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Determination of efficacy of some fungicides used in hazelnut orchards against *Ambrosiella hartigii* Batra symbiotically associated with *Anisandrus dispar* Fabricius (Coleoptera: Curculionidae: Scolytinae) under laboratory conditions

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ABSTRACT

Anisandrus dispar Fabricius (Coleoptera: Curculionidae: Scolytinae) cultivate symbiotic fungus *Ambrosiella hartigii* Batra in the galleries found in sapwood of host trees for feeding. Therefore, controlling the symbiotic fungus means depriving *A. dispar* of a source of food. In the study, the efficacy of five doses (0.25x, 0.5x, 1.0x, 2.0x and 4.0x; where x is the field rate recommended by the manufacturer) of five fungicides including active ingredients of boscalid+kresoxim methyl, fluopyram+tebuconazole, penconazole, sulphur and tetraconazole used in hazelnut orchards against powdery mildew caused by *Erysiphe corylacearum* was evaluated against *A. hartigii* (TR-Ah-06 isolate) under laboratory conditions. The results of the study showed that all doses of the fungicides significantly inhibited the mycelial growth of *A. hartigii* when compared to the control ($P<0.05$). Among them, fluopyram+tebuconazole, penconazole and tetraconazole even at the lowest dose completely inhibited the mycelial growth of the fungus. However, the mycelial growth was reduced at the rates of 29.56-100% depending on the increasing doses of boscalid+kresoxim methyl. Sulphur inhibited the mycelial growth of *A. hartigii* at the rates of 78.57-100% depending on the increasing doses. In addition, the ED_{50}, MIC and MFC values of fluopyram+tebuconazole, penconazole and tetraconazole were determined to be lower even than the 0.25x, which was the lowest dose at the experiment. The results showed that the fungicides, especially fluopyram+tebuconazole, penconazole and tetraconazole were effective against *A. hartigii.*

Keywords:
Ambrosia beetles
Ambrosiella hartigii
Effect
Fungicides
Hazelnut
Symbiotic fungus

Fındık bahçelerinde kullanılan bazı fungisitlerin *Anisandrus dispar* Fabricius (Coleoptera: Curculionidae: Scolytinae) ile simbiyotik ilişkili *Ambrosiella hartigii* Batra'ya karşı laboratuvar şartlarında etkinliklerinin belirlenmesi

ÖZET

Anisandrus dispar Fabricius (Coleoptera: Curculionidae: Scolytinae) beslenmek amacıyla konukçu ağaçların odun dokusunda açtıkları galerilerde simbiyoitk fungus *Ambrosiella hartigii* Batra'yı yetiştirmektedir. Bu nedenle simbiyotik fungusun kontrol edilmesi *A. dispar*'ı bir besin kaynağından mahrum etmek anlamına gelmektedir. Bu çalışmada fındık bahçelerinde fındık külleme hastalığına neden olan *Erysiphe corylacearum*'a karşı kullanılan boscalid+kresoxim methyl, fluopyram+tebuconazole, kükürt, penconazole ve tetraconazole etkili maddeli beş fungisitin beş dozunun (0.25x, 0.5x, 1.0x, 2.0x ve 4.0x; buradaki x üretici firma tarafından tavsiye edilen dozu ifade etmektedir) laboratuvar şartlarında *A. hartigii* (TR-Ah-06 izolatı) üzerindeki etkinliği test edilmiştir. Sonuç olarak, fungisitlerin tüm dozları kontrol ile kıyaslandığında bu fungusun misel gelişmesini önemli derecede engellemiştir ($P<0.05$). Bunlar arasında en düşük dozdaki fluopyram+tebuconazole, penconazole ve tetraconazole fungusun misel gelişmesini tamamen inhibe etmiştir. Ayrıca, boscalid+kresoxim methyl'in dozlarının artmasına bağlı olarak *A. hartigii*'nin misel gelişmesi %29.56-100 arasında azalmıştır. Kükürtün dozlarının artmasına bağlı olarak bu fungusun misel gelişmesi ise %78.57-100 oranında engellenmiştir. Buna ek olarak, fluopyram+tebuconazole, penconazole ve tetraconazole'nin ED_{50}, MIC ve MFC değerleri en düşük dozdan bile düşük bulunmuştur. Sonuçlar bu

Anahtar Sözcükler:
Ambrosia böcekleri
Ambrosiella hartigii
Etki
Fındık
Fungisitler
Symbiyotik fungus

fungisitlerin özellikle fluopyram+tebuconazole, penconazole ve tetraconazole'nin *A. hartigii*'ye karşı oldukça etkili olduğunu göstermiştir.

