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Antagonistic Effects of *Trichoderma* Species in Biocontrol of *Armillaria* Root Rot Disease *In Vitro* Conditions

Ayşe Mehtap ASLAN[1*], Beşir YÜKSEL[1], Nuray ÖZTÜRK[1]

[1]Düzce University, Faculty of Forestry, Düzce, TURKEY
*Corresponding Author: mehtapbayir34@hotmail.com

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Abstract

Aim of study: Armillaria root rot is a disease of fruit trees, some herbaceous plants and forest trees. *Armillaria* spp. which cause this disease, are among the most destructive fungal pathogens of trees. In this study, antagonistic effects of *Trichoderma* species in biocontrol of *Armillaria* were investigated *in vitro* conditions

Area of study: The study area was in the Düzce University, Konuralp Campus.

Material and methods: Trichoderma harzianum was provided from Düzce University, Faculty of Forestry, Department of Forestry Industrial Engineering (FS19) and *Trichoderma viride* was provided from Düzce University, Konuralp Campus. *Armillaria mellea* and *Desarmillaria tabescens* were isolated from the infected roots of hazelnut and oak trees in Konuralp Campus and cultured in potato dextrose agar. *Trichoderma* species were inoculated to confront with the pathogens in cultures of *Armillaria* and *Desarmillaria* to determine antagonistic effects for 15 days. Penetration of *Trichoderma* hyphae in rhizomorphs and sporulation of *Trichoderma* species in the surface of rhizomorphs were investigated by scanning electron microscopy.

Main results: The statistically significant decrease was observed for *A. mellea* and *Desarmillaria* species caused by *Trichoderma*. One day after *Trichoderma* inoculation, *Trichoderma* hyphae grew approximately twice as much towards the host rhizomorphs. Three days after colonization of *Armillaria* and *Desarmillaria* rhizomorphs by *Trichoderma*, growth of *Armillaria* and *Desarmillaria* seemed to completely stopped.

Highlights: Trichoderma species have been studied as the most widely used antagonists in biological control against many plant-pathogenic fungi worldwide. To develop an effective biological method for plant protection in the future, biological control studies are highly important in oak forest under natural stand conditions.

Keywords: Antagonistic effect, *Armillaria mellea*, *Desarmillaria tabescens*, Root rot, *Trichoderma harzianum*, *Trichoderma viride*

In Vitro Koşullarında *Armillaria* Kök Çürüklüğü Hastalığının Biyolojik Kontrolünde *Trichoderma* Türlerinin Antagonistik Etkileri

Öz

Çalışmanın amacı: Armillaria kök çürüklüğü, bazı otsu bitkiler, meyve ağaçları, ve orman ağaçlarında zarar yapan fungal bir hastalıktır. Bu hastalığa neden olan *Armillaria* türleri, ağaçlar üzerinde etkili en yıkıcı fungal patojenler arasında yer alır. Bu çalışmada *Armillaria* türlerinin biyokontrolünde *Trichoderma* türlerinin antagonistik etkileri araştırılmıştır.

Çalışma alanı: Çalışma alanı Düzce Üniversitesi Konuralp Kampüsü yerleşkesindedir.

Materyal ve yöntem: Çalışmada kullanılan *Trichoderma harzianum* Düzce Üniversitesi Orman Fakültesi Orman Endüstri Mühendisliği Bölümü'nden (FS19), *Trichoderma viride* ise Düzce Üniversitesi Konuralp Yerleşkesinden temin edilmiştir. *Armillaria mellea* ve *Desarmillaria tabescens* türleri ise Düzce Üniversitesi Konuralp Kampüsünde bulunan hastalıklı fındık ve meşe ağaçlarının köklerinden izole edilerek patates dekstroz agarında kültüre alınmıştır. Daha sonra *Trichoderma* türleri *Armillaria* ve *Desarmillaria* kültürlerine yerleştirilmiş ve 15 gün boyunca gelişimleri gözlemlenmiştir. *Trichoderma* hiflerinin rizomorflara girişi ve sporulasyonu taramalı elektron mikroskobu kullanılarak gözlemlenmiştir.

Temel sonuçlar: A. mellea ve *Desarmillaria* türlerinin büyümelerinde istatistiksel olarak anlamlı bir düşüş gözlenmiştir. Bir gün sonra yapılan ölçümde *Trichoderma* hiflerinin *Armillaria* ve *Desarmillaria* rizomorflarının yaklaşık iki katı kadar olduğu gözlemlenmiştir. *Trichoderma* türlerinin kolonizasyonundan sonra, *Armillaria* ve *Desarmillaria* funguslarının üçüncü günden itibaren gelişimleri sonlanmıştır.

