



## Evaluation of some fungicides against symbiotic fungus *Ambrosiella hartigii* associated with *Anisandrus dispar* Fabricius and *Xylosandrus germanus* Blandford (Coleoptera: Curculionidae: Scolytinae)

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### ABSTRACT

In this study, the efficacy of seven fungicides (azoxystrobin, captan, iprodione, imazalil, prochloraz, thiram and cyprodinil+fludioxonil) on two isolates (TR-202 and TR-205) of *Ambrosiella hartigii* was evaluated at *in vitro*. The fungicides at five concentrations (0.25x, 0.5x, 1.0x, 2.0x and 4.0x; where x is the field rate recommended by the manufacturer) were used in this study. Results showed that all doses of the fungicides significantly inhibited the mycelial growth of these isolates compared to the control ( $P < 0.05$ ). Captan, imazalil, prochloraz and cyprodinil+fludioxonil were found to completely inhibit the mycelial growth of *A. hartigii* isolates even at the lowest dose while thiram, iprodione and azoxystrobin depending on increased doses reduced the mycelial growth of these isolates at rates between 75–100%, 65–100% and 52–85%, respectively. The doses that caused 50% reduction ( $ED_{50}$ ), minimum inhibition concentration (MIC), and minimum fungicidal concentration (MFC) values indicated that imazalil, prochloraz and cyprodinil+fludioxonil were more effective against the fungus compared to other fungicides. There were no differences between two isolates with regard to  $ED_{50}$ , MIC and MFC values of the fungicides.

### 1. Introduction

Turkey is the world's biggest hazelnut producer, supplying about 80% of the total global production. Ambrosia beetles (Coleoptera: Curculionidae: Scolytinae), *Anisandrus dispar* Fabricius and *Xylosandrus germanus* Blandford are very widespread pests on hazelnut trees (Tuncer et al. 2017). If effected by the pests, the most of the hazelnut trees finally die, especially in hazelnut orchards at Black Sea region coastline in Turkey in which ground water level is high (Saruhan and Tuncer 2001; Ak 2016). *A. dispar* has been the most common species in the hazelnut orchards in Turkey for a long time as well as Italy (Ak et al. 2005; Bucini et al. 2005). Another species, *X. germanus* was recently detected in hazelnut orchards of Turkey and became one of major pests (Knižek 2011; Ak 2016). This beetle is also among the most significant ambrosia beetles in USA nurseries and orchards (Oliver and Mannion 2001). Afterwards, both *A. dispar* and

*X. germanus* were determined as pests of kiwi trees in Black Sea region of Turkey (Ak et al. 2011).

The control of ambrosia beetles is very difficult as they spend most of their lives in the sapwood of trees (Saruhan and Akyol 2012). Current control methods against these beetles in hazelnut orchards include the use of some insecticides to emerged adults, cultural methods (removing infested branches or trees) and mass trapping of emerged adults through red winged ethanol baited sticky traps. However the methods are not enough to eliminate spreading and damage of the pests. Therefore, alternative control methods should be included in the control strategies.

Ambrosia beetles including about 3400 species are insects that make tunnels through sapwood (xylem) of trees and cultivate symbiotic fungi in the tunnels for their food. Female adults of these beetles bore into the sapwood and inoculate the tunnels with symbiotic fungi that are carried within them by specialized pouches called as mycangia (Six 2003). Except in some species (xylomycetophagy; larvae also feed fungus-infested wood), both larvae and adults generally

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feed only on the fungi growing in the tunnels (Biedermann 2007). The symbiotic fungi provide very important organic molecules necessary for adults and larvae development (Norris 1979). The majority of these symbiotic fungi are in the genera *Ambrosiella* or *Raffaelea* (Harrington et al. 2010). Most species belonging to *Ambrosiella* have been associated with species in three tribes of Scolytinae; Xyleborini, Corthylini and Xyloterini (Harrington et al. 2010). Among them, *Ambrosiella hartigii* Batra has been reported symbiotically associated with *A. dispar* and *X. germanus* and carried in the mycangia located between the pronotum and mesothorax of female adults (Batra 1967; Bucini et al. 2005; Yang et al. 2008). The symbiotic fungi (*Ambrosiella*, *Raffaelea* etc.) generally are known as harmless to trees. But, they could disrupt the flow of water and nutrients because of their growth in the sapwood of host trees (Castrillo et al. 2011). Moreover, some species like *Raffaelea lauricola* T.C. Harr., Aghayeva, & Fraedrich associated with *Xyleborus glabratus* Eichh are very pathogenic to trees (Fraedrich et al. 2008; Harrington et al. 2008). On the other hand, the symbiotic fungi are indirectly harmful due to their role as source of food for ambrosia beetles. Many studies have indicated that females of the beetles do not begin ovipositing until their symbiotic fungus is growing within the galleries (French and Roeper 1972; Weber and McPherson 1983; Weber and McPherson 1984; Ranger et al. 2016).

