



ORIGINAL ARTICLE

Evaluation of some Fungicides for the Control of Armillaria Root Rot of Walnut Trees

Nima Khaledi*, Mahdi Rezaei

Seed and Plant Certification and Registration Institute, Agricultural Research, Education and Extension Organization (AREEO), Karaj, Iran

KEY WORDS

Chemical control;
Pathogenicity;
Soil-borne;
Thiophanate-methyl;
Walnut

A B S T R A C T

Armillaria root rot, primarily caused by the closely related species *Armillaria mellea*, is a prevalent disease affecting walnut trees globally, leading to stunted growth and plant mortality. Currently, no walnut rootstock genotypes have been identified that offer complete resistance to this disease. The application of chemical fungicides represents the most straightforward and accessible method to mitigate the damage caused by *Armillaria* root rot in walnut trees. This research aimed to assess the prevalence of *Armillaria* root rot in both traditional and commercial walnut orchards and to evaluate the efficacy of chemical fungicides, specifically thiophanate-methyl and triazole compounds (propiconazole, cyproconazole, and hexaconazole), in reducing the severity and incidence of root rot. Samples collected from various commercial and traditional orchards in Iran were analyzed for the presence of the causal agent of the disease. Using a combination of morphological and molecular techniques, five isolates of *A. mellea* were identified. Results indicated variability in pathogenicity among the *A. mellea* isolates, ranging from pathogenic to weakly pathogenic. Moreover, the fungicides tested were effective in completely inhibiting mycelial growth at the minimum inhibitory concentration (MIC). However, the effectiveness of each fungicide in reducing disease incidence varied. Notably, thiophanate-methyl significantly outperformed the triazole fungicides in inhibiting disease progression. The findings suggest that thiophanate-methyl, when applied at the minimum fungicidal concentration (MFC) upon the initial appearance of disease symptoms, holds promise for the management of *Armillaria* crown and root rot in walnut orchards.

Introduction

The walnut (*Juglans regia* L.) is a significant source of nutrition globally (Akca and Sahin, 2022; Sarikhani *et al.*, 2021; Pakrah *et al.*, 2021; Chatrabnous *et al.*, 2018; Farsi *et al.*, 2018; Jahanbani *et al.*, 2018). In 2022, world walnut production was approximately 3.874 million tons, with China, the United States, Iran, and Turkey contributing roughly

72% of this output. In Iran, walnuts are cultivated on around 52 thousand hectares, producing about 355 thousand tons (FAOSTAT, 2022). Hamedan Province is the largest producer in Iran, with a cultivated area of 21,600 hectares and a production of 67,145 tons, accounting for 23% of the country's walnut output (Ahmadi *et al.*, 2021).

*Corresponding author: Email address: n_khaledi@areeo.ac.ir

Received: 21 April 2024; Received in revised form: 5 June 2024; Accepted: 31 August 2024

DOI: 10.60680/jon.2024.07694

In recent years, losses in orchards due to soil-borne diseases—including damping-off, root rot, and vascular wilt—have significantly increased (Lee *et al.*, 2020). The growth and production of walnuts are adversely affected by both abiotic and biotic stresses (Liu *et al.*, 2020; Mahmoudian *et al.*, 2021; Arab *et al.*, 2022). Among the biotic agents, the disease caused by *Armillaria mellea* (Vahl ex Fr) Kummer poses a severe threat to traditional orchards worldwide. *Armillaria* species are necrotrophic fungal pathogens with a broad host range, affecting conifers, woody plants, shrubs, and various fruit trees, including walnuts, avocados, peaches, almonds, and apples (Elias-Roman *et al.*, 2019). The estimated annual yield loss due to *A. mellea* has been reported to range between 10% and 40% (Baumgartner, 2004). Most *Armillaria* species act as secondary pests, infesting trees primarily when they are weakened and taking advantage of various pathogenicity factors, particularly the production and secretion of enzymes during the infection period (Devkota and Hammerschmidt, 2020; Khodadadi *et al.*, 2016, 2020).

