Trends in Horticulture 2024, 7(2), 10273.
https://doi.org/10.24294/th10273

Article

Comparison of chemical and biological control methods for Armillaria root rot in olive

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CITATION

Naseri B, Jafary H, Kakhki SHN. Comparison of chemical and biological control methods for Armillaria root rot in olive. Trends in Horticulture. 2024; 7(2): 10273. https://doi.org/10.24294/th10273

ARTICLE INFO

Received: 12 November 2024
Accepted: 13 December 2024
Available online: 27 December 2024

Abstract: Olive production is threatened by a fungal pathogen, *Armillaria mellea* (Vahl. Fr.) P. Kumm.,causing decline in trees worldwide. Effectiveness of once and twice applications of fungicides hexaconazole, propicoconazole and thiophanate-methyl and application of biological agent (*Trichoderma harzianum*) to control *A. mellea* was studied at orchard scale during four years. *T. harzianum* inhibited the pathogen growth on agar media. This antagonistic fungus provided a 25% control efficiency of *A. mellea* on olive trees younger than 15 years which was the same as control efficiency of once application of hexaconazole. Control efficiencies as perfect as 100% were determined on younger (<15 years old) diseased olive trees treated with once applications of thiophanate-methyl and hexaconazole, and twice applications of thiophanate-methyl. Moreover, olive tree age was significantly effective on fungicidal control efficiency. Hence, this four-year research advanced our understanding of sustainable olive production in study region and other geographical areas with similar agro-ecological characteristics.

Keywords: biocontrol; dieback; honey fungus; orchard management; root rot; sustainability

1. Introduction

Olive (*Olea europaea* L.) cultivation is threatened by a number of fungal pathogens in particular *Armillaria mellea* (Vahl. Fr.) P. Kumm. causing decline (or dieback) in trees [1,2]. This disease has been known as the most predominant and destructive disease in olive orchards in Zanjan province, Iran [3]. Drip or sprinkler irrigation systems can restrict the disease spread as reported by Adaskaveg et al. [4] and Eguchi et al. [5]. Because, the collar is the most susceptible part of olive tree to *A. mellea*, which infects collar tissues depending on the rhizosphere wetness and temperature [6]. In Japan, inserting warm water (35 °C) into the soil of rhizosphere for three days via drip irrigation system eliminated *Rozellinianecatrix*, which similarly causes collar and root rots in trees [5]. Soil disinfestations using biocide fungicides such as methyl bromide can restrict these fungal pathogens, providing that this strategy is done before planting the orchard [6]. Removing the soil surrounding the collar reduced significantly *A. mellea* in orange and grape trees [7,8]. Otieno et al. [9] found inhibitory effects of applying *Trichoderma harzianum* formulations on wheat straw in the rhizosphere soil around the collar infected by *A. mellea*. In Iran, similar findings on this pathosystem have been documented [10]. Previous studies describe mechanisms including mycoparasitism, antibiosis, competition or

modification of the rhizospheric microbiota as being responsible for effective biocontrol of *A. mellea* [11,12]. Further research into unidentified mechanisms and plant characteristics will advance our understanding of this biocontrol agent to be used for sustainable olive production at orchard scale.

Comparisons of fungicides to limit *A. mellea* in grape orchard showed that injecting propiconazole into vessel tissues of trees reduced the decline disease [4,13]. Elsewhere, the fungicides cyproconazole, hexaconazole, propicoconazole and tetraconazole reduced the mycelia growth of *A. mellea*on agar medium under laboratory conditions [13]. Furthermore, among these four influential fungicides, cyproconazole applied once (at flowering-fruiting stages) and twice (at branch growth and flowering-fruiting stages) via injections of 5 L into soil surrounding the grape tree by 50 cm radial distance from the collar at a 40 cm depth eliminated the decline disease. However, the efficiency of this influential disease management method for grape trees should also be evaluated for olive *A.mellea* decline. Moreover, it is highly desired to compare the efficiency of fungicidal and biological methods in order to control this destructive disease of olive trees. Therefore, the current research aimed to compare the effectiveness of once and twice applications of hexaconazole, propicoconazole and thiophanate-methyl with the application of biological agent, *T. harzianum*, to control *A.mellea* decline at orchard scale during four years. Furthermore, associations of the age of olive trees with the antagonistic and fungicidal treatments were also explored in this research.

