Journal of Nuts

Journal homepage: sanad.iau.ir/journal/ijnrs/

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

K E Y W O R D S

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).

In recent years, losses in orchards due to soil-borne diseases—including damping-off, root rot, and

*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

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.

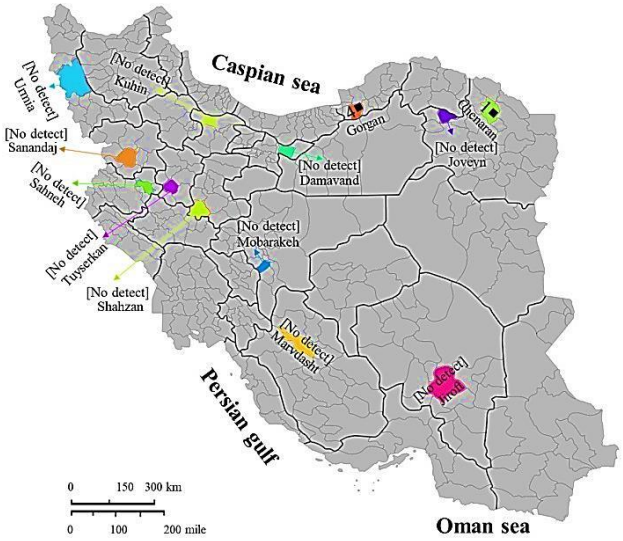

Fig 1. A map of Iran showing the geographical distribution of fungal isolates isolated from walnut orchards. The number next to the symbols indicates the number of *Armillaria mellea* (■) isolates isolated in the same region.

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 *Armillria* 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; 4 = rot affecting 51-75% of root and crown region, visible *A. mellea* mycelial colonization; 5 = 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):

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

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 (IC_{50}) (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. IC_{50} 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 IC_{50}) 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):

% Control efficacy = (Disease index of control - Disease index of treated group) / Disease index of control × 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 7.0-9.0 × 5.5-6.5 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 25.5-39.5 × 2.0-9.5 µm, cylindric-flexuous to clavate, often irregular, thin-walled and smooth. Pileipellis a cutis, elements 5.5-9.5 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.

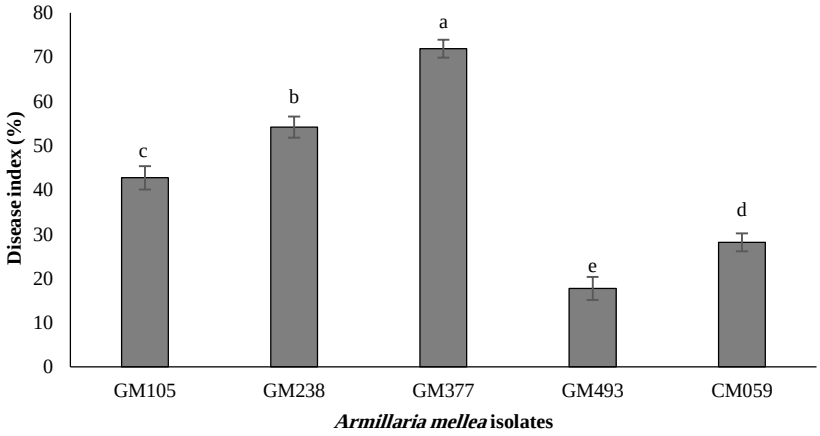

Fig 2. Pathogenicity of *Armillaria mellea* isolates on seedlings inoculated of walnut genotype Damavand.

Antifungal activities of synthetic fungicides in vitro

Minimum inhibitory concentration (MIC), minimum fungicidal concentration (MFC), and inhibitory concentration 50 (IC_{50}) 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 IC50 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)

*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.

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 Armillary *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.

References

Aguín O, Mansilla JP, Sainz JM (2006) In vitro selection of an effective fungicide against *Armillaria mellea* and control of white root rot of grapevine in the field. Pest Management Science. 62, 223-228. doi: 10.1002/ps.1149

Ahmadi K, Ebadzadeh HR, Hatami F, Mohammadnia-Afrozi S, Esfandiarpour E, Abbasi Taleghani R (2021) Agricultural Statistics. Ministry of Agriculture-Jahad. Available at Web site: https://maj.ir/Dorsapax/userfiles/Sub65/amarbaghi1400.pdf

Akca Y, Sahin U (2022) Responses of 'Chandler'

walnut variety grafted onto different rootstocks to salt stress. International Journal of Horticultural Science and Technology. 9, 1-13. doi: 10.22059/ijhst.2021.319247.444

