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Evaluation of some important woody plant species against wood destroying activity of honey fungus

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Abstract

Armillaria mellea is one of the most important pathogens of plant species in horticultural and forest regions of Iran. In order to management of Armillaria root rot disease by tolerant plant species, resistant of 11 woody plants species was investigated against *Armillaria mellea*. In this study, 27 isolates of *Armillaria* spp. were collected from forest regions of Mazandaran province in north of Iran. The fungal isolates were identified using of RFLP-PCR analysis of Internal Transcribed Spacer 1 (ITS1) region. Then, resistance of the plant species was studied against the pathogen *in vitro*. The data analysis proved that *Armillaria mellea* isolates A23 and A1 caused the highest and lowest level of wood destroying activity with 11.82 and 7.11% wood weight loss, respectively. The tested plant indicated significant difference against the fungus. *Citrus aurantium* L., *Ficus carica* L., and *Olea europaea* L. with 19.00, 16.25 and 15.85% wood weight loss showed susceptible reaction to *A. mellea*. However, *Zelkova carpinifolia* Dippel with 1.68% wood weight loss were introduced as most tolerant species to *A. mellea* which followed by *Prunus divaricate* Ledeb. and *Diospyrus lotus* L., with 5.6 and 7.46% of wood weight loss, respectively.

Keywords: *Armillaria mellea*, Armillaria root rot, resistance, RFLP-PCR, Iran.

Introduction

Armillaria (Fr.) Staude is a genus of Basidiomycetes in the family Tricholomataceae, Agaricales (Sicoli et al., 2003) with world-wide distribution and forty two described species (Fox, 2000). The genus *Armillaria* comprises a group of fungi causing the important disease known as Armillaria root rot. This disease can cause the substantial losses in natural forests, commercial forest plantations, and horticultural crops (Hood et al., 1991; Kile et al., 1991). Armillaria root rot attacks over 700 species of plants (http://www. chasehorticulturalresearch. com/pdfs/armillaria_ root.pdf). *Armillaria mellea* is the most common in milder temperate regions, particularly Mediterranean climates, where it is animportant agent of mortality of ornamental, fruit and forest trees (Sung et al., 1991; Harrington et al., 1992; Guillaumin et al., 1993). The disease was first reported on *Castanea crenata* in 1903 (Nomura, 1903). *Armillaria* is a primary pathogen causing root rot of tea (*Camellia sinensis*) in Kenya and yield losses is about 50% in small holder farms (Onsando et al., 1997). Armillaria root rot has been recorded on *Eucalyptus* and *Pinus* spp. from the northern parts of South Africa (Doidge et al., 1953; Wingfield and Knox-Davies, 1980). *Armillaria mellea* and *A. gallica* were important in hardwood forests of England (Rishbeth, 1985; Davidson and Rishbeth, 1988; Rishbeth, 1991) and California (Baumgartner and Rizzo, 2001). Armillaria root rot disease is an important disease of fruit, nut, and vine crops in California. Of all the deciduous fruit crops in California, pears have been considered among the least susceptible to infection by *Armillaria* (Thomas, 1934; Raabe, 1972: Ogawa and English, 1991). In Trentino region, in the north east of Italy, *A. mellea* root rot is a severe and increasing problem on grapevine (Gobbin et al., 2006). In northern Italy, results of the 4-year assessment were that 25% of the vineyards were infected by *Armillaria*. The causal agent was almost exclusively *A. mellea* but, in two cases, *A. gallica* was also isolated (Pertot et al., 2007). In Iran, *A. mellea* is widely distributed throughout the country and it has been reported as pathogen of fruit and forest tree species (Saber 1974; Ershad, 1995; Asef et al., 2003; Dalili et al., 2008). Results of several researchers reported considerable differences in reaction of plant species against the fungus. Pataky (2000) evaluated some plant species and introduced resistant woody plants to Armillaria root rot. Dalili et al. (2010) investigated resistance of horticultural and forest plant species and the results demonstrated there was significant difference. *Armillaria mellea* is the prevalent species of the genus in different parts of Iran and threaten susceptible horticultural and forest plant species in the infected areas. In this investigation, reaction of the 11 plant species was studied against the pathogen *in vitro* to find tolerant or resistant species to planting in high risk areas. Application of resistant species is one of the most important methods for managing the disease.