2. Materials and methods

2.1. The pathogen *Armillaria mellea*

Jafari et al. [3] isolated the pathogen from olive trees showing shoot decline and chlorotic leaves, and then, identified as *A. mellea* according to the standard key [14]. For the pathogen identification, samples of mycelium and/or rhizomorphs were collected from some of superficial roots and collar of symptomatic trees by carefully detaching the bark [8]. The presence of *A. mellea*was confirmed in all samples by culturing fungal mycelia samples on potato dextrose agar (PDA) at room temperature, approx. 24 ℃ [15,16] which were consistent with Jafari et al. [17] findings. Furthermore, identification of *A. mellea* isolates were also confirmed by characterizing the ITS region and detection of sequence [17].

2.2. The antagonist *Trichoderma harzianum*

Three isolates of *T. harzianum* were obtained from seven soil samples (approx. 3 kg each sample) collected from commercial olive orchards in Tarom, northern part of Zanjan. For fungal isolation, soil samples were cultured on PDA containing chloramphenicol, streptomycin and rosebengal [16]. The culture was grown at room temperature (approx. 24 ℃) and identified as *T. harzianum* according to the standard identification keys [18,19]. Dual cultures of *T. harzianum* and *A. mellea* were examined to verify the antagonistic activity of this fungal antagonist against the pathogen.

To prepare inoculum of the antagonist [9], a mixture of three isolates of *T.*

harzianum were cultured in rice bran as follows: a number of 1 L conical flasks were each filled with 500 g dry rice bran, moistened with 250 mL tap water and autoclaved twice on two consecutive days. 10 mL of conidial suspension prepared from seven-day old cultures of *T. harzianum* was added to autoclaved rice bran in each flask, incubated at room temperature and shaken every day for the uniform colonization. Flasks were shaken by hand several times a day to homogenize the fungal growth on the whole rice bran. After one week, the number of propagules produced on the rice bran was measured using a haemocytometer. Then, the number of propagules was adjusted to 5×10^4 colony-forming units (cfu) per g (wet weight) by adding an equal amount of fresh rice bran autoclaved. The inocula of *T. harzianum* were used in the experiments to inoculate the soil [9]. To meet this requirement, the soil of three spots, 10 cm apart from the collar and at the depth of 20 cm, was thoroughly mixed with 10 g of the antagonist inoculum per inoculation spot. The soil inoculation with the antagonist was conducted every year.

2.3. Fungicides preparation

The fungicidal formulations (50 L per tree) were used in experiments as follows: hexaconazole 50 g/L (Anvil SC 5%, Aria company), propiconazole 100 g/L (Tilt EC 25%, Aria company), and thiophanate-methyl 200 g/L (WP 70%, Mahan company).

2.4. Experimental treatments

The following nine treatments were arranged as randomized complete blocks with 6 replicates (54 trees in total): (1) healthy tree as control 1, (2) untreated tree infected by *A. mellea* as control 2, (3) application of *T. harzianum*, (4) once application of propiconazole, (5) twice applications of propiconazole, (6) once application of thiophanate-methyl, (7) twice applications of thiophanate-methyl, (8) once application of hexaconazole, (9) twice applications of hexaconazole. Fungicides were applied either once at flowering-fruiting stage (in July, early summer) or twice at branch growth (in April, early spring) and flowering-fruiting stages every year. The 6 replicates considered per treatment were divided into the groups according to the age as follows: three trees < 15 and three trees > 15 years old.

2.5. Orchard experiments for *Armillaria mellea*control in olive

The experiment was conducted for 4 years at Tarom district, Zanjan province, Iran in a commercial olive orchard (49º 05′ E, 36º 47′ N) which was determined based on having not only healthy trees but also trees infected by *A. mellea* [20]. Five types of olive trees were detected in the experimental orchard based on their decline level, naturally infected with the pathogen. Diseased trees were selected for this study with a severity scale ranging from 25 to 75%, irrespective of any specific sampling pattern. Decline ratings were determined at harvest (in October, early to mid-autumn) every year according to the disease severity scale [13] which was modified as follows: 0 = healthy plant; 1 = tree showing no aerial symptoms of olive decline but with *A. mellea*mycelium evident on roots; 2 = tree showing < 25% aerial

symptoms; 3 = 25%-50%; 4 = 50%-75%; 5 = highly diseased tree showing 75%-100% symptoms of decline and yellowing of leaves, and mycelia under the bark of roots.