Fungal pathogens are generally controlled by using of fungicides. There are very few studies on antifungal activity of fungicides against symbiotic fungi associated with ambrosia beetles in the world (Joseph et al. 2002; Mayfield et al. 2008; Kagezi et al. 2015). Mayfield et al. (2008) found that propiconazole inhibited mycelial growth of *Raffaelea* sp. associated with *X. glabratus* by 84% at 0.01 ppm. Similarly, Kagezi et al. (2015) demonstrated that all doses of tebucozanole inhibited the mycelial growth by 100%, followed all doses of chlorothalonil and dimethomorph + mancozeb by under 40% as a result of studying the efficacy of four doses of chlorothalonil, tebuconazole and dimethomorph + mancozeb against simbiotic fungus associated with black coffee twig borer, *Xylosandrus compactus* Eichhoff (Scolytinae) at *in vitro*.

Presently, sensitivity of the symbiotic fungus, *A. hartigii* associated with *A. dispar* and *X. germanus* to fungicides has not yet been determined. Thus, the aim of the present study was to evaluate the *in vitro* efficacy of five different doses of seven fungicides against two isolates belonging to *A. hartigii* isolated from mycangia of females of *A. dispar* and *X. germanus*.

## 2. Materials and Methods

### 2.1 Fungal isolates

Fungal isolates used in this study were isolated from females of *A. dispar* and *X. germanus*, collected from hazelnut orchards in Samsun province in Black Sea region of Turkey. Healthy females of *A. dispar* and *X. germanus* were cleaned with 1 mL PBS (phosphate buffered saline) + 0.1% Tween 80 (15 sec.) and 40% ethanol (5 sec.). Then, these beetles were dissected to obtain the mycangia below the mesonotum with forceps and scalpel under a Leica EZ4 stereomicroscope at 40-70X magnification. Each mycangium was individually placed in a 1.5 mL sterile microcentrifuge tube containing 0.5 mL PBS, and homogenized using a micropestle (Six et al. 2009). Aliquots of 100  $\mu$ L were taken from each tube and then spread evenly onto Potato Dextrose Agar (PDA: Merck Ltd., Darmstadt, Germany) in Petri dishes (6 cm dia.) amended with streptomycin (0.05 g L<sup>-1</sup>) (Six et al. 2009). The cultures incubated for 3-7 days at 25  $\pm$ 1°C in the dark. The isolates from mycangia of *A. dispar* and *X. germanus* were identified as *A. hartigii* according to the macroscopically and microscopically characteristics (Batra 1967). Two isolates of *A. hartigii* (TR-202 from *A. dispar* and TR-205 from *X. germanus*) were used in the study.

### 2.2 Fungicides

The fungicides tested for their antifungal activities were listed in Table 1. Since there is no licensed fungicide against *A. hartigii*, the fungicides entering different chemical groups have been selected. These fungicides were used at five doses (0.25x, 0.5x, 1.0x, 2.0x and 4.0x; where x is the field rate recommended by the manufacturer).

### 2.3. Antifungal effects of fungicides on mycelial growth of the isolates

A modified method of Mamza et al. (2008) was used to assay the effects of the fungicides on mycelial growth of two isolates belonging to *A. hartigii*. All doses of seven fungicides were added to autoclaved and cooled PDA medium at 50°C. For each dose, a 15 mL aliquot of ameliorated PDA medium was aseptically dispensed into a Petri dish (9-cm-dia.), with an unamended PDA dish used as a control. A mycelial disc (5 mm dia.) from 5-day-old culture was placed in the center of each dish. The Petri dishes were then sealed with parafilm, and 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 colony diameters were measured at two perpendicular points (4 days after inoculation). Mycelial growth values were recorded, and converted into the inhibition percentages of mycelial growth inhibition (MGI) in relation to the controls using the formula  $MGI (\%) = [(dc - dt)/dc] \times 100$ , where dc represents mycelial growth diameter of the control and dt represents mycelial growth diameter of the amended Petri dish. Treatment was replicated 5 times for each dose of the fungicides and repeated ones.

Table 1  
Fungicides used in the study.