Araştırma vurguları: Trichoderma türleri, pek çok bitki patojen funguslarına karşı biyolojik mücadelede en yaygın kullanılan antagonistler olarak bilinmektedir. Gelecekte etkin bir biyolojik mücadele yöntemi geliştirmek için doğal ortam koşullarında bu çalışmaların yapılması oldukça önemlidir.

Anahtar Kelimeler: Antogonistik etki, *Armillaria mellea*, *Desarmillaria tabescens*, Kök çürüklüğü, *Trichoderma harzianum*, *Trichoderma viride*

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Introduction

The plant pathogens such as fungi, bacteria, nematodes, viruses are organisms that cause reduction in crop quality and yields and have an impact for global food security (Jibril et al., 2016; Abdulkhair & Alghuthaymi, 2016).

Armillaria (Fr.) Staude (Agaricales, Physalacriaceae; honey fungi) is a genus of one of the plant pathogenic fungi (Sipos et al., 2018). *Armillaria* root rot, also known as oak root rot, is a disease of forest and fruit trees worldwide (Hood et al., 1991; Shaw & Kile, 1991; Downer & Lac´an, 2020; Miller et al., 2020). This disease causes serious damage in commercial forests (Lehtijarvi et al., 2012), orchards, vineyards and urban areas (Keča, 2009). The fungus colonizes the cambium in living roots and causes necrotic lesion under the root bark and feeds on the dead tissues after killing the cambium (Baumgartner et al., 2011). The first indication of the presence of disease is yellowing of the leaves with subsequent loss of the foliage (Doepel, 1962). Sometimes oleoresin production is stimulated and flow through cracks in the bark (Mitchell, 1950). Infected plants also show stunted shoots, dwarfed foliage, wilting, dwarfed fruit etc. (Baumgartner et al., 2011).

Armillaria species are recognized with strand-like fungal organs called rhizomorphs which grow root-like through soil (Sipos et al., 2018). The genus *Armillaria* has been reported from North America, Europe, South America, Australia, New Zealand and Africa (Baumgartner & Rizzo, 2001; Keca et al., 2009; Wingfield et al., 2009; Pildain et al., 2010; Kile & Watling, 1988). The first report of *Armillaria* species was on dead *Abies bornmülleriana* Mattf. in Turkey in 1937 (Schimitschek, 1937). Later, the genus *Armillaria* has been reported from many host plants in Turkey (Bremer et al., 1947; Erdem, 1956; Lohwag, 1957; Selik, 1973). Following these reports, *Armillaria* root disease caused by *Armillaria mellea* (Vahl) P. Kumm. (1871) was first reported on Carrizo citrange (*Citrus sinensis* L. Osb. x *Poncirus trifoliata* L. Raf.) and sour orange (*C. aurantium* L.) in Turkey (Baysal-Gürel & Çınar, 2014).

Desarmillaria tabescens (Scop.) R. A. Koch and Aime (synonyms *A. tabescens* (Scop.) Emel) was included to the genus *Armillaria*. Molecular phylogenetic analyses assigned this species to genus *Desarmillaria*. This fungus is another causative agent of *Armillaria* root disease (Amiri et al., 2008; Park et al., 2017). This disease has significantly caused enormous damage on a wide range of woody plants (Morrison et al., 1991; Baumgartner, 2004; Williams-Woodward, 2013).

Trichoderma spp. Pers. 1801 are known as one of most effective fungal bioagents against plant pathogens (Kumar & Ashraf, 2017; Mukhopadhyay & Kumar, 2020). In a previous research, it was reported that *Trichoderma* species could be used for biocontrol of *Armillaria* (Hagle & Shaw, 1991).

The objective of the present study was to investigate antagonistic effects of two *Trichoderma* species (*T. harzianum* Rifai, 1969, *T. viride* Pers., 1794) in biocontrol of *A. mellea* and *D. tabescens*.

Material and Method

Study Area

The study area was in the Düzce University, Konuralp Campus (40°54'N, 31°10'E). The altitude varies between 278 m and 307 m. *T. harzianum* was provided from Düzce University, Faculty of Forestry, Department of Forestry Industrial Engineering (FS19) and *T. viride* from Düzce University, Konuralp Campus. *A. mellea* and *D. tabescens* were isolated from the infected roots of *Corylus* sp. L. and *Quercus* spp. L. trees in Konuralp Campus.