1. Introduction

Ambrosia beetles (Coleoptera: Curculionidae; Platypodinae and Scolytinae) are closely tied to symbiotic fungi known commonly as ambrosia fungi (Harrington et al., 2014). They construct galleries in the nutrient-poor sapwood of host trees, and cultivate the symbiotic fungi (mostly *Ambroseilla* spp. and *Raffaelea* spp.) in the galleries for their nutrition (Harrington, 2005). Ambrosia beetles, which generally do not eat wood, feed solely on the symbiotic fungi growing in the galleries (Beaver, 1989; Biedermann, 2007). Exceptionally, the larvae of some species also feed on fungus-colonized wood as well as symbiotic fungi (Biedermann, 2007). The fungi provide important organic molecules necessary for developing of larvae and adults (Norris, 1979; Beaver, 1989). The symbiotic fungi are usually found in the genera, *Ambrosiella* and *Raffaelea* (Six et al., 2009; Harrington et al., 2010). Among them, *Ambrosiella hartigii* Batra has been identified as the symbiotic fungus associated with *Anisandrus dispar* Fabricius (Scolytinae) in Turkey and other some countries (Batra, 1967; Bucini et al., 2005; Kushiyev, 2015).

Ambrosia beetle, *A. dispar* is a widespread pest in hazelnut orchards in Turkey as well as some fruit trees (Ak et al., 2005; 2011; Tuncer et al., 2017). The beetle leads to significant yield losses by drying hazelnut branches and trees (Saruhan and Akyol, 2012). In addition to the direct beetle damage, the symbiotic fungus *A. hartigii*, which produce mycelium abundantly in the sapwood, could disrupt the flow of water and nutrients of host trees (Castrillo et al., 2011). Moreover, this fungus is indirectly harmful due to its role as food source for larvae and adults of *A. dispar*. Therefore, the control of the fungus may provide indirect control of the beetle. Because, controlling the symbiotic fungus means depriving the beetle of a food source.

Generally, plant pathogenic and saprophytic fungi are controlled by chemical fungicides. Previously, in a few studies, antifungal effects of different chemical fungicides on some symbiotic fungi such as *A. hartigii* and *Raffaelea* sp. associated with ambrosia beetles were tested (Joseph et al., 2002; Mayfield et al., 2008; Kagezi et al., 2015; Erper et al., 2018). In a study, it was determined that 0.01 ppm of propiconazole inhibited the mycelial growth of *Raffaelea* sp. associated with ambrosia beetle, *Xyloborus glabratus* Eichh. (Scolytinae) at the rate of 84% (Mayfield et al., 2008). Erper et al. (2018) found that the lowest dose (0.25x) of captan, cyprodinil+fludioxonil, imazalil and prochloraz completely inhibited the mycelial growth of two isolates of *A. hartigii* isolated from *A. dispar*, and also from *Xylosandrus germanus* Blandford (Scolytinae), which is another important ambrosia beetle in hazelnut orchards. Also, they showed that azoxystrobin, iprodione and thiram depending on increased doses reduced the mycelial growth of the symbiotic fungus at the rates of 50-85%, 64-100% and 75-100%, respectively.

Recently, a new and highly destructive powdery mildew caused by *Erysiphe corylacearum*, a member of the family Erysiphaceae and distinct from *Phyllactinia guttata*, has been reported in 16 provinces of Turkey licensed for hazelnut production, and its prevalence was 100% in most of them (Sezer et al., 2017). Some agronomic practices have been used to control the powdery mildew in Turkey, including the use of cultural treatments such as removal and disposal of infected leaves from orchards. Fungicide application including sulphur, carboxamides, strobilurin and DeMethylation Inhibitors [(DMI) -Triazoles)] is done intensely in all hazelnut areas where the disease is seen (Anonymous, 2018).

The object of this study was to evaluate the *in vitro* efficacy of boscalid+kresoxim methyl, fluopyram+tebuconazole, penconazole, sulphur and tetraconazole fungicides used to control of the powdery mildew in hazelnut orchards on the mycelial growth of *A. hartigii* for alternative control of *A. dispar*.