Chemical group	Active ingredient	Trade name	Manufacturer	Formulation <sup>a</sup>	Registered doses in Turkey
Strobilurin	Azoxystrobin	Caira	Hektaş	250 g L <sup>-1</sup> SC	0.75 mL L <sup>-1</sup> (grape)
Cyclic imides	Captan	Captan <sup>h</sup>	Hektaş	500 g kg <sup>-1</sup> WP	1.50 g L <sup>-1</sup> (apple)
Dicarboximide	Iprodione	Herodion 50	Hektaş	500 g kg <sup>-1</sup> WP	0.75 g L <sup>-1</sup> (grape)
DMI <sup>b</sup> (triazole)	Imazalil	Bestnate 50	Agrobrest	500 g L <sup>-1</sup> EC	0.30 mL L <sup>-1</sup> (tomato)
DMI (imidazole)	Prochloraz	Soufrex	Agrobrest	450 g L <sup>-1</sup> EC	1.00 mL L <sup>-1</sup> (wheat)
Dimethylthiocarbamate	Thiram	Pomarsol Forte	Bayer	800 g kg <sup>-1</sup> WP	1.50 g L <sup>-1</sup> (apple)
Mixture	Cyprodinil + Fludioxonil	Switch 62.5	Syngenta	375 g kg <sup>-1</sup> WG+ 250 g kg <sup>-1</sup> WG	0.60 g L <sup>-1</sup> (tomato)

<sup>a</sup> SC, suspension concentrate; WP, wettable powder; EC, emulsifiable concentrate; WG, water dispersible granule.

<sup>b</sup>Demethylation inhibitors.

#### 2.4. ED<sub>50</sub>, MIC and MFC values of the fungicides

Probit analysis was used to calculate doses of the fungicides causing 50% reduction (ED<sub>50</sub>) in mycelial growth of *A. hartigii* (IBM SPSS Statistic Program, New York, USA). Mycelial growth was determined in PDA amended with fungi at 0.25x, 0.5x, 1.0x, 2.0x and 4.0x doses as described above. The minimum inhibition concentration (MIC) value that completely inhibited the mycelial growth was also determined in parallel experiments. Toxic effects of fungicides were determined according to Thompson (1989) and Tripathi et al. (2004). PDA discs taken from ameliorated Petri dishes that exhibited no fungal growth were used to re-inoculate unameliorated PDA dishes, which were monitored for 9 days at 25°C revival of growth. The dose that completely inhibits the fungus and irreversibly when transferred to fresh medium was stated as minimum fungicidal concentration (MFC).

#### 2.5. Statistical analysis

Results obtained from the present 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 this study, five doses of azoxystrobin, captan, iprodione, imazalil, prochloraz, thiram and cyprodinil+fludioxonil were evaluated for their antifungal activity against TR-202 and TR-205 isolates of *A. hartigii*. All doses of these fungicides significantly inhibited the mycelial growth of the two isolates compared to the control (P<0.05) (Table 2). Especially, captan, imazalil, prochloraz and cyprodinil+fludioxonil completely inhibited the

mycelial growth of both isolates even at the lowest dose. There was no significant difference among the inhibitory effects of all doses of the four fungicides (P>0.05). Thiram reduced mycelial growth of the isolates at rates between 76–100% depending on the increasing fungicide doses, and there was statistically different among five doses of the fungicide on *A. hartigii* isolates (P<0.05). On the other hand, iprodione inhibited mycelial growth by 100% at the highest dose and over 60% at other doses on these isolates. Similarly, there was significant difference among all doses of iprodione (P<0.05). Azoxystrobin was found to be the lowest efficiency in inhibiting mycelial growth at rates between 52–85%. Additionally, anti-fungal activity of different doses of azoxystrobin were significantly different (P<0.05). Nevertheless, the anti-fungal effects of azoxystrobin, iprodione and thiram against two isolates of *A. hartigii* significantly increased due to the increased dose of the fungicides.

The ED<sub>50</sub>, MIC and MFC values of the seven fungicides for inhibiting mycelial growth of *A. hartigii* isolates were determined as <0.1875, >3.0, >3.0 for azoxystrobin; <0.375, 0.375, >6.0 for captan; <0.1875, 3, >3 for iprodione; <0.075, <0.075, <0.075 for imazalil; <0.25, <0.25, <0.25 for prochloraz; <0.375, 6, >6.0 for thiram; and <0.15, <0.15, 2.4 for cyprodinil+fludioxonil, respectively (Table 3). The ED<sub>50</sub> values of all fungicides were found to be low even than the 0.25x which is the lowest dose. The MIC and MFC values of three fungicides (azoxystrobin, imazalil and prochloraz) for both isolates were showed as same doses. The MFC values of imazalil and prochloraz for the each isolate were also determined to be lower than the 0.25x. Moreover, there were no differences between the two isolates with regard to ED<sub>50</sub>, MIC and MFC values of the fungicides.