Symptoms of *Armillaria* root rot observed in orchards include yellowing, premature leaf fall, sudden wilting, branch dieback, and reduced shoot growth, which can be severe enough to cause plant death. These symptoms, combined with the decay and destruction of wood tissue, the formation of white mycelial sheets, and the presence of rhizomorphs of the fungus on the roots, provide clear evidence for diagnosing *Armillaria* root rot disease (Mohammadi *et al.*, 2022; Baumgartner *et al.*, 2011).

Diagnosing the disease is challenging, as aerial symptoms are often unreliable and non-specific (Hoopen and Krauss, 2006). This pathogen is soil-borne and transmits through infested soil and plants to healthy plants via mycelial strands and rhizomorphs. Consequently, the pathogen can spread throughout the orchard, leading to successive infections in adjacent plants (Porter *et al.*, 2022). *Armillaria* species are found worldwide and can survive as saprophytes on

woody debris and organic matter in the soil. Thus, once an orchard is infested, eradicating the pathogen becomes extremely difficult (Kedves *et al.*, 2021).

Several management strategies have been implemented to control *Armillaria* crown and root rot disease, including cultural practices, chemical and biological control, the use of *Armillaria* root rot-tolerant walnut rootstocks, and healthy propagating materials, along with planting uninfected and healthy rootstocks (Rees *et al.*, 2022). Currently, there is no commercially available walnut rootstock proven to be resistant to *Armillaria* root rot (Vahdati *et al.*, 2021). As a result, managing the disease caused by *Armillaria* spp. is particularly challenging due to the pathogen's long survival and broad host range. Chemical control, which is readily accessible to farmers, is viewed as the best approach for disease management. Fungicides such as cyproconazole, tetraconazole, propiconazole, and hexaconazole, identified as sterol biosynthesis inhibitors, have been reported to be effective against *Armillaria* root rot in orchards (Amiri *et al.*, 2008; Aguín *et al.*, 2006). Soil-applied fungicides protect growing plants and can slow disease progression in both symptomatic and asymptomatic plants. The appropriate use of fungicides may play a crucial role in slowing the spread of infection within orchards (Amiri and Schnabel, 2012).

Despite the economic importance of *Armillaria* root rot disease in orchards, limited information is available regarding effective fungicides. Therefore, the objectives of this research were to: (i) assess the infection status of both commercial and traditional orchards regarding *Armillaria* root rot disease, and (ii) evaluate the effectiveness of selected chemical fungicides in reducing the development of the disease caused by *A. mellea* in walnut trees.

Materials and Methods

Sample collection and isolation of fungal isolates

A total of 65 walnut trees with and without

suspicious symptoms of *Armillaria* root and crown, including mycelial fans, rhizomorph signs or basidiocarps were sampled from commercial and traditional walnut orchards in different regions of Iran during the 2023 growing season (Fig. 1). For all plants from which crown and roots samples were taken, soil samples (approximately 1 kg) were also obtained by mixing rhizosphere soil at a depth of 5-30 cm. The samples were individually packaged in paper bags and then taken to the laboratory of the Seed and Plant Certification and Registration Institute (SPCRI) in Iran for the isolation of putative pathogens. For fungal isolations, samples were soaked in 96% ethanol for 1 min and then washed with distilled

water. Small pieces of tissue were removed (approximately 1 cm × 1 cm), surface-sterilized in 2% sodium hypochlorite solution for 15 min, rinsed with sterile distilled water, and air dried (Worrall, 1991). These were cultured in petri plates containing malt extract agar (MEA) amended with Benomyl 50% WP (14 mg mL⁻¹) and streptomycin sulfate (100 mg mL⁻¹) added after autoclaving (Soltantoyeh *et al.*, 2014). For isolation of fungal isolates from the soil, 1 gram of each soil sample was suspended in 9 ml of distilled water, then serially diluted and plated in petri dishes containing MEA medium amended as mentioned above (Lochman *et al.*, 2004a). Petri dishes were kept in dark at 22 ± 1 °C for 7 days.