Amiri A, Bussey KE, Riley MB, Schnabel G (2008) Propiconazole inhibits *Armillaria tabescens in vitro* and translocates into peach roots following trunk infusion. Plant Disease. 92, 1293-1298. doi: 10.1094/PDIS-92-9-1293

Amiri A, Schnabel G (2012) Persistence of propiconazole in peach roots and efficacy of trunk infusions for Armillaria root rot control. International Journal of Fruit Science. 12, 437-449. doi: 10.1080/15538362.2012.679183

Antonín V, Tomsovsky M, Sedlák P, Májek T, Jankovsky L (2009) Morphological and molecular characterization of the *Armillaria cepistipes* - *A. gallica* complex in the Czech Republic and Slovakia. Mycological Progress. 8, 259-271. doi: 10.1007/s11557-009-0597-1

Arab MM, Brown PJ, Abdollahi-Arpanahi R, Sohrabi SS, Askari H, Aliniaeifard S, Mokhtassi-Bidgoli A, Mesgaran MB, Leslie CA, Marrano A, Neale DB, Vahdati K (2022) Genome-wide association analysis and pathway enrichment provide insights into the genetic basis of photosynthetic responses to drought stress in Persian walnut. Horticulture Research, 1-38.

Avis TJ (2007) Antifungal compounds that target fungal membranes: applications in plant disease control. Canadian Journal of Plant Pathology. 29, 323-329. doi: 10.1080/07060660709507478

Baumgartner K (2004) Root collar excavation for postinfection control of Armillaria root disease of grapevine. Plant Disease. 88, 1235-1240. doi: 10.1094/PDIS.2004.88.11.1235

Baumgartner K, Coetzee MPA, Hoffmeister D (2011) Secrets of the subterranean pathosystem of Armillaria. Molecular Plant Pathology. 12, 515-534. doi: 10.1111/j.1364-3703.2010.00693.x

Chatrabnous N, Yazdani N, Vahdati K (2018) Determination of nutritional value and oxidative stability of fresh walnut. Journal of Nuts. 9(1), 11-20.

Chauhan C, Namdev S (2022) Spectrophotometric method development for thiophante methyl based systemic fungicide, Roko formulation. Materials Today: Proceedings. 68, 1106-1109. doi: 10.1016/j.matpr.2022.08.481

Chen L, Heng J, Qin S, Bian K (2018) A comprehensive understanding of the biocontrol potential of *Bacillus velezensis* LM2303 against Fusarium head blight. Plos One, 13(6), e0198560. doi: 10.1371/journal.pone.0198560

Coetzee MPA, Wingfield BD, Bloomer P, Ridley GS, Wingfield MJ (2003) Molecular identification and phylogeny of *Armillaria* isolates from South America and Indo-Malaysia. Mycologia. 95, 285-293.

Dalili SAR, Nanagulyan SG, Alavi SV (2008) Identification of *Armillaria* species on different hosts from Iran. Mycologia Balcanica. 5, 119-122. doi: 10.2307/3762039

Devkota P, Hammerschmidt R (2020) The infection process of *Armillaria mellea* and *Armillaria solidipes*. Physiological and Molecular Plant Pathology. 112, 101543. doi: 10.1016/j.pmpp.2020.101543

Elias-Roman RD, Galderon-Zavala G, Guzman-Mendoza R, Vallejo-Perez MR, Klopfenstein NB, Mora-Aguilera JA (2019) 'Mondragon': a clonal plum rootstock to enhance management of Armillaria root disease in peach orchards of Mexico. Crop Protection. 121, 89-95. doi: 10.1016/j. cropro. 2019.03.011

FAOSTAT (2022) FAOSTAT Database. Food and Agriculture Organization of the United Nations, Statistics Division. https://www.fao.org/faostat/en/#data/QCL

Farsi M, Fatahi Moghadam M, Zamani Z, Hassani D (2018) Effects of scion cultivar, rootstock age and hormonal treatment on minigrafting of persian walnut. International Journal of Horticultural Science and Technology. 5, 185-197. doi; 10.22059/ijhst. 2018.255460. 233

Freeman S, Sztejnberg A, Chet I (1986) Evaluation of *Trichoderma* as a biocontrol agent for *Rosellinia necatrix*. Plant and Soil. 94, 163-170.

Gohet E, Van Canh T, Louanchi M, Despreaux D (1991) New developments in chemical control of white root disease of *Hevea brasiliensis* in Africa. Crop Protection. 10, 234-238. doi: 10.1016/0261-2194(91)90049-W

Hoopen GMT, Krauss U (2006) Biology and control of *Rosellinia bunodes*, *Rosellinia necatrix* and *Rosellinia pepo*: A review. Crop Protection. 25, 89-107. doi: 10.1016/j.cropro.2005.03.009

Jahanbani R, Ghaffari SM, Vahdati K, Salami M, Khalesi MR, Sheibani N, Moosavi-Movahedi AA (2018) Kinetics study of protein hydrolysis and inhibition of angiotensin converting enzyme by peptides hydrolysate extracted from walnut. International Journal of Peptide Research and Therapeutics. 24(1), 77-85.