Control efficiency was calculated as follows: (mean decline severity before treatment—mean decline severity after treatment) × 100/mean decline severity before treatment. The statistical analyses, ANOVA and student's *T*-test, were performed using SAS software. Transformation of the severity data was not required due to the normal distribution of data according to the Kurtosis test.

3. Results

The ANOVA results demonstrated that the treatments of olive trees with the antagonist, *T. harzianum*, and once or twice applications of fungicides, propiconazole, thiophanate-methyl and hexaconazole, were significantly effective on Armillaria decline studied over four years under orchard conditions (**Table 1**). Over the four years of this research, the healthy trees showed the lowest decline disease development per study year from the second year onwards (**Table 2**). There was no significant difference in the decline severity ratings between the diseased control 2 and the antagonist application treatments in the four study years. Mean decline severity ratings detected for the antagonistic treatment were slightly lower in the third and fourth years. For fungicide treatments, the twice applications of the three fungicides studied often reduced the decline severity ratings more slightly than the once applications. Furthermore, the twice applications of each fungicide were the same effective on the decline disease development as the once applications (**Table 2**).

Table 1. Analysis of variance for Armillaria decline severity ratings determined on olive trees treated with antagonist (*Trichoderma harzianum*) and fungicides.

Variation sources	**df**	**Mean squares**			
		2014	**2015**	**2016**	**2017**
Replicate	5	0.0563	0.1248	0.0539	0.1914
Treatment	8	0.5856	0.3566	0.2992	0.4933
Experimental error	40	0.0249	0.0253	0.0247	0.0240
Variation coefficient (%)		8.81	9.57	10.06	10.77

Bold numbers are significant at the 1evel of 1%.
df = degree of freedom.

Table 2. Mean values of Armillaria decline severity ratings determined on olive trees treated with antagonist (*Trichoderma harzianum*) and fungicides.

Treatments	**Mean disease severity ratings**			
	2014	**2015**	**2016**	**2017**
Healthy tree (control 1)	0.00b	0.17c	0.17c	0.17c
Diseased tree (control 2)	2.00a	2.17ab	2.33a	2.83a
Antagonistic treatment	2.17a	2.17ab	2.17a	2.23a
Once applied propiconazole	3.00a	2.50a	1.83ab	1.17b
Twice applied propiconazole	2.67a	2.33a	1.50ab	1.00b

Table 2. (*Continued*).

Treatments	Mean disease severity ratings			
	2014	**2015**	**2016**	**2017**
Once applied thiophanate-methyl	2.83a	2.17ab	1.67ab	1.00b
Twice applied thiophanate-methyl	3.00a	2.00ab	1.17b	0.50bc
Once applied hexaconazole	2.67a	1.83ab	1.67ab	1.17b
Twice applied hexaconazole	2.67a	1.33b	1.17b	0.50bc

Numbers with the same alphabetical characters are significant at the 1evel of 1%.

For propiconazole, the once applications reduced significantly the mean decline severity ratings detected in the first and second years by 53%–61% compared to the fourth year (**Table 2**). The twice applications of propiconazole reduced significantly the mean decline severity detected in the first and second years by 57%–63% compared with the fourth year. For each year of this study, there was no significant difference in the mean decline severity ratings between the once and twice applications of propiconazole. Irrespective of the first year, mean decline severity ratings determined for the twice applications of propiconazole were slightly lower than the once applications (**Table 2**).

For thiophanate-methyl, the once applications reduced significantly the mean decline severity ratings detected in the first year by 65% compared to the fourth year (**Table 2**). The twice applications of thiophanate-methyl reduced significantly the mean decline severity detected in the first year by 61% and 83% compared with the third and fourth years, respectively. For each year of this study, there was no significant difference in the mean decline severity ratings between the once and twice applications of thiophanate-methyl. Mean decline severity ratings determined for the twice applications of thiophanate-methyl were slightly lower than the once applications, excepting for the first year (**Table 2**).