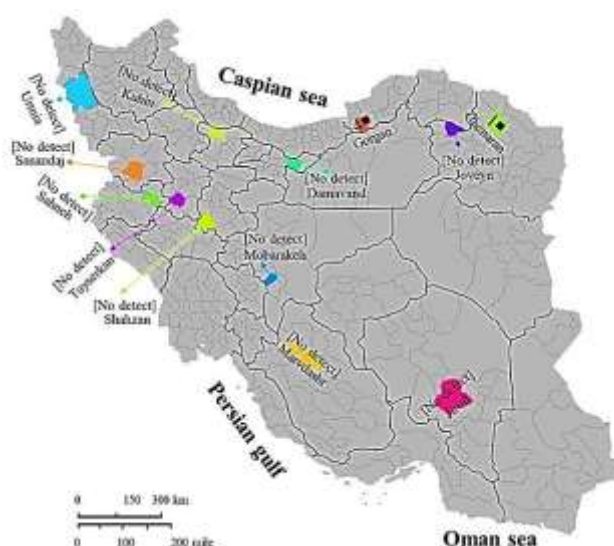


Fig 1. A map of Iran showing the geographical distribution of fungal isolates isolated from walnut orchards. The number next to the symbols indicates the number of *Armillaria mellea* (■) isolates isolated in the same region.

Morphological and molecular identification of fungal

isolates

Identification of *Armillaria* isolates was performed using a standard key as described by Volk (2005). For total nucleic acid extraction, mycelial plugs of *Armillaria* isolates (5 mm diameter) were picked up from potato dextrose agar (PDA) plates and transferred into 250 mL Erlenmeyer flasks containing 100 ml potato dextrose broth (PDB) medium, then incubated at 25 °C in shaking incubator at a rotary speed of 120 rpm for 10 days. The mycelial tissue was dried with sterile filter paper and ground with liquid

nitrogen to a fine powder. Total genomic DNA was extracted with the Genomic DNA isolation kit (Pishgam Biotech, Iran). DNA quantity was measured using a NanoDrop spectrophotometer and its quality was checked through agarose gel electrophoresis. DNA samples were diluted with deionized water to a final concentration of 50 ng µL⁻¹.

Validation of morphological features of fungal isolates at the identified species level was performed using the species-specific primers of *Armillaria*

mellea (forward: 5'-CTG ACC TGT TAA AGG GTA TGT GC-3' and reverse: 5'-AAG CTG AAT CCT TCT ACA AAG TCA A-3') according to Lochman *et al.* (2004b). The PCR reaction was performed in a volume of 25 µl, each reaction containing 1 µL of 2.5 µM each reverse and forward primers, 12.5 µL of PCR Master Mix (CinnaGen, Iran), and 3 µL of sample DNA and 7.5 µL PCR grade water. The PCR program included pre-incubation step for 3 min at 94 °C, followed by 35 cycles of 30 s at 94 °C, 40 s at 55 °C, 30 s at 72 °C and final extension for 5 min at 72 °C. The amplified products were separated by electrophoresis on a 1.5% agarose gel at 90 V for 30 min and visualized with GelRed staining. Negative control with molecular grade water instead of template DNA and positive control containing DNA of *A. mellea* in PCR reaction mix were included with all PCR runs.

Plant materials

Twelve-month-old seedlings of Damavand walnut genotype obtained from Iranian Horticulture Research Institute, were used as host in virulence tests. 'Damavand' is a popular walnut genotype planted due to its ability to grow in the climatic conditions of the country. Walnut seedlings were planted individually in 15 cm diameter (× 11 cm height) plastic pots filled by sterilized peat moss, perlite, and field soil in a 1:1:1 ratio (v/v/v). The pots were maintained at 30 ± 4 °C; 16/8h light/dark photoperiod; 50 ± 5 % relative humidity and irrigated when needed.

