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***Orijinal araştırma* (*Original article*)**

The interaction of the mycorrhizae of the fungus *Rhizophagus irregularis* (Walker & Schüßler, 2010) (*Glomerales: Glomeraceae*) and the stem and bulb nematode (*Ditylenchus dipsaci* Kühn, 1857) (Tylenchida: Anguinidae) on the onion plant (*Allium cepa* L.) (Asparagales: Amaryllidaceae)

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Soğan bitkisinde mikoriza (*Rhizophagus irregularis* Walker & Schüßler, 2010) ve soğan sak nematodunun (*Ditylenchus dipsaci* Kühn, 1857) (Nematoda: Anguinidae) etkileşimi

Öz: Soğan sak nematodu ana konukçusu olan soğan bitkisinde önemli ekonomik kayıplara neden olan bir bitki paraziti nematod türüdür. Mikoriza bitki gelişimini ve sistemik dayanıklılığını arttırarak bitkileri çoğu hastalık ve zararlı etmenlerine karşı dayanıklı hale getirmektedir. Ancak soğan sak nematodu ile ilişkisine yönelik ayrıntılı bir çalışmaya rastlanmamıştır. Soğan bitkisinde mikorizanın soğan sak nematodu üzerine etkisi araştırılmıştır. Mikoriza soğanda nematod penetrasyonu ve üremesini önemli oranda etkilememiştir. Ortalama penetrasyon oranları mikorizalı ve mikorizasız bitkilerde sırasıyla %13.5 ve %7.5 olarak belirlenmiştir. Üreme oranı büyütme dolabında 0.6-1.3 kat, serada 0.7-3.6 kat olarak elde edilmiştir. Bitki ağırlığı uygulamalarda 0.9-2.2 g arasında kayıt edilmiştir. Mikorizanın soğan bitkisinin gelişimini arttırarak soğan sak nematoduna toleransını arttırması nedeniyle, soğan sak nematodunun bulaşık olduğu soğan yetiştirme alanlarında yaygınlaştırılması faydalıdır.

Anahtar Kelimeler: Mikoriza, Soğan sak nematodu, nematod bitki matar interaksiyonu, nematod penetrasyon oranı, nematod üreme oranı

Abstract: The stem and bulb nematode, *Ditylenchus dipsaci*, is a plant parasite that causes significant economic losses to growers of the main host plant, onion. Fungal mycorrhizae can increase plant growth and induce systemic resistance against many diseases and pests. However, no evidence of a detailed study was found regarding the relationship of mycorrhizae with *D. dipsaci*. In this study, the effects of the mycorrhizae of the fungus, *Rhizophagus irregularis*, on *D. dipsaci*, the stem and bulb nematode, on the onion plant, *Allium cepa*, were investigated. The mycorrhizae did not significantly reduce nematode penetration and multiplication on onion plant roots. Mean penetration rates for mycorrhizal and non-mycorrhizal plants were 13.5% and 7.5%, respectively. The

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multiplication rate was between 0.6 and 1.3 in a growth chamber and 0.7 and 3.6 in a greenhouse. Fresh plant weight was 0.9-2.2 g in the greenhouse. Since the mycorrhizae of *R. irregularis* increased the growth of the onion plant by increasing its tolerance to *D. dipsaci*, it would be beneficial to increase mycorrhizal levels in onion growing areas where *D. dipsaci*, the stem and bulb nematode, is present.

Key words: mycorrhiza, stem and bulb nematode, nematode-plant-fungi interaction, nematode penetration rate, nematode reproduction factor

Introduction

The stem and bulb nematode (*Ditylenchus dipsaci* Kühn, 1857) (Tylenchida: Anguinidae) causes damage to more than 500 plant species (Sturhan & Brzeski 1991). Yield losses of up to 80% occur across the world, including Turkey (Mennan & Ecevit 2002; Duncan & Moens 2006; Yavuzaslanoglu et al. 2015). The stem and bulb nematode develops in the intercellular spaces in the aboveground parts of the onion plant, causing the disintegration of the tissues and changes in the plant's physiology. It decreases market value by causing rotting of onion tubers (Yavuzaslanoglu 2019).

Mycorrhizal species of fungi form a symbiotic relationship with plant roots; fungi transport water and nutrients from the deep layers of the soil to the plant and have a growth environment within the plant for itself (Fitter et al. 2011). It has been reported that mycorrhizal fungi are particularly effective in transporting phosphorus and zinc to plants (Özdemir et al. 2010).