For the hexaconazole fungicide, the once applications decreased significantly the mean decline severity ratings determined in the first year by 56% compared to the fourth year (**Table 2**). The twice applications of hexaconazole decreased significantly the mean decline severity determined in the first year by 50%, 56% and 81% in comparison with the second, third and fourth years, respectively. For each year of this study, there was no significant difference in the mean decline severity ratings between the once and twice applications of hexaconazole. Mean decline severity ratings determined for the twice applications of hexaconazole were slightly lower than the once applications, excepting for the first year (**Table 2**).

In **Figure 1**, trend lines represented Armillaria decline severity ratings determined during four years on olive trees treated with the antagonist, *T. harzianum*, and fungicides, propiconazole, thiophanate-methyl and hexaconazole. The probability associated with the student's *T*-test for this four-year study demonstrated the significant effect of year on the decline severity ratings determined across the experimental treatments (**Table 3**). This suggested that the mean disease severity ratings detected for the nine experimental treatments varied from 2014 to 2015 (T-prob. $\leq$ 0.05) from 2014 to 2016 (T-prob. $\leq$ 0.05) and from 2014 to 2017 (T-prob. $\leq$ 0.05). For the second year onwards, the mean disease severity ratings

determined for the experimental treatments varied from 2015 to 2016 (T-prob. ≤ 0.05), from 2015 to 2017 (T-prob. ≤ 0.05) and from 2016 to 2017 (T-prob. ≤ 0.05; **Table 3**).

Table 3. Probability associated with paired student's *T*-test for four-year study of olive decline severity ratings determined across nine experimental treatments.

Variation sources	Study years			Tree age	
	2014	**2015**	**2016**	**< 15 years**	**> 15 years**
2015	0.013			0.020	
2016	0.007	0.015			
2017	0.010	0.014	0.021		

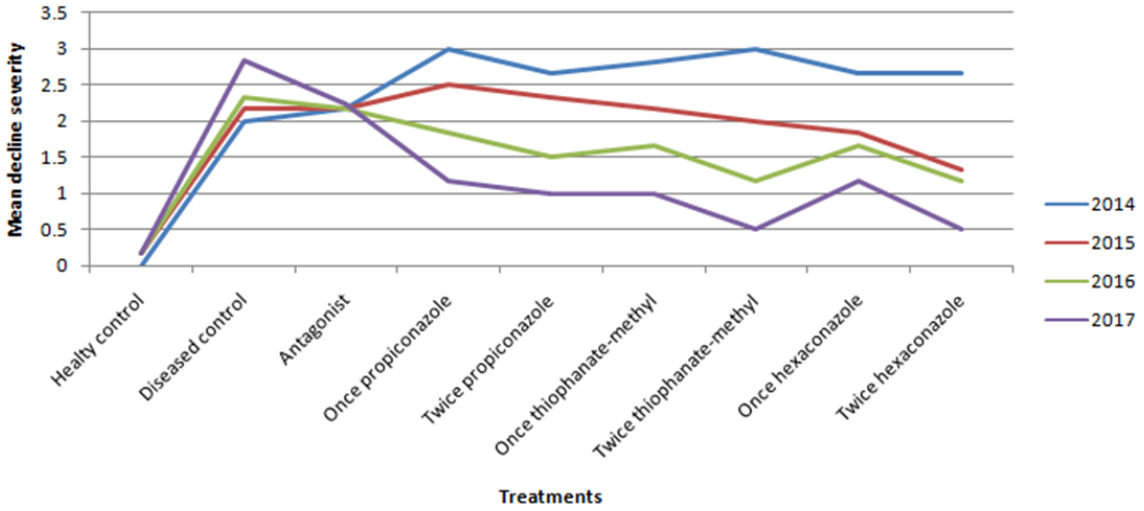

Figure 1. Trend lines representing Armillaria decline severity ratings determined during four years on olive trees treated with antagonist (*Trichoderma harzianum*) and fungicides.