Inoculum preparation

Fungal inoculum was prepared on wheat grains as described by Freeman *et al.* (1986). Briefly, wheat grains were soaked in distilled water for 12 h. Then, the grains were autoclaved in two consecutive days at 121 °C. The inoculum of *A. mellea* was produced by inoculation and colonization of 100 g sterilized wheat grains in 250 ml Erlenmeyer flasks with five mycelial plugs (5 mm diameter) of a 2-week-old culture and

incubated at 25 ± 1 °C in the dark condition for three weeks and shaken at least twice for better colonization. Artificial inoculation was carried out by 30 g of fungal inoculum per ~1.0 kg of substrate soil in each pot. The sterilized wheat grains without the fungus served as the control.

Pathogenicity test

To confirm Koch's postulates, fungal isolates were inoculated into twelve-month-old walnut trees under controlled conditions. In the experimental design, four replicate walnut trees were planted for each isolate in a completely randomized design and the experiment was repeated twice. Three months after inoculation, aerial symptoms of chlorosis and leaf fall as well as necrotic zones on root and root collar were surveyed, in comparison with control plants. Disease severity was estimated at 90 days after inoculation based on aerial symptoms and percentage of crown plus root rot infection according to modified scale of Rees *et al.* (2022), where: 0 = healthy plant; 1 = chlorosis and yellowing of leaves, superficial and scattered lesions on root surface; 2 = branch necrosis, less than 10% root surface with lesions; 3 = dieback, restricted rot at crown region and extensive lateral lesions affecting 11-25% of root surface; 4 = rot affecting 26-50% of root and crown region; 5 = rot affecting 51-75% of root and crown region, visible *A. mellea* mycelial colonization; 6 = extensive rot affecting >76% of root and crown region, visible *A. mellea* mycelial colonization; 6 = dead plant. The disease index (DI) was calculated by the following formula (Elias-Roman *et al.*, 2019):

$$\text{Disease index} = \left[\frac{(\sum (\text{Number of diseased plants at all levels}) \times (\text{The value of relevant level}))}{((\text{Total number of investigated plants}) \times (\text{Highest disease level}))} \right] \times 100$$

Chemical fungicides

The following fungicide formulations were used in experiments: thiophanate-methyl (Topsin M® [WP

70 %], Ariashimi), cyproconazole (Alto® [SL 10%], Ariashimi), propiconazole (Tilt® [EC 25 %], Ariashimi) and hexaconazole (Aria [SC 5%], Ariashimi) (Nourbakhsh, 2022; Aguín *et al.*, 2006). The International Plant Protection Convention (IPPC) has recommended thiophanate-methyl to control soil-borne diseases, including root and crown rot, as soon as the symptoms of the disease are observed (Nourbakhsh, 2022).

Determination of effective inhibitory concentrations

The fungal isolate with the highest level of pathogenicity was selected for further experiments. The commercial fungicides were examined for antifungal activity against *A. mellea*. The broth micro-dilution method was employed in order to determine the minimum inhibitory concentration (MIC), minimum fungicidal concentration (MFC), and half maximal inhibitory concentration (IC₅₀) (Plodpai *et al.*, 2013). The MIC values were defined as the minimum dose of fungicide that completely inhibited visible fungal growth. The MFC values were defined as the lowest dose at which no fungal growth was observed after subculturing into fresh PDA medium. IC₅₀ values were calculated graphically from the dose-response curves. The nature of toxicity (fungistatic and/or fungicidal effect) of the synthetic fungicides against *A. mellea* was determined as described by Thompson (1989). The inhibited fungal mycelial plugs on fungicide-amended PDA plates were sub-cultured into PDA medium, to determine which concentration of each fungicide had fungicidal effects on *A. mellea*.

Effects of chemical fungicides on the progress of diseases caused by *A. mellea* isolates on walnut seedlings

The experiments were conducted as described by Freeman *et al.* (1986). Fungicide treatments including various concentrations (MIC, MFC and IC₅₀) were sprayed in near the tree crown as soon as the

symptoms of the disease appeared. The inoculated plants were maintained at 25 ± 2 °C, with 16:8 h light: dark photoperiods cycle and irrigated when needed. After three weeks following inoculation, the percentage of disease control was evaluated using the formula described by Plodpai *et al.* (2013):

$$\% \text{ Control efficacy} = (\text{Disease index of control} - \text{Disease index of treated group}) / \text{Disease index of control} \times 100$$

Statistical analysis

The trials were organized in a completely randomized design with four replicates and carried out twice. The obtained data were statistically analyzed via SAS software (version 9.2; SAS Institute, Cary, NC, USA) and mean comparison was conducted using the Duncan's multiple range test at the level $P \leq 0.05$.