Endomycorrhizal fungi adapt well to cultivated plants, especially colonizing the onion plant well and producing significant increases in onion yield (Ames 1989; Bolandnazar 2009; Rozpadek et al. 2016). Elsen et al. (2008) found that arbuscular mycorrhizae reduced the populations of the plant parasitic nematodes, *Rodopholus similis* (Cobb 1893) Thorne, 1949 and *Pratylenchus coffeae* Goodey, 195 by more than 50%. Jaisme-Vega et al. (1997) reported that *Glomus mosseae* (T.H.Nicolson & Gerd.) Gerd. & Trappe reduced root knot nematode galls by 34%-64% in the banana plant. Also, Jaisme-Vega & Rodriguez-Romero (2004) reported that mycorrhizae reduced the lesion rate due to *Pratylenchus goodeyi* Sher & Allen, 1953, but the nematode population did not differ from the control.

Mycorrhizae are found widely in agricultural areas (Ortaş 1997). They are environmentally friendly plant protection agents. However, no detailed study was located regarding the interaction of mycorrhizae and the stem and bulb nematode. In this study, the aim was to determine whether mycorrhizae are effective in controlling the stem and bulb nematode under controlled conditions. For this purpose, the effect of mycorrhizae on the penetration and reproduction of the stem and bulb nematode on onion plants was investigated under growth chamber and greenhouse conditions.

Materials and methods

Origin and identification of the stem and bulb nematode

The efficacy of mycorrhizal inoculation against the stem and bulb nematode was investigated using a population of the stem and bulb nematode. Culture of stem and bulb nematodes was established on sterile carrot discs from one female and one male nematode from a garlic plant obtained from Karaman Province (latitude: 37,10351; longitude: 33,117) and maintained (Yavuzaslanoglu & Aksay 2021). Nematodes were extracted by washing the carrot discs with sterile tap water for the experiments.

The identification of the stem and bulb nematode was performed according to the methodology of Yavuzaslanoglu et al. (2018). It was identified as *Ditylenchus dipsaci* by obtaining bands at 327, 396, 967, 256, 325 and 245 bp using the species specific primers, PF1/PR1, PF2/PR2, 18S/26S, Dip1R/ DipUF, Dit2F/Dit2R and Dit5F/Dit5R, respectively.

Origin and application of mycorrhizae

A commercial preparation named "Great White Granular 1" (132 propagules / g) (Hidroteks Agricultural Products Industry and Trade Limited Company, Istanbul, Turkey) of the endomycorrhizal fungal species, *Rhizophagus irregularis* (Walker **&** Schüßler, 2010) (formerly *Glomus intraradices*), was used in the experiments. The mycorrhizal product was applied at the rate of 2.376 propagules / 7 L of pot soil, as per the producer's instructions.

Growing onion plants for the experiments

Onion plants of the variety Betapanko (Beta Seeds, Konya, Turkey), which is susceptible to the stem and bulb nematode (Yavuzaslanoglu 2019), were grown from seed sown in two plastic pots (17x15x15 cm) containing 6 L of field soil autoclave-sterilised at 121 ℃ for 120 minutes, then treated or untreated with mycorrhizae, at 20 ℃, 70% humidity and 16: 8 hours day: night period, in a growth chamber.

Determination of mycorrhizal infection rate of onion roots

Mycorrhizae infected plants were grown for 9 weeks or 20 weeks for the experiments. To determine the rate of mycorrhizal infection at 9 and 20 weeks, 5 of the mycorrhizal plants were carefully pulled out of pot; the roots were then gently washed in running water and cut into 1 cm lengths. The cut root pieces were placed in 9 cm diameter glass Petri dishes and stained according to the methodology of Koske & Gemma (1989) to determine the mycorrhizal infection rate. Potassium hydroxide (KOH, 10%) was added to the cut roots in the Petri dishes which were placed in an incubator at 65 ℃ for 1 hour. After that, the Petri dishes were removed from the incubator and the KOH was drained from them. The roots were then washed with distilled water. Then, 2 N hydrochloric acid (HCl) was added to the roots in the same Petri dishes. The Petri dishes were then placed in the incubator for 10 minutes at 65 ℃. After draining the HCl, sufficient 0.1% trypan blue stain was added to the remaining roots in the Petri dishes. The Petri dishes

were then placed in the incubator for 15 minutes at 65 ℃. In the last stage, the trypan blue was drained from the Petri dishes and lactic acid was added. For the last time, the Petri dishes were placed in the incubator (65 ℃ for 15 minutes). The stained roots in the Petri dishes were examined under a stereo microscope (Olympus SZ61). Numbers of infected mycorrhizae hyphae were counted on a template marked with a 1 cm^2 grid, according to the gridline intersection method of Giovannetti & Mosse (1980). The percentage infection rate was determined with the following formula:

