air-dried under ambient temperatures for four weeks and then passed through 2 mm and 250 μm sieves, respectively after removal of stones and plant residues. The number of microsclerotia per gram of soil was estimated by Kabir *et al.* (2004). The inoculum density was 25 microsclerotia per gram of soil in 2007 and 40 in 2008. The soil texture in the experimental field was measured as sandy clay with pH 7.46-7.62, 1.59-1.25% organic matter, 1.4-7.2% lime and 0.34-0.13% salt, 4.81-1.57 mg kg^{-1} phosphorus, 108-99.3 mg kg^{-1} potassium, 0.23%-0.20 nitrogen in 2007 and 2008, respectively before starting the experiments. Each year 70 kg N and 0, 40 kg and 80 kg P_2O_5 per hectare were incorporated with a harrow before cotton planting. The same amount nitrogen was also applied between rows just before flowering. The average temperatures, rainfall and humidity for both 2007 and 2008 growing seasons were obtained from Diyarbakir Region Meteorological Station.

The experiments were arranged as randomized complete block design with four replications. Each plot consisted of four rows of 5 m in length. The plants were grown 70 cm apart between the rows with 20 cm spacing in each row. Intervals between plots were 2 m and intervals between blocks were 3 m. Banks were created around the plots for preventing phosphorus to pass through. The cotton seeds were sown on 10 May 2007 in the first year and on 15 May 2008 in the second year. An AMF inoculum (*Glomus intraradices*) which we ourselves produced using maize as trap plant was tested in the study. Inocula consisted of spores, extra radical mycelium and mycorrhizal roots. In the AMF inoculated plots, 10 g (10 spores g^{-1}) of inoculum was applied below 5 cm of seeds in each row before sowing while sterile sand was placed in the Non-AMF plots. The cultural managements such as irrigations and hoes were programmed according to recommendations made for cotton growing South eastern Anatolia region.

Parameters and methods of measurement: Five plants chosen randomly in the AMF inoculated plots were removed and dried after harvesting, then roots were dyed to detect AMF presence, which was determined using Phillips and Hayman's method (Phillips and Hayman, 1970). The percentage of mycorrhizal colonization was estimated applying the Grid Line Intersect Method in each treatment 8 weeks after planting (Giovannetti and Mosse, 1980). Total dry weights of cotton plants were determined after harvesting. Samples were then oven-dried at 7°C for 48 h, ground and phosphorous (P) content of shoots was measured using the vanadate molybdate-yellow procedure with spectrophotometer (Kacar, 1984). Mycorrhizal Dependency (MD) of cotton plants was expressed as the difference between the dry weight of the mycorrhizal plant and the dry weight of the nonmycorrhizal plant as a percentage of the dry weight of the mycorrhizal plant (Declerck *et al.*, 1995).

Verticillium wilt in each sampled plot was assessed three times (each year during the period beginning early September and ending mid-October or November), in the 5-10, 50-60 and 75% of the bolls open stage. At each recording date, each individual plant was examined for the green ports of cotton plants and the disease severity was estimated for each plant using 0-3 scale (0 = healthy, 1 = 1-50%, 2 = 51-89%, 3 = dead plant) (Barrow, 1970) and the incidence (%) of infected plants was then determined for each plot. A Disease Severity Index (DSI) was calculated for each plot by: (mean severity×incidence %)/maximum severity rating). In addition, disease severity index of the stem sections of plants were examined for presence of internal browning or discoloration in the stem-root-neck of plants using by 0-3 scale (0 = healthy plant, 1 = 1-33%, 2 = 34-67%, 3 = 68-100%) (Erwin *et al*., 1975).

At the end of the growing season (17 October and 10 November 2007; 10 October and 5 November 2008) cotton yield was determined by hand harvesting from each plot.

Statistical analysis: Data were subjected to analysis of variance with the general linear models procedure of JMP 5.0.1. statistical software. Mean separations were determined by Fisher's protected least-significant-difference (FLSD) test at 0.05 probability level.

RESULTS

AMF root colonization and mycorrhizal dependency: Variations occurred between AMF colonization and mycorrhizal dependency rates in cotton variety of Sayar-314 depending on the dosages of phosphorus under the field conditions (Table 1). Colonization and mycorrhizal dependency rates of *G. intraradices* in cotton variety of Sayar-314 were determined as the highest as 56, 20 and 54 and 17% in the years of 2007 and 2008, respectively at the dosage of 0 (control) and as the lowest as 40, 0 and 41, 0% at the dosage of 80 kg ha^{-1} kg da in the same years, respectively. The most important finding in the table, to which it is worth paying attention, is the fact that mycorrhizal dependency occurred as 0% at the dosage of 80 kg ha^{-1} in both of the years.