Results

Morphological and molecular characteristics of *Armillaria* isolates

Morphological studies indicated that five isolates were classified into *Armillaria* genus. The color of the colony was initially white and later changed to mahogany brown mahogany. All isolates of *A. mellea* produce rhizomorphs. Basidiospores $7.0\text{-}9.0 \times 5.5\text{-}6.5$ μm (mean = 7.5×6.0 μm), mostly ellipsoid, with a prominent apiculus, smooth, hyaline to brownish in KOH solution (2%). This is the only species of *Armillaria* that lacks clamp connections at the base of the basidia. Basidia 2- and 4-sterigmate. Pleurocystidia not found. Cheilocystidia $25.5\text{-}39.5 \times 2.0\text{-}9.5$ μm , cylindric-flexuous to clavate, often irregular, thin-walled and smooth. Pileipellis a cutis, elements $5.5\text{-}9.5$ μm wide, septate, terminal cells cylindric with rounded or subclavate apices. After presumptive identification, the fungal isolates were submitted to molecular identification by species-specific primers. The results of molecular identification were confirmed by morphological

characteristics of *A. mellea* isolates. All fungal isolates were identified by PCR amplification with *A. mellea*-specific primers AR1 and AR2, which yielded about 720 bp.

Distribution and frequency of isolates

In this research, 130 samples including 65 soil samples and 65 root samples were collected from approximately 13 walnut orchard plots in different regions of Iran for screening of causal agent of Armillaria crown and root rot disease (on average 2 to 5 samples per orchard plot). In this study, five fungal isolates were found from root and soil samples in the studied orchards. A total of four isolates were isolated from root sections of walnut trees collected in two traditional orchards (Gorgan and Chenaran) and one isolate was isolated from soil samples collected in one traditional orchard (Gorgan) (Fig. 1). Among the samples collected from the soil and roots, no *Armillaria* infection was observed and detected in all commercial orchards (Kuhin and Tuyserkan) and 9 traditional orchards (Mobarakeh, Urmia, Damavand, Marvdasht, Sahneh, Sanandaj, Jiroft, Shahzan and Joveyn) from different regions of Iran (Fig. 1). The

results of this research revealed that four and one isolates recovered of Gorgan and Chenaran samples, respectively (Fig. 1). The sample collected from Gorgan had a higher prevalence of fungal infection than other samples examined in this study.

Pathogenicity assay

The results of pathogenicity test indicated that different *A. mellea* isolates had different pathogenicity in walnut seedlings (Fig. 2). According to the results, there are significant differences in the disease index among isolates tested. The results of this study provide support that all *A. mellea* isolates were pathogenic or weakly pathogenic. The Koch's postulates were demonstrated by the re-isolation of the same fungus from the diseased roots. The results of virulence tests of different *A. mellea* isolates on walnut seedlings indicated that the highest and lowest disease index values were recorded for the isolates of GM377 and GM493, respectively (Fig. 2). The virulence test showed that the disease index for fungal isolates ranged from 17.7 ± 2.6 to 71.8 ± 1.9 (Fig. 2). Based on pathogenicity test, the GM377 was selected for further experiments.