2. Materials and Methods

2.1. Isolation of the symbiotic fungus

Fungal isolate (TR-Ah-06) isolated from *A. dispar*'s female adult was used in the present study. The beetle was obtained from hazelnut orchard in Samsun province of Black Sea region (Turkey) during April 2018. The obtained female adult was disinfected by 1 mL PBS (phosphate buffered saline) containing 0.1% Tween 80 (15 sec.) and 40% ethanol (5 sec.) and put on sterile filter paper to dry (Erper et al., 2018). After the beetle was dissected, and it was placed on Petri dishes (6 cm dia.) containing Potato Dextrose Agar (PDA; Merck Ltd., Darmstadt, Germany) The Petri dishes were incubated for 4 days at 25 ±1°C under dark conditions. Then the isolate was identified as *A. hartigii* according to the macroscopic and microscopic characteristics (Batra, 1967).

2.2. Chemical fungicides

The fungicides used in the study were given in Table 1. Antifungal efficacy of five fungicides at five doses (0.25x, 0.5x, 1.0x, 2.0x and 4.0x; where x is the field rate recommended against powdery mildew caused by *E. corylacearum* on hazelnut by the manufacturer in Turkey) were evaluated to *A. hartigii* under laboratory conditions.

Table 1. Chemical fungicides selected for *in vitro* testing

Chemical group	Active ingredient	Trade name	Manufacturer	Registered doses in Turkey
Mixture	200 g L^{-1} boscalid+ 100 g L^{-1} kresoxim methyl	Collis SC	Basf	0.30 mL L^{-1}
	200 g L^{-1} fluopyram+ 200 g L^{-1} tebuconazole	Luna Experience SC 400	Bayer	0.25 mL L^{-1}
DMI[a]- Triazoles	100 g L^{-1} penconazole	Fullpas 100 EC	Agrobest	0.35 mL L^{-1}
	100 g L^{-1} tetraconazole	Domark 10 EC	Hektaş	0.50 mL L^{-1}
Sulphur	80% sulphur	Saupolo 80 WG	Astranova	4.0 g L^{-1}

[a]DeMethylation Inhibitors (DMI)

2.3. Effect of the fungicides on mycelial growth

Antifungal effect of the five fungicides on mycelial growth of *A. hartigii* were tested according to Erper et al. (2018). The five doses of the fungicides were added to autoclaved PDA medium at 50°C, and then the ameliorated PDA medium were dispensed aseptically into 9-cm Petri dishes (20 mL per Petri). The same amount of unamended PDA medium was dispensed into Petri dishes for control treatment. A mycelial disc (6-mm-dia.) cut from 5-day-old culture of *A. hartigii* was placed in the center of each Petri dish, and the Petri dishes were incubated at 25°C in the dark for 4 days. When the control fungal colonies had grown to the point of nearly covering the Petri dishes, all the dishes were measured at two perpendicular points. Mycelial growth values were converted into percentage of mycelial growth inhibition (MGI), in relation to the control treatment by using the formula MGI (%) = [(dc – dt)/dc] × 100, where dc and dt represented mycelial growth diameter in the control and amended Petri plates, respectively. Each treatment was replicated five times, and the experiment was repeated ones.

2.4. ED_{50}, MIC and MFC values of the fungicides

Probit analysis was used to calculate the doses of five fungicides causing 50% reduction (ED_{50}) in the mycelial growth of *A. hartigii* (IBM SPSS Statistic Program, New York, USA). The minimum concentration [minimum inhibition concentration (MIC)] that completely inhibited the mycelial growth was also determined in parallel experiments. Toxic effects of the fungicides on the fungus were determined according to Thompson (1989) and Tripathi et al. (2004). Mycelial discs taken from ameliorated Petri dishes that exhibited no fungal growth were used to re-inoculate unameliorated dishes containing PDA, which were observed for 9 days at 25°C for revival of the fungal growth. The minimum fungicide concentration required to completely and irreversibly inhibit fungal growth after transference to unameliorated PDA medium was referred to as the minimum fungicidal concentration (MFC).

2.5. Statistical analysis

The results of this study were separately subjected to analysis of variance (One-Way ANOVA) using the IBM SPSS Statistics Program, and significant differences between the means were determined by using Tukey's HSD test ($P<0.05$).