Disease assessment: Disease severity indexes in green parts and stem sections in *V. dahliae*-contaminated cotton plantations were determined. The lowest value in disease index of green parts occurred when AMF and phosphorus dosage of 40 kg ha^{-1} were applied together in both of the years. Furthermore, it was observed that AMF application at every dosage of phosphorus was more effective from the point of view of green parts' disease index compared with the divisions not treated with AMF and it reduced disease index. Furthermore, the said reductions were found statistically significant. The highest green parts' disease index was determined in 2007 in plants which took place in divisions which were not treated with phosphorus and mycorrhizal fungus. With respect to stem sections, it was seen that AMF *G. intraradices* was effective in suppressing the disease in both of the years. In the years of 2007 and 2008, the application of AMF along with phosphorus dosage of 40 kg ha^{-1} yielded the lowest disease severity in the divisions like index values of 0.58 and 0.63, respectively. As seen in the table, disease indexes were obtained as higher in cases of phosphorus dosage of 40 kg ha^{-1} with no AMF and furthermore, the variation was found statistically significant (Table 2).

Table 1: Percentages of Root Colonization (RC) and Mycorrhizal Dependency (MD) in cv Sayar-314 inoculated *G. intraradices* with different phosphorus doses in naturally infested fields with *V. dahliae* during 2007 and 2008 growing seasons

	Years			
	2007		2008	
Doses of P (kg ha^{-1})	RC (%)	MD (%)	RC (%)	MD (%)
0 (Control)	56a	20a	54a	17a
40	48b	18a	45b	16a
80	40ab	0c	41b	0c

Values are means of four replications; In each column, mean values followed by the same letter are not significantly different according to FLSD at p = 0.05 level

The incidences of disease in cotton plants were determined by investigating 30 subsequent plants in total by considering symptoms of the disease in stem sections. Accordingly, the incidence of disease was found lower in AMF (+) cotton plants in both of the years compared with AMF (-) plants except for applied to cotton plants that received the highest doses of P either in 2007 or 2008. The lowest incidence occurred in the divisions to which AMF and phosphorus dosage of 40 kg ha^{-1} were applied together (42.85%) in both of the years while the highest incidence occurred with the application including phosphorus dosage of 0 kg ha^{-1} with no mycorrhizal inoculation as 55.56 and 53.96% in 2007 and 2008, respectively (Table 3).

Effect of AMF *G. intraradices* on disease severity of *V. dahliae* in cotton plant varied depending on phosphorus dosages used in stem and green sections (Table 4). According to the obtained findings, AMF application was very effective in suppressing the diseases at phosphorus dosage of 40 kg ha^{-1} in both of the years and this effect occurred as 29.22% (in 2007) in stem section and as 25.39% in green sections (in 2008). The effect of AMF inoculation in suppressing the disease without phosphorus application occurred within a range of 0.30-2.54% in stem and green sections in the years, respectively (Table 4). It was found that applying the highest phosphorus dosage which was 80 kg ha^{-1} had a negative effect in suppressing the disease and this

Table 2: Disease index of green parts and stem sections of mycorrhizal or non-mycorrhizal cotton plants (cv Sayar-314) with different phosphorus doses in naturally infested fields with *V. dahliae* during 2007 and 2008 growing seasons

	Disease index (DSI) in 2007				Disease index (DSI) in 2008			
	Green part		Stem section		Green part		Stem section	
Doses of P (kg ha^{-1})	AMF^-	AMF^+	AMF^-	AMF^+	AMF^-	AMF^+	AMF^-	AMF^+
0 (Control)	1.07a	0.75d	0.97a	0.78c	1.03a	0.73cd	1.02a	0.79ab
40	1.00ab	0.62e	0.93ab	0.63d	1.08a	0.58d	1.04a	0.63b
80	0.93bc	0.84cd	0.88abc	0.82bc	0.92ab	0.78bc	0.95a	0.96a

Values are means of four replications; In each column, mean values followed by the same letter are not significantly different according to FLSD at p = 0.05 level. AMF^-: Non-applied arbuscular mycorrhizal fungus, AMF^+: Applied arbuscular mycorrhizal fungus

Table 3: Incidence of disease (%) based vascular system of mycorrhizal or non-mycorrhizal cotton plants (cv Sayar-314) with different phosphorus doses in naturally infested fields with *V. dahliae* during 2007 and 2008 growing seasons

	2007		2008	
Doses of P (kg ha^{-1})	AMF^-	AMF^+	AMF^-	AMF^+
0 (Control)	55.56a	45.72d	53.96a	47.74c
40	53.88ab	42.85d	52.10ab	44.25d
80	50.01bc	50.63c	50.63abc	48.75bc

Values are means of four replications; In each column, mean values followed by the same letter are not significantly different according to FLSD at p = 0.05 level. AMF^-: Non-applied arbuscular mycorrhizal fungus, AMF^+: Applied arbuscular mycorrhizal fungus.