Laboratory Studies

Samples of *Trichoderma*, *Armillaria* and *Desarmillaria* species were cultured on Potato Dextrose Agar (PDA) in petri dishes (90 x 15 mm) and grown at 25 ± 2 °C, 34-40% humidity in the dark. Pure cultures were obtained and observed using Olympus BX51 microscopes. The isolated samples were identified using previous studies (Kubicek & Harman, 2002; Yüksel & Tekel, 2020).

Approximately 1 x 1 cm discs cut from *D. tabescens* and *T. harzianum* cultures were set

at one side of PDA in same petri dishes about 3-4 cm apart at the same time.

Similar procedures also were applied to *T. viride* and *A. mellea* to evaluation of colonization by *Trichoderma* species. Inoculation study was performed in 15 replicates.

Measurement of growth of *Trichoderma*, *Armillaria* and *Desarmillaria* species were recorded daily for 15 days. Penetration and sporulation of *Trichoderma* in rhizomorphs, colonization of rhizomorphs by *Trichoderma* mycelia, discharge of rhizomorph tissue were observed by Scanning Electron Microscopy (SEM).

Statistical analysis was performed using SPSS V22. statistic software package. Growth data were analyzed with one way ANOVA. Games-Howell test was chosen for the post hoc analysis.

Results and Discussion

The statistically significant (p<0.05) decrease was observed for *A. mellea* and *Desarmillaria* species [F (7,496) = 3.86, p = 0.0001]. According to the results of Games-Howell multiple comparison test, significant differences were found between groups (Table 1).

Table 1. Games-Howell multiple comparison results

Dependent Variable		Mean Difference (I-J)	Std. Error	Sig.	Interval	
					Lower Bound	Upper Bound
A. mellea vs. *T. harzianum*	Control *A. mellea* vs. *T. harzianum*	-3,46012*	.34887	.000	-4.5659	-2.3544
A. mellea vs. *T. viride*	Control *A. mellea* vs. *T. viride*	-3,29503*	.34537	.000	-4.3916	-2.1985
D. tabescens vs. *T. harzianum*	Control *D. tabescens* vs. *T. harzianum*	-1,87820*	.48327	.013	-3.4737	-.2827
D. tabescens vs. *T. viride*	Control *D. tabescens* vs. *T. viride*	-2,38354*	.48676	.001	-3.9867	-.7803

* The mean difference is significant at the 0.05 level

According to laboratory measurements, *A. mellea* and *D. tabescens* species continued to grow in the first three days (Figure 1a, d). Three days after colonization of these fungus, growth of *A. mellea* and *D. tabescens* species stopped due to antagonistic effect of *Trichoderma* spp. *Trichoderma* hyphae grew towards the rhizomorphs twice as much within 24 h. *T. harzianum* and *T. viride* hyphae have completely covered the surface of *D. tabescens* since the 6th day (Figure 1a, b). However, rhizomorphs of *A. mellea* species completely covered with these hyphae from the 8th day (Figure 1c, d).

The interaction of *Trichoderma* hyphae with *A. mellea* and *D. tabescens* rhizomorph was observed using SEM (Figure 2a, d). *Armillaria* rhizomorphs were colonized by *Trichoderma* hyphae. It was observed that *Trichoderma* mycelium coiled around the rhizomorphs (Figure 2b, d). Besides, sporulation of *T. viride* was observed in surface of infected *D. tabescens* rhizomorph (Figure 2a). After *Trichoderma* hyphae entered the rhizomorph, holes were occurred on the surface of rhizomorph (Figure 2c).

These results are similar to previous studies that on antagonistic effects of *Trichoderma* species in biocontrol of *Armillaria* (Asef et al., 2008). As a result of the study, it was observed that *Trichoderma* hyphae grew 10 mm towards the rhizomorphs in less than 40 hours. Degeneration of rhizomorphs was observed about two weeks after inoculation. A study on biological control of *Armillaria gallica* Marxm. & Romagn. with *Trichoderma polysporum* (Link: Fr.) Rifai, *T. harzianum* and *T. viride* has been done. These species penetrated outer tissue of the rhizomorphs of *A. gallica* and killed the hyphae (Dumas & Boyonoski, 1992). Antagonistic abilities of 62 *Trichoderma* isolates were tested against 25 *Armillaria* isolates *in vitro* (Chen et al., 2019). Some *Trichoderma* isolates have been reported to effective against 25 *Armillaria* isolates examined.