3. Results

In the present study, antifungal effects of boscalid+kresoxim methyl, fluopyram+tebuconazole, penconazole, sulphur and tetraconazole against *A. hartigii* were tested under laboratory conditions. The results showed that all doses of the five fungicides significantly inhibited the mycelial growth of *A. hartigii* compared with the control ($P<0.05$) (Table 2). Especially, the mycelial growth of the fungus was completely inhibited even at the lowest dose of fluopyram+tebuconazole, penconazole and tetraconazole. There was no significant difference between the inhibitory effects of all doses of fluopyram+tebuconazole, penconazole and tetraconazole ($P<0.05$). On the other hand, boscalid+kresoxim methyl reduced the mycelial growth of the fungus at the rates of 29.56-100% depending on the increasing fungicide doses in comparison with the control treatment. There was significant differences among five doses of boscalid+kresoxim methyl ($P<0.05$). In addition, sulphur inhibited the mycelial growth of *A. hartigii* at rates of 78.57-100% depending on increasing doses, and there was statistically difference among five doses of the fungicide ($P<0.05$) (Table 2).

The ED_{50}, MIC and MFC values of the fungicides on *A. hartigii* were determined as 0.151, 1.2, >1.2 for boscalid+kresoxim methyl; <0.0625, <0.0625, <0.0625 for fluopyram+tebuconazole; <0.0875, <0.0875, <0.0875 for penconazole; 0.27, 16.0, 16.0 for sulphur; and <0.125, <0.125, <0.125 for tetraconazole, respectively (Table 3).

The results showed that the ED_{50}, MIC and MIC values of the fungicides were varied against the fungus. For example; the ED_{50} values of

fluopyram+tebuconazole, penconazole, sulphur and tetraconazole were found to be lower even than the 0.25x which is the lowest dose, but this value of boscalid+kresoxim methyl was found as higher than the lowest dose. On the other hand, the MFC values of fluopyram+tebuconazole, penconazole and tetraconazole, which showed fungitoxic activity against the fungus, were determined to be lower than the 0.25x dose. However, the MFC values of boscalid+kresoxim methyl and sulphur on *A. hartigii* were found as >4.0x and 4.0x, which is the highest dose, respectively (Table 3).

Table 2. Antifungal effects of the fungicides on the mycelial growth of *Ambrosiella hartigii*

Fungicides	Doses (g/mL L^{-1})	Inhibition of mycelial growth (%)
Boscalid+kresoxim methyl	0.075	29.56[a]±0.44[b]h[c]
	0.15	34.44±0.86 g
	0.3	81.78±0.27 e
	0.6	95.53±0.44 b
	1.2	100.00±0.00 a
Fluopyram+tebuconazole	0.0625	100.00±0.00 a
	0.125	100.00±0.00 a
	0.25	100.00±0.00 a
	0.5	100.00±0.00 a
	1.0	100.00±0.00 a
Penconazole	0.0875	100.00±0.00 a
	0.175	100.00±0.00 a
	0.35	100.00±0.00 a
	0.7	100.00±0.00 a
	1.4	100.00±0.00 a
Sulphur	1.0	78.57±0.43 f
	2.0	83.93±0.47 d
	4.0	92.17±0.36 c
	8.0	95.53±0.12 b
	16.0	100.00±0.00 a
Tetraconazole	0.125	100.00±0.00 a
	0.25	100.00±0.00 a
	0.5	100.00±0.00 a
	1.0	100.00±0.00 a
	2.0	100.00±0.00 a
Control	0	0.00±0.00 i

[a]Values represent the mean of five replications of fungicides doses used for *A. hartigii*
[b]Mean values followed by standard error of the mean
[c]Means followed by the same letter are not significant different according to the Tukey's HSD ($P<0.05$)

Table 3. The ED_{50}, MIC and MFC values of the fungicides inhibiting mycelial growth of *Ambrosiella hartigii*

	Fungicides				
	Boscalid+ kresoxim methyl	Fluopyram+ tebuconazole	Penconazole	Sulphur	Tetraconazole
ED_{50}[a]	0.151	<0.0625	<0.0875	0.27	<0.125
MIC[b]	1.2	<0.0625	<0.0875	16.0	<0.125
MFC[c]	>1.2	<0.0625	<0.0875	16.0	<0.125

[a]The concentration that caused 50% reduction
[b]Minimum inhibition concentration
[c]Minimum fungicidal concentration

4. Discussion

Ambrosia beetles are currently uncontrollable threat to forest ecosystems and fruit industries throughout the world (Hulcr and Dunn, 2011). The control of these beetles is very limited because the majority of their life is spent under the bark of trees, where they are physically protected from sprayed insecticides (Reding et al., 2010). Therefore, there is a need to find new alternative control methods against ambrosia beetles. The management of symbiotic fungi which are solely food source of ambrosia beetles could provide an important alternative control approach for the beetles. But, studies on symbiotic fungi have been ignored so far. Some studies have indicated that females of ambrosia beetles do not begin ovipositing until their symbiotic fungus is growing within their tunnels (French and Roeper, 1972; Weber and McPherson, 1983; Weber and McPherson, 1984; Ranger et al., 2016). This feature, which is seen in ambrosia beetles, is significantly advantage for the development of alternative control methods. If the development of symbiotic fungi in the galleries can be prevented by chemical fungicides, an effective control against ambrosia beetles may be achieved.