Table 4: Percentage efficacy of arbuscular mycorrhizal fungus (AMF) on *Verticillium* wilt on susceptible (cv Sayar-314) cotton plant with different phosphorus doses in naturally infested fields with *V. dahliae* during 2007 and 2008 growing seasons

	Efficacy (%)*			
	Stem section		Green part	
Doses of P (kg ha^{-1})	2007	2008	2007	2008
0 (Control)	2.54	0.82	0.30	0.91
40	29.22	27.11	22.41	25.39
80	-5.58	-9.05	-9.70	-4.68

*Percentage of reduction in wilt severity compared with the pathogen alone. Efficacy was calculated by: (disease index of non-mycorrhizal cotton plants-disease index of mycorrhizal cotton plants / disease index of non-mycorrhizal cotton plants×100)

Table 5: Contents of phosphorus (P) of mycorrhizal or non-mycorrhizal cotton plants (cv Sayar-314) leaves with different phosphorus doses in naturally infested fields with *V. dahliae* during 2007 and 2008 growing seasons

	Content of p (mg kg^{-1})			
	2007		2008	
Doses of P (kg ha^{-1})	AMF^-	AMF^+	AMF^-	AMF
0 (Control)	0.27c	0.45a	0.25a	0.29a
40	0.33bc	0.44a	0.28a	0.31a
80	0.29c	0.39ab	0.28a	0.29a

Values are means of four replications; In each column, mean values followed by the same letter are not significantly different according to FLSD at p = 0.05 level. AMF^-: Non-applied arbuscular mycorrhizal fungus, AMF^+: Applied arbuscular mycorrhizal fungus

negative effect occurred within a range of -4.68 -9.70% in stem and green sections in the years, respectively (Table 4).

Table 6: Effect of arbuscular mycorrhizal fungus with different phosphorus doses on cotton yield in naturally infested fields with *V. dahliae* during 2007 and 2008 growing seasons

	Cotton yield (kg ha^{-1})			
	2007		2008	
Doses of P (kg ha^{-1})	AMF^-	AMF^+	AMF^-	AMF
0 (Control)	3930.0d	4525.0b	3050.0d	4352.5ab
40	3822.5d	4917.5a	3550.0cd	4587.5a
80	4035.0cd	4300.0bc	3570.0bcd	3987.5abc

Values are means of four replications; In each column, mean values followed by the same letter are not significantly different according to FLSD at p = 0.05 level. AMF^-: Non-applied arbuscular mycorrhizal fungus, AMF^+: Applied arbuscular mycorrhizal fungus

Content of phosphorus in leaves: Phosphorus content of green sections of cotton plants were determined within a range of 0.39 mg kg^{-1}-0.45 mg kg^{-1} in the divisions, which had been treated with AMF and 0.27 mg kg^{-1} -0.33 mg kg^{-1} in the divisions, which had not been treated with AMF, in 2007 depending on the applied phosphorus dosages (Table 5). The highest phosphorus content was obtained in 2007 in the divisions, which had been treated with mycorrhizal with no phosphorus fertilization (0.45 mg kg^{-1}) (Table 5). Furthermore, variations between phosphorus contents in mycorrhizal-treated and non-treated divisions were found statistically significant. Green parts' phosphorus contents in AMF-treated divisions in 2008 were higher than those in non-treated divisions but the variation was not found statistically significant (Table 5).

Yield: It was also found that cotton yield varied between 4917.5 and 3050.0 kg ha^{-1} depending on years and cotton yield increased considerably especially in AMF-treated divisions (Table 6). The highest cotton yield was achieved in 2007 as 4917.5 kg ha^{-1} in AMF-treated divisions along with phosphorus fertilization of 40 kg ha^{-1} while the yield was significantly reduced in general in the divisions which had not been treated with AMF (Table 6).

DISCUSSION

The effects of arbuscular mycorrhizal fungus (AMF), *G. intraradices* and different phosphorus dosages on

Verticillium wilt which is a serious disease for cotton, with limited number of ways for overcoming it, were investigated in this study.

In this study, first of all, root colonization and mycorrhizal dependency rates of the combination of Sayar-314x *G. intraradices* at different phosphorus dosages were determined under the field conditions. The highest colonization and mycorrhizal dependency rates were found in the divisions which had been treated with mycorrhizal with no phosphorus fertilization while the lowest rates occurred in the divisions fertilized with phosphorus at a dosage of 80 kg ha^{-1}. In some studies implemented in P-poor soils, it was reported that AM fungi better colonized in such type of soils and increased more phosphorus uptake of the plant (Kothari *et al*., 1991). In a similar study, it was reported that high phosphorus levels prevented colonization of AM fungi (Srivastava *et al*., 1996).