Kedves O, Shahab D, Champramary S, Chen L, Indic B, Bóka B, Nagy VD, Vágvölgyi C, Kredics L, Sipos G (2021) Epidemiology, biotic interactions and biological control of Armillarioids in the Northern Hemisphere. Pathogens. 10(1), 76. doi: 10.3390/pathogens10010076

Khodadadi F, Tohidfar M, Mohayeji M, Dandekar AM, Leslie CA, Kluepfel D, Butterfield T, Vahdati K (2016) Induction of polyphenol oxidase in walnut and its relationship to the pathogenic response to bacterial blight. Journal of the American Society for Horticultural Science. 141(2), 119-124.

Khodadadi F, Tohidfar M, Vahdati K, Dandekar AM, Leslie CA. (2020). Functional analysis of walnut polyphenol oxidase gene (*JrPPO1*) in transgenic tobacco plants and PPO induction in response to walnut bacterial blight. Plant Pathology. 69, 756-764.

Liao TZ, Chen YH, Tsai JN, Chao C, Huang TP, Hong CF, Wu ZC, Tsai IJ, Lee HH, Klopfenstein, NB, Kim MS, Stewart JE, Atibalentja N, Brooks FE, Cannon P, Mohd Farid A, Hattori T, Kwan HS, Lam YCR, Ota Y, Sahashi N, Schlub RL, Shuey LS, Tang AMC, Chung CL (2023) Translocation of fungicides and their efficacy in controlling Phellinus noxius, the cause of brown root rot disease. Plant Disease. 107, 2039-2053. doi: 10.1094/PDIS-06-22-1285-RE

Lee SH, Shin H, Chang WB, Ryu KY, Kim HT, Cha B, Cha JS (2020) Dieback reality of apple trees resulting from soil-borne fungal pathogens in South Korea from 2016 to 2019. Research in Plant Disease. 26, 8-94. doi: 10.5423/RPD.2020.26.2.88

Liu XJ, Meng PP, Yang GY, Zhang MY, Peng SB, Zhai MZ (2020) Genome-wide identification and transcript profiles of walnut heat stress transcription factor involved in abiotic stress. BMC Genomics. 21, 474. doi: 10.1186/s12864-020-06879-2.

Lochman J, Serý O, Jankovský L, Mikes V (2004a) The rapid identification of European *Armillaria* species from soil samples by nested PCR. FEMS Microbiology Letters. 237, 105-110. doi: 10.1016/j.femsle.2004.06.019

Lochman J, Serý O, Jankovský L, Mikes V (2004b) Variations in ITS of ribosomal DNA of Czech *Armillaria* species determined by PCR and high performance liquid chromatography. Mycological Research. 108, 1-9. doi: 10.1017/s0953756204000644

Mahmoudian M, Rahemi M, Karimi S, Yazdani N, Tajdini Z, Sarikhani S, Vahdati K (2021) Role of kaolin on drought tolerance and nut quality of Persian walnut, Journal of the Saudi Society of Agricultural Sciences, 20(6), 409-416.

Metaliaj R, Sicoli G, Luisi N (2006) Pathogenicity of *Armillaria* isolates inoculated on five *Quercus* species at different watering regimes. Phytopathologia Mediterranea. 45, 3-9.

Mohammadi AH, Haghdel M, Mirabolfathy M, Alaei H (2022) Armillaria root and crown rot disease in pistachio orchards. Plant Pathology Science. 11, 92-102. doi: 10.52547/pps.11.2.92

Mwenje E, Wingfield BD, Coetzee MPA, Wingfield MJ (2003) Molecular characterisation of *Armillaria* species from Zimbabwe. Mycological Research. 107, 291-296.

Najafi S, Jafary, H, Nazer Kakhki SH (2018) Chemical management of Armillaria rot diseases of olive trees in Tarom region of Zanjan province. In Proceedings of the 23rd Iranian Plant Protection Congress, Gorgan, Iran 27-30 August.

Nourbakhsh S (2022) List of important pests, diseases and weeds of major agricultural products, chemicals and recommended ways for their control. Plant Protection organization, Ministry of Jihad-e Agriculture.