In contrast to the lack of variations in the interaction of *T. harzianum* with *A. mellea* on olive tree collars and roots, fungicides efficiently reduced the decline severity ratings over the four years (**Figure 1**). In diseased control 2, infections of olive trees by *A. mellea* increased as the study progressed over the four years. Furthermore, the study year showed no variation on the olive decline in healthy trees. The probability associated with the student's *T*-test for this four-year study demonstrated the significant effect of year on the decline severity ratings determined across the diseased control 2 and six fungicidal treatments (**Figure 1**).

In **Figure 2**, the antagonistic treatment showed 25% control efficiency on younger olive trees under 15 years old. For olive trees older than 15 years, the application of *T. harzianum* was ineffective on the decline disease development which increased by 60% in the current four-year research. For fungicidal treatments, the perfect control efficiencies (100%) were determined on younger (< 15 years old) diseased olive trees treated with the once applications of thiophanate-methyl and hexaconazole, and the twice applications of thiophanate-methyl. For all the antagonistic and fungicidal treatments, the control of decline disease was more efficient on younger olive trees than older ones. Moreover, the 25% control efficiency of *T. harzianum* recorded on olive trees younger than 15 years was the

same as the control efficiency of the once application of hexaconazole under orchard conditions (**Figure 2**).

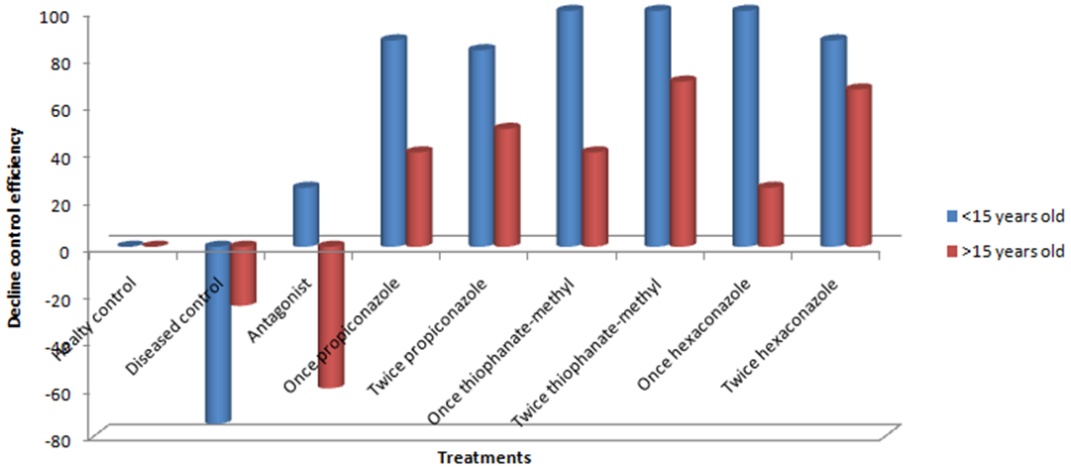

Figure 2. Control efficiency of antagonistic and fungicidal treatments applied to Armillaria declined olive trees in fourth year of study.

In addition, the development of olive decline disease on the untreated-diseased trees was significantly greater in younger trees than older ones (> 15 years old) according to the *T*-test results (**Table 3**). Based on the paired *T*-test, t-probability of comparing the control efficiency percentage values determined for younger and older olive trees treated with the antagonist and fungicides was significant. This suggested that the olive tree age was significantly effective on the control efficiency of treatments applied during this four-year research (**Figure 2**).

4. Discussion

Drip or sprinkler irrigation restricted the decline disease caused by *A. mellea* as reported by Adaskaveg et al. [4] and Eguchi et al. [5]. It is previously known that these two irrigation systems can lower soil wetness around the collar and roots, resulting in reductions in aggressiveness of root rot pathogens [21]. In addition, inserting warm water (35 °C) into the rhizosphere soil for three days via drip irrigation system eliminated *R. necatrix*, another collar and root rot pathogen in trees [22]. Applying biocide fungicides such as methyl bromide decreased these fungal pathogens if performed before planting the orchard [23]. Removing the soil surrounding the collar reduced significantly *A. mellea* in orange and grape trees [7,8]. However, it was still desired to find alternate easier and cheaper methods to manage the decline disease and improve productivity in olive orchards. Therefore, the current study examined the potential of antagonistic and fungicidal treatments on the development of *A. mellea* decline under olive orchard conditions during the four years. To the best of our knowledge, this the first report of comparing the control efficiency of an antagonistic fungus with fungicides found influential on the disease earlier.