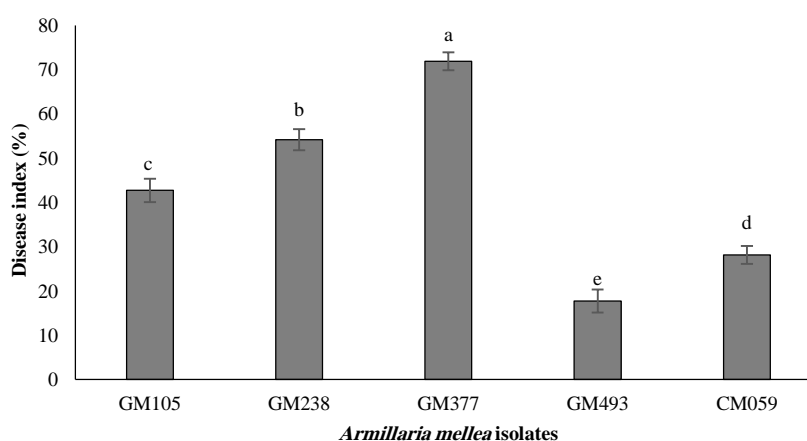


Fig 2. Pathogenicity of *Armillaria mellea* isolates on seedlings inoculated of walnut genotype Damavand.

Antifungal activities of synthetic fungicides in vitro

Minimum inhibitory concentration (MIC), minimum fungicidal concentration (MFC), and inhibitory concentration 50 (IC₅₀) values of chemical

fungicides are reported in Table 1. The MIC values of fungicides were between 815 and 1725 ppm. The lowest MIC value was related to thiophanate-methyl

with 815 ppm. In addition, the lowest and highest IC_{50} values for thiophanate-methyl and hexaconazole were recorded as 428 ppm and 945 ppm, respectively (Table 1). The MFCs of synthetic fungicides including thiophanate-methyl, propiconazole, cyproconazole, and hexaconazole against *A. mellea*

were found to be 865, 1655, 1780, and 1860 ppm, respectively (Table 1). The lowest MIC, MFC, and IC_{50} levels were found for thiophanate-methyl. The results of this study on fungistatic and/or fungicide activity have shown that all fungicides have had fungicidal activity on *A. mellea*.

Table 1. Antifungal activity of the synthetic fungicides against mycelial growth of *Armillaria mellea* in vitro

Treatments	IC_{50} * [ppm]	MIC‡ [ppm]	MFC† [ppm]	Activity
Thiophanate-methyl	420 ± 4 d	815 ± 3 d	865 ± 6 d	Fungicidal effect
Propiconazole	800 ± 4 c	1495 ± 5 c	1655 ± 5 c	Fungicidal effect
Cyproconazole	905 ± 6 b	1685 ± 3 b	1780 ± 4 b	Fungicidal effect
Hexaconazole	945 ± 2 a	1725 ± 6 a	1860 ± 7 a	Fungicidal effect

* IC_{50} = Inhibitory concentration with 50% fungal growth inhibitory effect (ppm); ‡ MIC = Minimum inhibitory concentration (ppm); † MFC = Minimum fungicidal concentration (ppm); Means followed by the same letter in the same column are not significantly different from each other at $P \leq 0.05$, according to Duncan's multiple range test. The experiment was repeated twice with similar results.

Efficiency of synthetic fungicides on disease index

The data in Table 2 indicated that treatment with fungicides, especially thiophanate-methyl, significantly reduced the development of *Armillaria* root rot disease. The root rot disease index caused by *A. mellea* on walnut seedlings was significantly reduced by the application of thiophanate-methyl at MFC concentrations followed by MIC and IC_{50} concentrations (Table 3). Similar results were obtained for the effect of the MFC concentration of synthetic fungicides on *Armillaria* root rot disease

compared to MIC and IC_{50} concentrations (Table 3). The comparison of the thiophanate-methyl treatments showed that no significant differences in reducing disease were observed between the MFC and MIC concentrations. In this study, no phytotoxicity was observed in plant leaves at low doses of thiophanate-methyl. In general, higher levels of inhibition were found for thiophanate-methyl compared to propiconazole, cyproconazole, and hexaconazole (Tables 2 and 3).