Pakrah S, Rahemi M, Nabipour A, Zahedzadeh F, Kakavand F, Vahdati K (2021) Sensory and nutritional attributes of Persian walnut kernel influenced by maturity stage, drying method, and cultivar. Journal of Food Processing and Preservation, e15513.

Pildain M, Coetzee M, Rajchenberg M, Petersen R, Wingfield M, Wingfield B (2009) Molecular phylogeny of Armillaria from the Patagonian Andes. Mycological Progress. 8, 181-194. doi: 10.1007/s11557-009-0590-8

Plodpai P, Chuenchitt S, Petcharat V, Chakthong S, Voravuthikunchai SP (2013) Anti-*Rhizoctonia solani* activity by *Desmos chinensis* extracts and its mechanism of action. Crop Protection. 43, 65-71. doi: 10.1016/j.cropro.2012.09.004

Porter DL, Bradshaw AJ, Nielsen RH, Newell P, Dentinger BTM, Naleway SE (2022) The melanized layer of *Armillaria ostoyae* rhizomorphs: its protective role and functions. Journal of the Mechanical Behavior of Biomedical Materials. 125, 104934. doi: 10.1016/j.jmbbm.2021.104934

Rees HJ, Drakulic J, Cromey MG, Bailey AM, Foster GD (2022) Endophytic Trichoderma spp. can protect strawberry and privet plants from infection by the fungus *Armillaria mellea*. Plos One. 17(8), e0271622. doi: 10.1371/journal.pone.0271622

Sarikhani S, Vahdati K, Ligterink W (2021) Biochemical properties of superior persian walnut genotypes originated from southwest of Iran. International Journal of Horticultural Science and Technology. 8, 13-24. doi: 10.22059/ijhst.2020.309363.392

Sipos G, Prasanna AN, Walter MC, O'Connor E, Bálint B, Krizsán K, Kiss B, Hess J, Varga T, Slot J, Riley R, Bóka B, Rigling D, Barry K, Lee J, Mihaltcheva S, LaButti K, Lipzen A, Waldron R, Moloney NM, Sperisen C, Kredics L, Vágvölgyi C, Patrignani A, Fitzpatrick D, Nagy I, Doyle S, Anderson JB, Grigoriev IV, Güldener U, Münsterkötter M, Nagy LG (2017) Genome expansion and lineage-specific genetic innovations in the forest pathogenic fungi

Armillaria. Nature Ecology & Evolution. 1(12), 1931-1941. doi: 10.1038/s41559-017-0347-8

Soltantoyeh R, Dalili A, Borhani A (2014) Evaluation of some important woody plant species against wood destroying activity of honey fungus. Australian Journal of Crop Science. 8(6), 881-886.

Thomidis T, Exadaktylou E (2012) Effectiveness of cyproconazole to control Armillaria root rot of apple, walnut and kiwifruit. Crop Protection. 36, 49-51. doi: 10.1016/j.cropro.2012.02.003

Thompson DP (1989) Fungitoxic activity of essential oil components on food storage fungi. Mycologia. 81, 151-153. doi: 10.2307/3759462

Vahdati K, Sarikhani S, Arab MM, Leslie CA, Dandekar AM, Aletà N, Bielsa B, Gradziel TM, Montesinos Á, Rubio-Cabetas MJ, Sideli GM, Serdar Ü, Akyüz B, Beccaro GL, Donno D, Rovira M, Ferguson L, Akbari M, Sheikhi A, Sestras AF, Kafkas S, Paizila A, Roozban MR, Kaur A, Panta S, Zhang L, Sestras RE, Mehlenbacher S (2021) Advances in rootstock breeding of nut trees: objectives and strategies. Plants 10 (11), 2234.

Volk TJ (2005). Key to North American *Armillaria* species. Retrieved May 18, 2017 from the Tom Volk's Fungi Web site: http://botit.botany.wisc.edu/toms_fungi/armkey.html

West JS, Fox RTV (2002) Stimulation of *Armillaria mellea* by phenolic fungicides. Annals of Applied Biology - AAB. 140, 291-295. doi: 10.1111/j.1744-7348.2002.tb00184.x

Woodward JE, Brenneman TB (2008) Development of an inoculation method for quantifying fungicide residues on peanut foliage. Peanut Science. 35, 25-31. doi: 10.3146/PS06-045.1

Worrall JJ (1991) Media for selective isolation of hymenomycetes. Mycologia. 83, 296-302. doi: 10.1080/00275514.1991.12026013

Yousefi Hamedani E, Sharifnabi B, Bahar M (2012) Application of classical and molecular techniques in detection of *Armillaria mellea* the causal agent of root and crown rot disease from soil and wood. Iranian Journal of Plant Pathology. 48, 223-235.