Results and discussion

Analysis of ITS1 region for identifying A. mellea species

The ITS1 region amplification of Iranian and test strains isolates was conducted with primers ITS1 and ITS2 (Fig. 1). After restricting by *HinfI*, two clearly distinct patterns were obtained in the test strains, one specific for all *A. mellea* isolates (mellea pattern) and the other common to the remaining *Armillaria* spp. (non-mellea pattern). Both patterns

Table 1. *Armillaria* isolates from Iran: origins, source tissues, host trees, identifications based on ITS1 RFLP-PCR patterns.

Isolate No.	Derivation	Host	Localities	ITS RFLP patterns	Species
A1	Wood fragment	*Fagus orientalis*Lipsky	Mazandaran-Behshar	I	*A. mellea*
A2	Wood fragment	*Carpinus betulus*L.	Mazandaran-Behshar	I	*A. mellea*
A3	Wood fragment	*Carpinus betulus*L.	Mazandaran-Behshar	I	*A. mellea*
A4	Wood fragment	*Crataegus pentagyna* Walds. Et Kit	Mazandaran-Behshar	I	*A. mellea*
A5	Wood fragment	*Fagus orientalis*Lipsky	Mazandaran-Behshar	I	*A. mellea*
A6	Wood fragment	*Carpinus betulus*L.	Mazandaran-Behshar	I	*A. mellea*
A7	Wood fragment	*Abies alba* Mill.	Mazandaran-Neka	I	*A. mellea*
A8	Basidiocarp	*Parrotia persica* C. A. Mey	Mazandaran-Neka	I	*A. mellea*
A9	Basidiocarp	*Parrotia persica* C. A. Mey	Mazandaran-Neka	I	*A. mellea*
A10	Basidiocarp	*Parrotia persica* C. A. Mey	Mazandaran-Neka	I	*A. mellea*
A11	Basidiocarp	*Parrotia persica* C. A. Mey	Mazandaran-Neka	I	*A. mellea*
A12	Wood fragment	*Fagus orientalis*Lipsky	Mazandaran-Neka	II	*Armillaria* sp.
A13	Wood fragment	*Diospyros lotus* L.	Mazandaran-Neka	II	*Armillaria* sp.
A14	Wood fragment	*Carpinus betulus*L.	Mazandaran-Neka	I	*A. mellea*
A15	Wood fragment	*Carpinus betulus*L.	Mazandaran-Sangedeh	I	*A. mellea*
A16	Wood fragment	*Carpinus betulus*L.	Mazandaran-Sangedeh	I	*A. mellea*
A17	Basidiocarp	*Carpinus betulus*L	Mazandaran-Sangedeh	I	*A. mellea*
A18	Basidiocarp	*Carpinus betulus*L.	Mazandaran-Sangedeh	I	*A. mellea*
A19	Basidiocarp	*Picea abies*L.	Mazandaran-Sangedeh	II	*Armillaria* sp.
A20	Basidiocarp	*Citrus aurantium*L.	Mazandaran-Tonekabon	I	*A. mellea*
A21	Basidiocarp	*Alnus subcordata* C. A. Mey	Mazandaran-Chamestan	I	*A. mellea*
A22	Wood fragment	*Carpinus betulus*L.	Mazandaran-Chamestan	I	*A. mellea*
A23	Wood fragment	*Gleditsia caspia*Desf.	Mazandaran-Chamestan	I	*A. mellea*
A24	Wood fragment	*Acer* sp.	Mazandaran-Chamestan	I	*A. mellea*
A25	Basidiocarp	*Quercus castaneifolia* C. A.Mey	Mazandaran-Chamestan	II	*Armillaria* sp.
A26	Wood fragment	*Gleditsia caspia*Desf.	Mazandaran-Chamestan	I	*A. mellea*
A27	Wood fragment	*Acer* sp.	Mazandaran-Chamestan	I	*A. mellea*

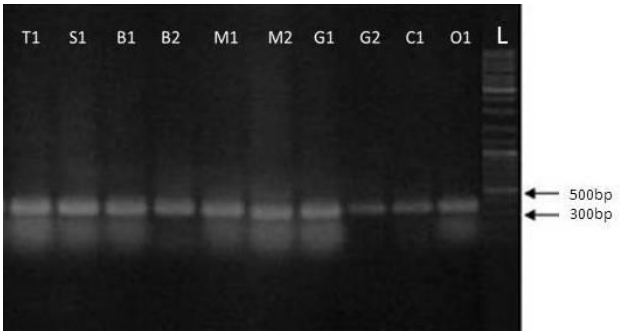

Fig 1. Intact ITS1 amplicons (360 bp.) of the *Armillaria* test strains. T (*A. tabescens*), S (*A. Sinapina*), B (*A. borealis*), M (*A. mellea*), G (*A. gallica*), C (*A. cepistipes*), O (*A. ostoyae*).