Table 2. Efficiency of fungicides treatment to control *Armillaria* root rot disease caused by *Armillaria mellea*

Treatment	Concentrations	Disease index	Suppression efficacy [%]
Untreated control	-	71.87 ± 1.9 a	-
Thiophanate-methyl	IC_{50}	59.37 ± 1.0 f	17.39 ± 1.4 e
	MIC	34.37 ± 1.9 j	52.17 ± 2.8 a
	MFC	31.25 ± 1.2 j	56.52 ± 1.7 a
Propiconazole	IC_{50}	63.54 ± 1.0 de	11.59 ± 1.4 fg
	MIC	45.83 ± 2.4 h	36.23 ± 3.3 c
	MFC	41.66 ± 1.7 i	42.02 ± 2.4 b
Cyproconazole	IC_{50}	67.71 ± 1.0 bc	5.79 ± 1.4 hi
	MIC	58.33 ± 0.0 f	18.84 ± 0.0 e
	MFC	52.08 ± 1.2 g	27.53 ± 1.6 d
Hexaconazole	IC_{50}	70.83 ± 0.0 ab	1.44 ± 0.0 i
	MIC	65.62 ± 1.0 cd	8.69 ± 1.4 gh
	MFC	60.42 ± 1.2 ef	15.94 ± 1.7 ef

* IC_{50} = Inhibitory concentration with 50% fungal growth inhibitory effect (ppm); ‡ MIC = Minimum inhibitory concentration (ppm); † MFC = Minimum fungicidal concentration (ppm); Results are means standard errors of the means from two independent experiments. Means followed by the same letter in the same column are not significantly different from each other at $P \leq 0.05$, according to Duncan's multiple range test. The experiment was repeated twice with similar results.

Table 3. Effect of chemical fungicides on percentage reduce disease index of *Armillaria* root rot disease (mean ± standard error) caused by *Armillaria mellea* on walnut seedlings.

Treatments	Thiophanate-methyl	Propiconazole	Cyproconazole	Hexaconazole
MFC† [ppm]	56.52 ± 1.67 a (a)	42.03 ± 2.16 a (b)	27.54 ± 1.67 a (c)	15.94 ± 1.69 a (d)
MIC‡ [ppm]	52.17 ± 2.77 b (a)	36.23 ± 3.04 b (b)	18.84 ± 0 b (c)	8.69 ± 1.45 b (d)
IC ₅₀ * [ppm]	17.39 ± 1.45 c (a)	11.59 ± 1.44 c (b)	5.797 ± 1.44 c (c)	1.45 ± 0 c (d)

*IC₅₀ = Inhibitory concentration with 50% fungal growth inhibitory effect (ppm); ‡ MIC = Minimum inhibitory concentration (ppm); † MFC = Minimum fungicidal concentration (ppm); Means followed by the same letter in the same column are not significantly different from each other at $P \leq 0.05$, according to Duncan's multiple range test. The experiment was repeated twice with similar results.

Discussion

This study is the first report to determine the health status of commercial and traditional walnut orchards to *Armillaria* root rot disease. The study also investigated *in vitro* and *in vivo* antifungal activity of the thiophanate-methyl and triazole compounds including propiconazole, cyproconazole and hexaconazole against *A. mellea*. The results indicated that using thiophanate-methyl was effective on reducing *Armillaria* root rot disease on walnut when compared to other triazole fungicides.

In this study, five *Armillaria* isolates collected from different traditional orchards in Iran revealed molecular and morphological similarity with *A. mellea*. Similar findings were also reported by Dalili *et al.* (2008), who demonstrated that the fungal isolates isolated from walnut trees in the Isfahan and East Azerbaijan orchards of Iran were identified as *A. mellea*. Similar morphological and molecular results of *A. mellea* in nurseries and orchards of different fruit crops were reported by Yousefi Hamedani *et al.* (2012), Pildain *et al.* (2009), Antonín *et al.* (2009), Lochman *et al.* (2004a), Mwenje *et al.* (2003), and Coetzee *et al.* (2003).

The *Armillaria* infection was only observed from sample collected in the traditional orchards. Our results showed that the highest percentage of fungal infections in root crown and roots was seen in the Gorgan region (80%), followed by the Chenaran region (20%). *Armillaria* root rot disease may be caused by cultivated sensitive genotypes, weak management of irrigation water, improper drainage, environmental condition, lack of proper cultural practices, soil infection to *A. mellea*, and lack of use of appropriate fungicides in the traditional orchard of

Gorgan region.