Table 2

Effects of fungicides on the mycelial growth of isolates of *Ambrosiella hartigii*.

Fungicides	Doses (g/mL L <sup>-1</sup> )	Inhibition of mycelial growth (%)	
		TR-202	TR-205
Azoxystrobin	0.1875	56.94±0.58 <sup>b</sup> i <sup>c</sup>	52.20±0.60 i
	0.375	63.41±1.26 h	62.04±1.35 h
	0.75	69.38±0.53 g	69.69±1.37 g
	1.5	75.29±1.41 f	76.81±0.99 f
	3.0	85.87±1.15 c	87.95±0.98 cd
Captan	0.375	100.00±0.00 a	100.00±0.00 a
	0.75	100.00±0.00 a	100.00±0.00 a
	1.5	100.00±0.00 a	100.00±0.00 a
	3.0	100.00±0.00 a	100.00±0.00 a
	6.0	100.00±0.00 a	100.00±0.00 a
Iprodione	0.1875	64.90±1.07 h	71.14±1.51 g
	0.375	74.21±1.42 f	78.03±1.08 ef
	0.75	81.66±1.66 de	84.99±0.96 d
	1.5	88.09±0.92 bc	89.92±1.07 c
	3.0	100.00±0.00 a	100.00±0.00 a
Imazalil	0.075	100.00±0.00 a	100.00±0.00 a
	0.15	100.00±0.00 a	100.00±0.00 a
	0.3	100.00±0.00 a	100.00±0.00 a
	0.6	100.00±0.00 a	100.00±0.00 a
	1.2	100.00±0.00 a	100.00±0.00 a
Prochloraz	0.25	100.00±0.00 a	100.00±0.00 a
	0.5	100.00±0.00 a	100.00±0.00 a
	1.0	100.00±0.00 a	100.00±0.00 a
	2.0	100.00±0.00 a	100.00±0.00 a
	4.0	100.00±0.00 a	100.00±0.00 a
Thiram	0.375	75.35±1.17 f	76.32±0.77 f
	0.75	79.46±1.04 e	80.59±0.81 e
	1.5	84.79±1.03 cd	87.67±0.62 cd
	3.0	91.30±0.89 b	93.65±1.61 b
	6.0	100.00±0.00 a	100.00±0.00 a
Cyprodinil+Fludioxonil	0.15	100.00±0.00 a	100.00±0.00 a
	0.3	100.00±0.00 a	100.00±0.00 a
	0.6	100.00±0.00 a	100.00±0.00 a
	1.2	100.00±0.00 a	100.00±0.00 a
	2.4	100.00±0.00 a	100.00±0.00 a
Control	0	0.00±0.00 j	0.00±0.00 j

<sup>a</sup>Values represent the mean of five replications of fungicides doses used for each isolates<sup>b</sup>Mean values followed by standard error of the mean<sup>c</sup>Means followed by the same letter are not significant different according to the Tukey's HSD (P < 0.05)

Table 3

ED<sub>50</sub>, MIC and MFC values of fungicides inhibiting mycelial growth of isolates of *Ambrosiella hartigii*.

Fungicides	TR-202			TR-205		
	ED <sub>50</sub> <sup>a</sup>	MIC <sup>b</sup>	MFC <sup>c</sup>	ED <sub>50</sub>	MIC	MFC
Azoxystrobin	<0.1875 <sup>d</sup>	>3.0	>3.0	<0.1875	>3.0	>3.0
Captan	<0.375	0.375	>6.0	<0.375	0.375	>6.0
Iprodione	<0.1875	3	>3	<0.1875	3	>3
Imazalil	<0.075	<0.075	<0.075	<0.075	<0.075	<0.075
Prochloraz	<0.25	<0.25	<0.25	<0.25	<0.25	<0.25
Thiram	<0.375	6	>6.0	<0.375	6	>6.0
Cyprodinil+Fludioxonil	<0.15	<0.15	2.4	<0.15	<0.15	2.4

<sup>a</sup>The concentration that caused 50% reduction.<sup>b</sup>Minimum inhibition concentration.<sup>c</sup>Minimum fungicidal concentration.<sup>d</sup>The ED<sub>50</sub> value of all fungicides was found to be low even than the lowest dose (0.25x).

#### 4. Discussion

Invasive fungus-farming ambrosia beetles (*A. dispar*, *X. germanus* etc.) are very important pests for many fruit and forest trees in the World (Hulcr and Dunn 2011). In addition, since these beetles spend most of their lives in the sapwood of trees, this is a bottleneck to control of them. Although a lot of studies have been performed on ambrosia beetles, there is an ignored topic about the control of the symbiotic fungi. One of them is *A. hartigii*, solely food source of the ambrosia beetles.