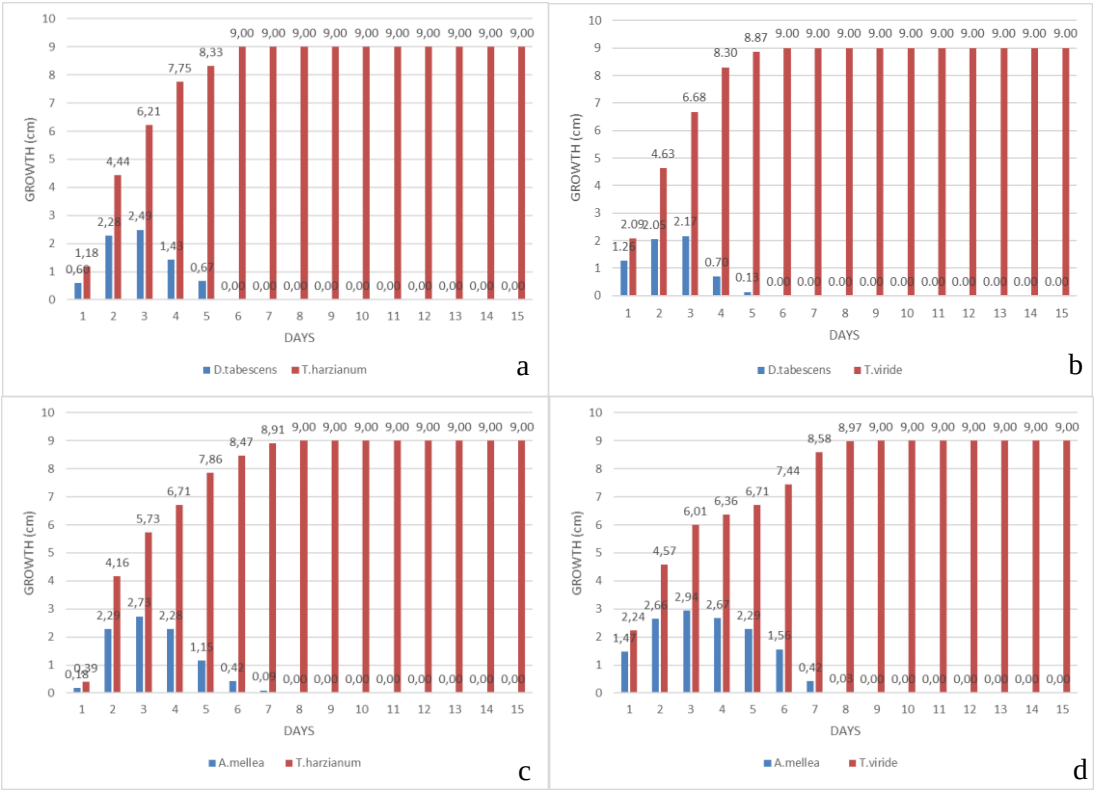

Figure 1. Comparative growth of species; (a) *D. tabescens* vs. *T. harzianum*, (b) *D. tabescens* vs. *T. viride*, (c) *A. mellea* vs. *T. harzianum*, (d) *A. mellea* vs. *T. viride*