Since AMF can benefit plant growth and health, there is an increasing interest in ascertaining its effectiveness in particular plant production situations to incorporate them into production practices. In fact, the mutualistic symbiosis between AMF and plant roots plays a crucial role in nutrient cycling in the ecosystem and can protect plants against soil-borne pathogens. In the present study, it was found that AM fungus, *G. intraradices*, reduced disease severity of *V. dahliae* in cotton plants and suppressed incidence of disease in the plants. Mycorrhizal plants provide higher mechanical power by increasing transportation of nutrients through their powerful transportation systems (xylem) and thus, reduced harmful effects of the transported pathogens. *V. dahliae* is a factor causing tracheomycosis and introduces into a plant through direct penetration through young roots or injuries (Tjamos and Beckman, 1989). Considering the point at which the pathogen introduced into the plant, because there was no injury on the plants, it may be said that it directly penetrated through young roots and, in the meanwhile, it met with the cells which had been physiologically and morphologically modified by *G. intraradices*. It was determined in some of studies also that arbuscular mycorrhizal fungi increased resistance against fungal wilt and root deterioration, stem and leaf diseases (Tylka *et al*., 1991; Mukerji, 1996).

The effects of different phosphorus dosages in combination with AMF on severity of the disease varied. In general, the effects of AM fungus in combination with phosphorus dosage of 40 ha kg^{-1} were found significant in suppressing the disease in stem and green sections of cotton plant in both of the years. It was also found that AMF inoculation reduced severity of the disease in the divisions lacking phosphorus fertilization. Another important finding is that high phosphorus dosage (80 kg ha^{-1}) increased severity of the disease despite AMF treatment and had negative effect in suppressing the disease. The results of a study which was conducted by Demir and Onogur (1999), correlate with those of present study. The obtained findings may be associated with the theory that high P rates or intensive P fertilization prevent AMF colonization on the plant and thus, has a negative effect in suppressing the disease (Davis *et al*., 1979; Kothari *et al*., 1991). In the present study, the effect of AM fungus on P uptake of cotton plants was also investigated. According to the results of P analyses conducted on leaves during the first flower formation stage, it was seen that mycorrhizal cotton plants were able to absorb more phosphorus compared with non-mycorrhizal plants. Also, soil analyses showed that mycorrhizal plants could absorb sufficient phosphorus even in very poor soils in phosphorus. In recent studies, it has been reported that AMF which lives symbiotically in more than 80% of plant populations in nature, has a determinant role in absorption of phosphorus existing in soil by plants (Srivastava *et al*., 1996; Smith and Read, 2008). In addition, as AMF can improve plant nutrition in part due to P uptake by external hyphae, mycorrhizal plants could be better prepared to overcome attacks of pathogens than non-mycorrhizal ones (Demir and Akkopru, 2007).

In this study, positive effects of AMF treatment on yield were also found and it was discovered that losses of 14.22 and 21.32% in yield occurred, respectively in both of the years. The findings show that *Verticillium* wilt is also effective on cotton yield (Bejorano-Alcazar *et al*., 1997; Erdogan *et al*., 2006). In addition, the yield did not decrease in AMF-treated divisions despite the disease. Liu (1995) reported that there is a competition and an antagonistic effect between AMF and *V. dahliae*. Also mentioned study emphasized that mycorrhizal fungi suppresses *V. dahliae* during the whole growth process of cotton plant, induces growth of cotton seedlings, increases the number of flowers and capsules and the increase has a positive effect on yield. The reason why AMF suppresses *V. dahliae* is that *Verticillium* may be prevented by AMF from entering into the plant through the root and thus, the plant is protected against the disease in early stages. Bejorano-Alcazar *et al*. (1997) stated that if symptoms of the leaf disease are seen before the first flowering stage, yield will decrease; on the other hand, if the symptoms are seen after capsules are opened, yield will not decrease. Thus, when a plant is infected, it has an effect on yield. Moreover, Gore *et al*. (2009)

reported that *V. dahliae*'s virulence is low in Southeastern Anatolia and its evergreen pathotype is dominant and this shows its effect in periods close to the harvest.

CONCLUSION

Consequently, considering the aforementioned data, it may be seen that promising different cotton×AMF combinations may be used successfully against pathogens like *Verticillium* wilt which cannot be prevented easily. Accordingly, it is believed that testing suitable plant×AMF combinations in different pathosystems may be effective in suppressing the mentioned disease. Furthermore, it was concluded that further studies on this matter should be carried and steps should be taken for practicing in larger fields.

ACKNOWLEDGMENTS

This study is a M.Sc. Thesis by Aysel Bars Orak under supervision of Dr. Semra Demir. It was supported by Scientific Research Foundation of Yuzuncu Yil University (2008-FBE-YL029).

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