The results of the pathogenicity test indicated that all isolates were pathogenic or weakly pathogenic on Iranian walnut genotype Damavand. Numerous reports indicated that differences in pathogenicity among *A. mellea* isolates (Elias-Roman *et al.*, 2019; Sipos *et al.*, 2017), and these indications support our results. Metaliaj *et al.* (2006) reported that there was variation in disease severity caused by different *Armillaria* isolates, which is in accordance with our research findings.

The antifungal activity results revealed that all fungicides tested had negative effects on mycelial growth of *A. mellea* *in vitro*. Among all fungicides tested, the lowest MIC, MFC, and IC₅₀ levels were observed for thiophanate-methyl, followed by propiconazole, cyproconazole, and hexaconazole, respectively. Similar results were obtained by Aguín *et al.* (2006), who reported that the mycelial growth of *A. mellea* was completely inhibited by propiconazole, cyproconazole, hexaconazole, and tetraconazole at MIC concentration compared to the control.

The results obtained at effective concentrations of a propiconazole and cyproconazole contrast with those reported by others (Thomidis and Exadaktylou, 2012; Aguín *et al.*, 2006). The reason for this difference is the various levels of pathogenicity of fungal isolates investigated as well as the quality of chemical fungicides used. The antifungal activity of the fungicide increases with elevating concentrations. The minimum level of the chemical fungicides required to inhibit growth of *A. mellea* was different. The differences in the fungicides' activity may be related to systemic or protectant activity of the

compounds, differential uptake by the fungus or some other consequences of the intrinsic biochemical properties of the fungicides evaluated (Woodward and Brenneman, 2008).

The results of the efficiency of synthetic fungicides on disease index indicated that use of chemical fungicides as sprayed in near the tree crown as soon as the symptoms of the disease appear was effective on reducing *Armillaria* root rot disease. Basipetal translocation for all fungicides tested were not observed, therefore soil drenching rather than instead of trunk injection should be considered to prevent *Armillaria* root rot disease. In our investigations, the thiophanate-methyl was the most effective fungicide in inhibiting the growth of *Armillaria* *in vitro*. Thiophanate-methyl is a thioureas based systemic fungicide for crop protection (Chauhan and Namdev, 2022). Similar results were obtained by Najafi *et al.* (2018), who showed that the application of thiophanate-methyl significantly reduced *Armillaria* root rot disease of olive.

The use of propiconazole as a control strategy to protect roots from *Armillaria* colonization and lower the spread of infection has been previously reported (Amiri and Schnabel, 2012). Studies on mechanisms of plant diseases suppression by fungicides have shown that the active components of fungicides may act on the pathogen or trigger defense mechanisms, resulting in disease reduction (Chen *et al.*, 2018; Avis, 2007). Thomidis and Exadaktylou (2012) reported that cyproconazole is an effective fungicide against *Armillaria* root rot disease in walnut, apple, and kiwifruit orchards, which is in accordance with our research results. In addition, cyproconazole appeared to be very effective in suppressing *Armillaria* root rot disease on *Hevea brasiliensis* (Gohet *et al.*, 1991), which is in accordance with our results. Likewise, application of phenolic fungicides significantly lowered the incidence of *Armillaria* root rot disease (West and Fox, 2002). Similar results were obtained by Liao *et al.* (2023), who suggested that some of the triazole compounds including cyproconazole,

tebuconazole, and epoxiconazole have potential for control brown root rot disease.

In conclusion, this study provided an overview and new insight into the health status of traditional and commercial orchards to *Armillaria* crown and root rot disease in Iran. Although *Armillaria* root rot infection was found at an acceptable level in the commercial fields, it is recommended to pay attention to maintenance and reduce the level of infection in order to enhance quality and health of the seedlings. The results of current research clearly revealed fungicides can slow down disease progression in plants. The findings provide new perspectives on the effect of the chemical fungicides on the rate of soil-borne disease progression caused by *A. mellea*.

Acknowledgements

The research was financially supported by Seed and Plant Certification and Registration Institute (SPCRI), Iran (Grant number: 2-08-08-001-020013).

Conflict of interest

The authors declare that they have no conflict of interest.

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