Otieno et al. [9] and KhabazandAsadi [10] observed the application of *T. harzianum* in the rhizosphere soil around the collar restricted *A. mellea*decline in trees. Elsewhere, fungicidal treatments decreased *A. mellea* in decline trees [4,5].

Furthermore, fungicides reduced the hyphal growth of *A. mellea*on agar media under laboratory conditions [13]. The current findings added to our knowledge the inhibitory effects of *T. harzianum* on the pathogen *A. mellea* on agar media. Moreover, a 25% control efficiency of this antagonistic fungus was recorded on olive trees younger than 15 years which was the same as the control efficiency of the once application of hexaconazole under orchard conditions. This appears to be the first finding reporting the significant effects of olive tree age and the equal control efficiency of *T. harzianum* and a fungicide on the pathogen *A. mellea*under orchard conditions. The present research also advanced our understanding of the noticeable effect of olive tree age on the control efficiency of antagonistic and fungicidal applications. Such novel information helps us to improve the effectiveness of this bioagent and develop biofungicides applied well-timed according to the tree age in future.

The efficiency of various fungicides to control *A. mellea* in grape orchards using propiconazole [4] and cyproconazole, hexaconazole, propicoconazole and tetraconazole [13] have been reported previously. It was still greatly needed to evaluate the efficiency of these fungicides applied to *A. mellea* in grape trees for controlling the same disease in olive trees. Therefore, the present findings support earlier findings on the fungicides, hexaconazole, propicoconazole and thiophanate-methyl, being influential on *A. mellea* in olive orchards. Furthermore, the control efficiencies as perfect as 100% were determined on younger (< 15 years old) diseased olive trees treated with the once applications of thiophanate-methyl and hexaconazole, and the twice applications of thiophanate-methyl. Moreover, this seems to add the significant effect of olive tree age on the effectiveness of fungicidal control of *A. mellea* under olive orchard conditions to the literature. It should be noted that a greater efficiency of the disease control detected for the twice applications of these three fungicides, at branching and fruiting stages of olive growth, was little understood. Hence, this four-year research provided novel information valuable to olive producers worldwide for sustainable olive production purposes. Future research may examine the control efficiency of further antagonistic agents in order to reduce fungicides usage in horticulture and improve sustainability as much as possible.

Recently, the biological fungicide Serenada AS0 containing the antagonist, *Bacillus amyloliquefaciens* (formerly *B. subtilis*), which has been genetically modified, has been recommended for the control of fungal diseases in particular, *Verticillium* spp., in olive orchards [11]. Poveda et al. [12] reviewed the literature for *Trichoderma* spp. as a potential tool to restrict biotic and abiotic stresses in plants. For instance, this fungus can produce organic acids which solubilise nutrients like P and K, siderophores which chelate Fe, Cu and Zn, and phytohormones which induce systemic changes in plants [12]. However, most of earlier findings on the biocontrol of *A. mellea* by *Trichoderma* spp. have been obtained in the lab or greenhouse. Therefore, the orchard scale biocontrol of this olive pathogen and its responsible mechanisms used by *Trichoderma* spp. received little consideration worldwide. Hence, the current study provided novel promising and orchard-scale outcomes for future research on more effective biocontrol of *A. mellea* by *T. harzianum*, seeking appropriate basic or adjective materials for the bioagent application and also more

antagonistic local populations of *T. harzianum*.

Author contributions: Conceptualization, BN; methodology, BN; software, SHNK; validation, HJ; formal analysis, SHNK; investigation, SHNK; resources, BN; data curation, HJ; writing—original draft preparation, BN; writing—review and editing, BN; visualization, HJ; supervision, BN; project administration, BN; funding acquisition, BN. All authors have read and agreed to the published version of the manuscript.

Conflict of interest: The authors declare no conflict of interest.

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