Infection (%) = Roots Infected with Mycorrhizae / Total Number of Roots x 100

Nematode penetration experiment

Nine-week-old mycorrhizal and non-mycorrhizal onion plants were placed in Petri dishes containing 20 mL of water agar (2% agar, w: v), with one plant per Petri dish and 10 replications. Immediately afterwards, 400 individuals of all stages of *D. dipsaci* were inoculated in 10 µL of sterile tap water to the root collar of each plant, i. e., where the root and stem meet. The Petri dishes were then incubated at 20 ℃ in darkness for 3 days.

At the end of the experiment, the mycorrhizal and non-mycorrhizal plants were removed from the Petri dishes and separately stained with acid fuchsin stain. The staining process was carried out as follows; the plants in each treatment were placed in 4% NaOCl for 5 minutes to lighten their colour. The plants were then washed 3 times with sterile tap water. Following that, the plants were placed in a 250 ml beherglass beaker containing 100 ml of stain solution comprising 87.5 ml of lactic acid, 6.3 ml of glycerol, 6.2 ml of water and 0.01 g of acid fuchsin stain. The contents of each beaker were then heated in a microwave oven for 30 seconds. The plant material was then placed in a Petri dish and allowed to cool. After cooling it was washed several times with tap water. In order to remove the excess stain, equal volumes (2-3 ml) of glycerol and distilled water and a few drops of lactic acid were added onto the plant in the Petri dish. The plant was then squeezed between two slides and the nematodes were counted under the microscope, as per the methodology of Hooper et al. (2005). The trial was repeated twice in the same way.

Nematode reproduction experiment under growth chamber conditions

In order to determine the effect of mycorrhizal treatment on the reproduction of the pest nematode, 300 ml of sterile sand: field soil (1: 2 v: v) was added to pots (7x7x7 cm). The sterilization of the soil was carried out in an autoclave for 120 minutes at 121 ℃. Nine-week-old mycorrhizal and non-mycorrhizal plants were both transplanted into 20 pots, one plant per pot, at the 2-3 leaf stage. In each mycorrhizal treatment, 10 of the plants were inoculated between two leaves and 10 of them were inoculated via the soil around the plant 2 times with 200 nematodes in 10 µl water with a 4 day interval.

The labelled pots were then placed in a plant growth chamber in a factorial completely randomized plot arrangement. The factors were two nematode application treatments and one mycorrhizal treatment. The plants were grown for 6

weeks at 20 ℃, 70% humidity, and 16 hours light and 8 hours darkness. The plants were watered weekly with 40 ml of water and with “Hoagland solution” at three weeks intervals to provide plant nutrients (Hothem et al. 2003). Finally, the nematodes were extracted from the plants and soil in each pot over a 24 hour period by using a modified "Baermann Funnel Method" and counted at 20x magnification under a microscope (details described earlier) (Hooper et al. 2005).

Nematode reproduction experiment under greenhouse conditions

Reproduction of the stem and bulb nematode was also investigated under greenhouse conditions from March to June 2021 for 14 weeks. Two parallel experiments with the same experimental design were set up at the same time using 20 weeks old mycorrhizal and non-mycorrhizal onion seedlings. The experiments were established with a factorial completely randomized plot design with 5 replications. The two factors were mycorrhizal treatment and nematode treatment. Two plants were transplanted into each round pot (19 x19 cm) containing 6 L of a non-sterile sand: field soil mixture (1:2 v:v).

The nematode treated plants were inoculated after transplanting; 400 nematodes were inoculated onto each plant, with the same procedure as in the growth chamber experiment.

The pots were irrigated weekly with 250 ml of water and nutrients were supplied with “Hoagland solution” at three week intervals (Hothem et al. 2003).