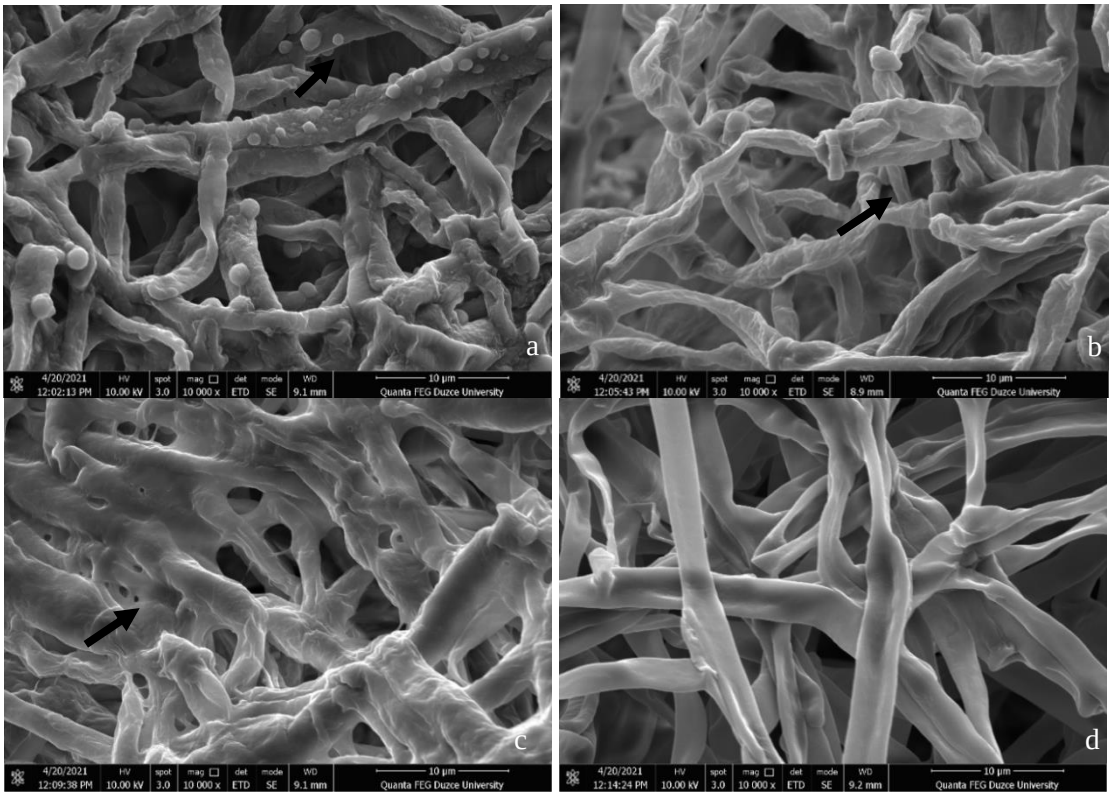

Figure 2. SEM of growth of *Trichoderma* hyphae on the surface of rhizomorphs (a) *D. tabescens* vs. *T. viride*, (b) *A. mellea* vs. *T. viride*, (c) *A. mellea* vs. *T. harzianum*, (d) *D. tabescens* vs. *T. harzianum*

Conclusion

Biocontrol methods which are considered to be environmentally friendly, widely used against pathogenic organisms to control plant diseases around the world (Emmert & Handelsman, 1999).

Armillaria fungi is one of the common wood-rotting pathogens on both angiosperm and gymnosperm in native forest ecosystems, planted forests, orchards and vineyards (Worrall, 2004; Keča et al., 2009).

Yüksel (2016) reported that 1097 diseases and pests have been detected in oak forests in Turkey. 902 (82%) of these pests are insects, 183 are diseases and 12 are other harmful groups. *Armillaria* fungi is one of the most important causes of oak (*Quercus* sp.) decline in worldwide (Luisi et al., 1996).

In previous studies, bark damagewere observed by infection of *A. mellea* and other fungi in *Q. cerris* L. in northeastern Bulgaria during 1991-99 (Rossnev & Petkov, 2000). *Armillaria* (*A. mellea*, *A. gallica* Marxm. & Romagn., *A. tabescens*) has been associated with oak mortality in the Ozark Mountains forests of northern Arkansas (Kelley et al., 2009). *A. mellea* has been one of the most dangerous and common species and associated with stems of standing *Q. robur* L.1753 in Ukraine and Russia (Dunaev & Afanasenkova, 2009).

Trichoderma spp. are known to play an important role in the biocontrol of *Armillaria* species (Sharma & Sankaran, 1988; O'Brien, 2017). *T. viride* has been reported to caused decrease of *Armillaria* species growth between 63-81% (Keča, 2009). It has also been reported that *Armillaria* rhizomorphs die 2-3 weeks after contact with *T. viride* (Keča, 2009).

Results of this study provided insights into use of *Trichoderma* species for control of *Armillaria* root rot *in vitro* conditions. Therefore, it is better to carry out more studies under natural stands related this study.

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Author Contributions

Conceptualization: B.Y.; Investigation: B.Y., A.M.A., N.Ö.; Material and Methodology: B.Y., A.M.A., N.Ö.; Supervision: B.Y., A.M.A.; Visualization: B.Y., A.M.A., N.Ö.; Writing-Original Draft: N.Ö.; Writing-review & Editing: N.Ö., B.Y.; Other: All authors have read and agreed to the published version of manuscript.

Conflict of Interest

The authors have no conflicts of interest to declare.

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