consisted of two fragments as follows: fragments with 290 bp and 70 bp in length (non-mellea pattern; T_1, S_1, B_1, B_2, G_1, G_2, C_1, O_1 in Fig.2) and fragments with 230 bp and 130 bp in length (mellea pattern; M_1, M_2). The results showed that 23 isolates (85.18%) were identified as *A. mellea* and the species was the most prevalent species in north of Iran. *Armillaria mellea* was isolated from different plant species such as *Fagus orientalis*, *Parrotia persica*, *Citrus aurantium*, *Carpinus betulus*, *Crataegus pentagyna*, *Abies alba*, *Alnus subcordata*, *Acer* sp. and *Gleditsia caspia*. Four isolates (A12, A13, A19 and A25) indicated pattern non-mellea species (Table 1). *Armillaria mellea* was important pathogen of the roots of many woody perennial plants (Farr et al., 1989). The species was common in mixed hardwood forests of California (Baumgartner and Rizzo, 2001). Baucom et al. (2005) investigated causal agent of oak decline in the Missouri Ozark Mountains and the results showed that 52%, 38% and 10% of the isolates were belonged to *A. mellea*, *A. gallica* and *A. tabescens*, respectively. In Europe, two species, namely *A. mellea* and *A. ostoyae* were regarded to be highly pathogenic and able to act as primary pathogens causing lethal disease (Gregory et al., 1991). Five *Armillaria* species were found during a survey of forest ecosystems in Serbia. Combinations of *A. gallica*, *A. mellea*, *A. ostoyae* and *A. cepistipes* have been most frequently observed, and on some mountain sites the combination of *A. ostoyae*, *A. cepistipes* and *A. gallica* were common (Keça and Solheim, 2006). In the north east of Italy, *A. mellea* is one of the most important fungal pathogens on grapevine (Gobbin et al., 2006).

Table 2. ANOVA table for wood weight loss (%) of different plant species caused by *A. mellea* isolates.

Source of variances	Degree of freedom	Sum of square	Means of square	F value
Isolates	2	402.55	201.27	212.82**
Plant species	10	2316.03	231.60	244.88**
Isolates × Plant species	20	164.08	8.20	8.67
Error	66	62.42	0.94	
Total	99	2945.09		
CV	9.83%			

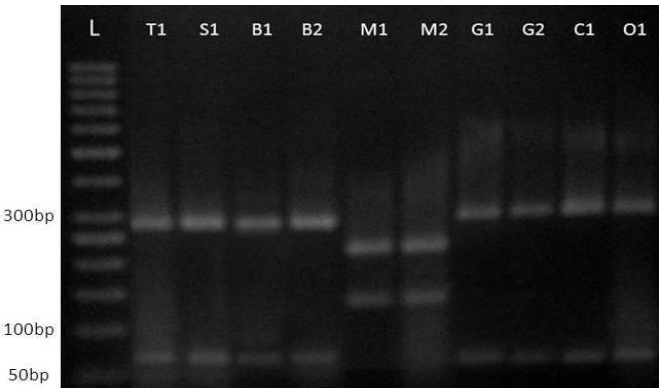

Fig 2. Restriction profiles of the *Armillaria* test strains ITS1 region with *HinfI*:mellea pattern (M1 andM2) with 230 and 130 bp;non-mellea pattern (T1, S1, B1, B2, G1, G2, C1 and O1) with 290 and 70 bp.in PCR-RFLP analyses.