The nematodes were extracted together from the two plants from each nematode inoculated pot over a 3 day period by using the modified "Baermann Funnel” method. Nematodes were counted at 20x magnification under a microscope, (details described earlier) (Hooper et al. 2005). Data are presented as the number of nematodes per plant. The fresh plant weight of all plants was also measured.

Statistical analysis

In order to determine the effect of mycorrhizal treatment on the nematode penetration rate in the Petri dish experiment, ANOVA was performed.

Differences in the reproduction factor (R_f) of nematodes from the effects of mycorrhizal and nematode inoculation treatments in the growth chamber were investigated with ANOVA.

The effects of mycorrhizal and nematode treatments on the fresh weight of onion plants and nematode reproduction in the greenhouse experiment were also investigated with ANOVA.

The two replications of the experiments were evaluated separately. Prior to statistical analysis, nematode counts in the nematode reproduction experiments in the growth chamber and greenhouse were transformed to $\ln(x+1)$ values to normalise the data. Statistical analysis was performed with JMP© 5.0 software (JMP, 2020). The differences between means were accepted as significantly different at the 5% level ($p < 0.05$).

Results and discussion

Mycorrhizal colonization was calculated as 60% and 79.5% on 9- and 20-weeks old plant roots, respectively. A microscope image of fungal mycorrhizal hyphae colonizing the roots of onion plants is presented in Figure 1.

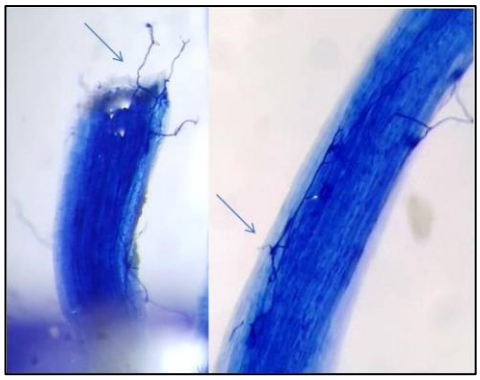

Figure 1. Microscopic view of fungal mycorrhizal hyphae on the roots of onion plants (arrows indicate mycorrhizal hyphae).

The numbers of nematodes that penetrated the mycorrhizal and non-mycorrhizal plants in both Petri dish experiments were not significantly different statistically. In the first experiment, a mean of 47 nematodes (11.8%) penetrated each mycorrhizal plant, whereas a mean of 29 (7.3%) nematodes penetrated each non-mycorrhizal plants. In the second experiment, means of 61 (15.3%) and 31 (7.8%) nematode penetrations were observed in the mycorrhizal and non-mycorrhizal plants, respectively (Table 1).

Table 1. Number of nematodes that penetrated onion plants, with and without mycorrhizal inoculation, after 3 days in two Petri dish experiments (mean ± std error of mean and t test group)

Experiment 1		Experiment 2	
Mycorrhiza +	Mycorrhiza -	Mycorrhiza +	Mycorrhiza -
47±8.3 a	29±8.3 a	61±8.3 a	31±8.3 a

There was no significant difference in the nematode reproduction factors of the mycorrhizal and nematode inoculation treatments in both trials under growth chamber conditions at 6 weeks after experiment.

In the first experiment, the reproduction factor (R_f) for nematodes inoculated onto the soil containing mycorrhizal plants was 1.1, and the mean reproduction factor of nematodes inoculated onto leaves was 1.3. For the non-mycorrhizal plants, the R_f values for nematodes inoculated onto the soil and the leaves were both 1.3 (Table 2).

In the second experiment, the R_f values for nematodes inoculated onto the soil and the leaves of mycorrhizal plants were both 0.8. For the non-mycorrhizal onion

plants, the R_f values for nematode inoculation onto the soil and leaves were 0.7 and 0.6, respectively (Table 2).