The current study indicated that the five fungicides (boscalid+kresoxim methyl, fluopyram+tebuconazole, penconazole, sulphur and tetraconazole) were effective on the mycelial growth of *A. hartigii* associated with *A. dispar* at *in vitro*. Especially, fluopyram+tebuconazole, penconazole and tetraconazole even at the lowest dose showed the fungitoxic effect on the mycelial growth of the fungus. Moreover, boscalid+kresoxim methyl and sulphur considerably reduced the mycelial growth depending on increased doses. These findings are in line with those of various previous studies on antifungal activity of fungicides against the mycelial growth of symbiotic fungi associated with ambrosia beetles (Joseph et al., 2002; Mayfield et al., 2008; Kagezi et al., 2015; Erper et al., 2018). In a study, Mayfield et al. (2008) determined that propiconazole fungicide used at 0.01 ppm inhibited significantly mycelial growth of symbiotic fungus *Raffaelea* sp. associated with *X. glabratus*. Also, they showed that the MIC and MFC values of propiconazole determined were 0.1 ppm and 1 ppm, and the same values of thiabendazole were found as <10 ppm and <50 ppm, respectively. In another study, Kagezi et al. (2015) tested antifungal effects of four doses (1.5x, 1.25x, 1.0x and 0.5x) of chlorothalonil, tebuconazole and dimethomorph+mancozeb on symbiotic fungus associated with *Xylosandrus compactus* Eichhoff (Scolytinae) at *in vitro*. In this study, it was stated that all doses of tebucozanole inhibited the mycelial growth of the fungus by 100%, followed by all doses of chlorothalonil and dimethomorph+mancozeb by under 40%. Similarly, in our previous study, it was determined that the lowest dose (0.25x) of captan, cyprodinil+fludioxonil, imazalil and prochloraz completely inhibited the mycelial growth of two isolates of *A. hartigii* isolated from *A. dispar* and *X. germanus* (Erper et al., 2018). It was also demonstrated that azoxystrobin, iprodione and thiram inhibited the mycelial growth of the symbiotic fungus at the rates of 50-85%, 65-100% and 75-100% depending on increased doses, respectively.

However, in a few studies, some researchers tested the effects of fungicides to symbiotic fungi associated with ambrosia beetles in field conditions (Mayfield et al., 2008; Fettig et al., 2014; Ranger et al., 2016; Jones et al., 2017). Mayfield et al. (2008) found that propiconazole, which was enjected into redbay (Persea borbonia) trees, inhibited the growth of *Raffaelea* sp. in the sapwood during approximately 30 weeks. Similarly, the development of symbiotic fungus in the trees treated with azoxystrobin and potassium phosphite was reduced and so eggs were not released (Ranger et al., 2016). On the other hand, some previous studies have stated that pesticide combinations (insecticides and fungicides) are usually more effective than only fungicide or insecticide applications for controlling ambrosia beetles and symbiotic fungi in the field (Fettig et al., 2014; Ranger et al., 2016; Jones et al., 2017). Fettig et al. (2014) determined that pesticide combinations [emamectin benzoate (insecticide) and propiconazole (fungicide)] injected with arborjet was more effective than single pesticide treatments for protecting pine trees from bark beetle, *Dendroctonus ponderosae* Hopkins (Scolytinae). Jones et al. (2017) found that the combination of a systemic insecticide (emamectin benzoate), a contact insecticide (bifenthrin), and a fungicide (metconazole) provided the best control against ambrosia beetle, *Euwallacea* sp. (Scolytinae) and its symbiotic fungus.

5. Conclusion

In conclusion, boscalid+kresoxim methyl, fluopyram+tebuconazole, penconazole, sulphur and tetraconazole, which are currently used against hazelnut powdery mildew caused by *E. corylacearum* in Turkey, significantly inhibited the mycelial growth of *A. hartigii* symbiotically associated with *A. dispar* under laboratory conditions. But, the efficacy of the fungicides alone or combined with insecticides against *A. hartigii* and *A. dipsar* should be determined in field conditions. Thus, successful fungicides may be used in controlling of the symbiotic fungus and its associated ambrosia beetle.

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