To our knowledge, this is the first report on the anti-fungal activity of the fungicides against symbiotic fungus, *A. hartigii* associated with *A. dispar* and *X. germanus* *in vitro*. This study demonstrated that azoxystrobin, captan, iprodione, imazalil, prochloraz, thiram and cyprodinil+fludioxonil are significantly effective on the mycelial growth of *A. hartigii*. Among these, captan, imazalil, prochloraz and cyprodinil+fludioxonil completely inhibited the mycelial growth of the isolates even at the lowest dose. Moreover, azoxystrobin, iprodione and thiram considerably inhibited the mycelial growth of the fungal isolates depending on increased doses. These findings are in line with those of several previous studies on antifungal activity of fungicides against the mycelial growth of symbiotic fungi associated with ambrosia beetles in the world (Joseph et al. 2002; Mayfield et al. 2008; Kagezi et al. 2015). Propiconazole was found to inhibit mycelial growth of *Raffaelea* sp. associated with *X. glabratus* by 84% at 0.01 ppm. Also the MIC and MFC values of this fungicide were determined as 0.1 ppm and 1 ppm, respectively (Mayfield et al. 2008). Additionally, they demonstrated that the MIC and MFC values of thiabendazole on *Raffaelea* sp. were found as <10 ppm and <50 ppm, respectively. In another study, different doses (1.5x, 1.25x, 1.0x and 0.5x) of chlorothalonil, tebuconazole and dimethomorph + mancozeb against symbiotic fungus associated with *X. compactus* Eichhoff (Scolytinae) were evaluated at *in vitro*. As a result, they showed that all doses of tebucozanole inhibited the mycelial growth of this fungus by 100%, followed all doses of chlorothalonil and dimethomorph + mancozeb by under 40% (Kagezi et al. 2015).

There are some studies on effects of fungicides against 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). Some researchers determined that propiconazole injected into redbay (*Persea borbonia*) trees was prevented the growth of *Raffaelea* sp. in the sapwood during about 30 weeks (Mayfield et al. 2008). Ranger et al. (2016) also observed that in azoxystrobin and potassium phosphite treated trees, the development of symbiotic fungi in the galleries of ambrosia beetles was reduced and thus the eggs were not released. Moreover, many researchers indicated that pesticide combinations (insecticides and

fungicides) were generally more effective than single pesticide treatments for controlling wood boring beetles and symbiotic fungi in the field conditions (Fettig et al. 2014; Ranger et al. 2016; Jones et al. 2017). Fettig et al. (2014) found that emamectin benzoate in combination with propiconazole injected by arborjet was more effective than emamectin benzoate alone for protecting pine trees from *Dendroctonus ponderosae* Hopkins (Scolytinae). Similarly, Jones et al. (2017) showed 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. But, there have not been any fungicide evaluated against symbiotic fungus *A. hartigii* associated with *A. dispar* and *X. germanus*.

In previous studies, some researchers have reported that azoxystrobin, captan, iprodione, imazalil, prochloraz, thiram and cyprodinil+fludioxonil significantly inhibited the mycelial growth of various plant pathogenic fungi (Rego et al. 2006; Luque et al. 2008; Gramaje et al. 2009; Kumari et al. 2012; Kaş and Özgönen Özkaya 2017). Rego et al (2006) indicated that prochloraz was the most effective fungicide (EC<sub>50</sub> values ≤0.09 mg L<sup>-1</sup>), followed by cyprodinil + fludioxonil (EC<sub>50</sub> values ≤0.75 mg L<sup>-1</sup>) on mycelial growth of *Cylindrocarpon destructans* isolates (Cy1, Cy21, Cy32 and Cy68). In another study, Gramaje et al. (2009) found that azoxystrobin, imazalil and prochloraz were inhibited significantly mycelial growth of *Phaeomoniella chlamydospora* isolates (<0.1 mg L<sup>-1</sup>). Finally, Kaş and Özgönen Özkaya (2017) determined that 200 ppm and 250 ppm of iprodione completely inhibited the growth of *Alternaria mali*.

Consequently, the present study showed that the fungicides like captan, imazalil, prochloraz and cyprodinil+fludioxonil significantly inhibited the mycelial growth of *A. hartigii* associated with *A. dispar* and *X. germanus*. However, the effectiveness of these fungicides alone or combined with insecticides against *A. hartigii* should be determined in field conditions. Thus, successful fungicides may be used in controlling of the symbiotic fungus and its associated beetles.

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