Table 2. Reproduction factor (R_f) of nematodes inoculated onto soil and leaves of mycorrhizal and non-mycorrhizal onion plants after 6 weeks in two experiments in a growth chamber (mean ± std error of mean and t test group)

Experiment Number	Mycorrhizae +		Mycorrhizae -	
	Soil Inoc.	Leaf Inoc.	Soil Inoc.	Leaf Inoc.
1	1.1±0.3a	1.3±0.3a	1.3±0.4a	1.3±0.3a
2	0.8±0.2a	0.8±0.1a	0,7±0.1a	0.6±0.1a

Gera Hol & Cook (2005) reported that mycorrhizae reduced the numbers of root knot nematodes by an average of 14% and cyst nematodes by 5%. However, the number of migratory endoparasitic nematodes increased with the application of mycorrhizae. The same authors also reported that sedentary endoparasitic nematodes have competitive relationships with mycorrhizal fungi for sites and nutrients in roots. Elsen et al. (2008) determined that arbuscular mycorrhizae reduced the numbers of *Rodopholus similis* and *Pratylenchus coffeae*, which are migratory endoparasitic nematodes, by more than 50% in pot experiment. On the other hand, as in our study, Jaisme-Vega & Rodriguez-Romero (2004) reported that there was no difference between the number of *Pratylenchus goodeyi* in mycorrhizae-inoculated and control banana plants.

Plant roots infected with mycorrhizae release compounds with antioxidant properties, such as phytoalexin, phenolics (Morandi 1996), and arginine and isoflovanoids (Caron 1989). In the present study, the aim was to determine whether these compounds, which are secreted from roots in response to mycorrhizal infection; have an inhibitory effect on the infection of onion plants by nematodes inoculated into the soil. However, in this study, a statistically significant difference was not observed between soil inoculation and plant tissue inoculation in the nematode reproduction factor.

Investigation of the effects of mycorrhizal treatment on nematode damage and yield of onion plants in a non-sterile soil medium in a greenhouse did not demonstrate a statistically significant difference due to mycorrhizal treatment in two experiments.

In the first experiment, the mean fresh weight of onion plants in mycorrhizae and nematode treatments were between 1.1 g and 1.4 g at 14 weeks after nematode inoculation. The mean numbers of nematodes at harvest were 276 and 1445 in mycorrhizae infected and non-infected plants (Table 3).

In the second experiment, the fresh weight of onion plants at 14 weeks after nematode inoculation was between 0.9 g and 2.2 g (Table 4). The fresh weight of onion plants was higher in mycorrhizae infected plants in both nematode inoculated and non-inoculated treatments in both greenhouse experiments but it was not significant ($p < 0.05$). The nematode numbers of mycorrhizae infected and non-infected plants were 1074 and 1383 nematodes/ plant, respectively (Table 4).

A positive effect of mycorrhizal treatment on onion growth was also demonstrated in earlier studies (Ames 1989; Bolandnazar 2009; Rozpadek et al. 2016).

Table 3. Fresh plant weight of mycorrhizal and non-mycorrhizal onion plants in nematode inoculated and non-inoculated treatments in first greenhouse experiment (mean ± std error of mean and t test group)

Treatments	Fresh Plant Weight (g)		
	Nematodes +	Nematodes-	Number of nematodes/ plant
Mycorrhizae +	1.4±0.2aA	1.3±0.2aA	276±235a
Mycorrhizae -	1.4±0.5aA	1.1±0.4aA	1445±708a

"A" indicates comparisons within columns and "a" indicates comparisons within rows with the t test.

In the biological control of plant parasitic nematodes, mycorrhizae cause direct effects through competition for space and nutrients and indirect effects due to the response of the plant to the mycorrhizal infection. The indirect effects include stimulation of the plant defence system and increased plant tolerance to biotic and abiotic stress factors, increased root secretions and changes in microorganism interactions in the rhizosphere (Schouteden et al. 2015).

Table 4. Fresh plant weight of mycorrhizal and non-mycorrhizal onion plants in nematode inoculated and non-inoculated treatments in second greenhouse experiment (mean ± std. error of mean and t test group)

Treatments	Fresh Plant Weight (g)		
	Nematode +	Nematode -	Number of nematodes/ plant
Mycorrhizae +	1.3±0.3aA	2.2±0.3aA	1074±715a
Mycorrhizae -	0.9±0.5aA	1.9±0.3aA	1383±678a

"A" indicates comparisons within columns and "a" indicates comparisons within rows by t test.

In the current study, mycorrhizal infection of onion plants did not inhibit the penetration and multiplication of the stem and bulb nematode. However, onion yield losses caused by the stem and bulb nematode could be decreased by the growth-enhancing effects of mycorrhizal infection. In the onion growing areas infested with the stem and bulb nematode, applications of mycorrhizae would be beneficial by increasing the tolerance of onion plant to the stem and bulb nematode.

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