In northern Italy, results of the 4-year assessment were that 25% of the vineyards were infected by *Armillaria*. The causal agent was almost exclusively *A. mellea* (Pertot et al., 2007). Twelve vineyards in North West of Spain were studied to assess the incidence of white root rot during 1995 and 1997. In both years, diseased plant material was collected and the *Armillaria* species responsible was identified on the basis of compatibility testing. During 1997, 83.33 percent of investigated vineyards were affected and three indicated a marked increase in the number of plants with white root rot (to 43%, in the vineyard in which 17% were affected in 1995). The results exhibited *A. mellea* was detected in samples from 10 of the 12 vineyards, and *A. gallica* in samples from two vineyards (Aguín-Casal et al., 2004). Keča et al. (2006) identified *A. mellea* by RFLP analysis on *Coprinus betulus* and *Quercus petraea* in Serbia and Montenegro. Coetzee et al. (2001) identified *A. mellea* on *Quercus* spp. by RFLP analysis of ITS region. Otieno et al. (2003) reported ITS PCR-RFLP profiles of *A. mellea* digested with *Alu*I, *Hinf*I and *Nde*II**.** The ITS1 region of Portuguese and European reference isolates was amplified with primers ITS5' and ITS2 and the length of the amplicons was estimated as 370 bp for *A. mellea* and 360 bp for the remaining *Armillaria* species. Different size of fragment can be used for direct identification of *A. mellea.* When *HinfI* was used to digest the ITS1 amplicon, two clearly distinct patterns were obtained, one specific fragment for all *A. mellea* isolates and the other common to the remaining *Armillaria* spp. (Bragança *et al.*, 2004). In Iran *Armillaria* has widely distributed throughout the country and was a well-known causal agent of root rot diseases. *Armillaria mellea* were reported on *Amygdalus communis*, *Cerasus avium*, *Crataegus sp*., *Cydonia oblonga*, *Platanus orientalis*, *Prunus* sp., *P. spinosa*, *Pyrus communis*, *Rosa* sp. and *Vitis vinifera* (Saber, 1974). Armillaria rot disease was reported in association with many cultivated and forest tree species (Ershad, 1995). Dalili et al. (2007, 2008b, 2008c, 2008d, 2009) reported new hosts of *A. mellea* and *A. gallica* from different regions of Iran and introduced *A. mellea* as the most prevalent species.

Wood destroying activity analysis

For evaluating the wood destroying activity of the isolates, the bars were taken out of test-tubes after 60 days the outer mycelia were peeled off. The samples were dried in 105 ºC during 24 hours and weighed. To survey ability of wood destroying activity of different isolates, the percent of weight loss was calculated. The data were analyzed with MSTAT-C statistical program. Analysis of variance of wood destroying activity of the isolates exhibited significant difference ($P<0.01$) among isolates and plant species (Table 2). The means comparison of different isolates indicated that isolate A23 with 11.82% weight loss had the highest level of wood destroying activity (class A) followed by A15, and A1 with 10.75, and 7.11% weight loss, respectively were settled in the next rank. The host plants indicated different reaction to *A. mellea* wood destroying activity. Comparison of the means of different plants species exhibited, there was significant difference ($P<0.01$) on the rate of wood destroying activity, and the plants species placed in different groups. *Citrus aurantium* with 19.00% weight loss had the lowest level of resistance to wood destroying activity (class A) and followed by *Ficus carica*, *O. europaea*, and *Fagus orientalis* with 16.25, 15.88 and 9.34 % weight-loss respectively. *Zelkova carpinifolia* and *Prunus divaricata* with 1.68% and 5.60% weight-loss were resistant to *A. mellea* (Fig.3). Study on the interaction among hosts and isolates showed that isolate A15 had the highest level of wood destroying activity on *Citrus aurantium* with 21.78 and followed by A23 on C*itrus aurantium* and O. *europaea* with 20.78 and 20. 10% weight loss respectively. The isolates indicated high level of wood destroying activity on *Ficus carica*. Isolate A1 exhibited the highest level of wood destroying activity on *Citrus aurantium* and *Ficus carica* (Table 3). This study indicated the ability of isolates for wood destroying activity was different while the reaction of hosts was constant. *Citrus*

Table 3. Mean wood weight loss (%) of different plant species caused by *A. mellea* isolates.

Plant species	*A. mellea* isolates		
	A1	A15	A23
Zelkova carpinifolia	1.43P	1.24P	2.34OP
Quercu scastaneifolia	6.13LMN	8.70GHIJK	9.87FGH
Ficus carica	12.78DE	17.33C	18.54BC
Carpinus betulus	5.36MN	8.20 GHIJKL	9.35FGHI
Parrotia persica	6.59KLM	10.04 FGH	11.37EF
Fagus orientalis	6.73JKLM	10.05FGH	11.22EF
Diospyros lotus	5.93LMN	7.64 HIJKLM	8.79GHIJK
Olea europaea	3.95NO	5.75MN	7.08IJKLM
Prunus divaricata	8.23GHIJKL	18.40BC	20.10AB
Crataegus sp.	6.54KLM	9.09FGHIJ	10.59FG
Citrus aurantium	14.44D	21.78A	20.78A

Means within each column having the same letters are not significantly different

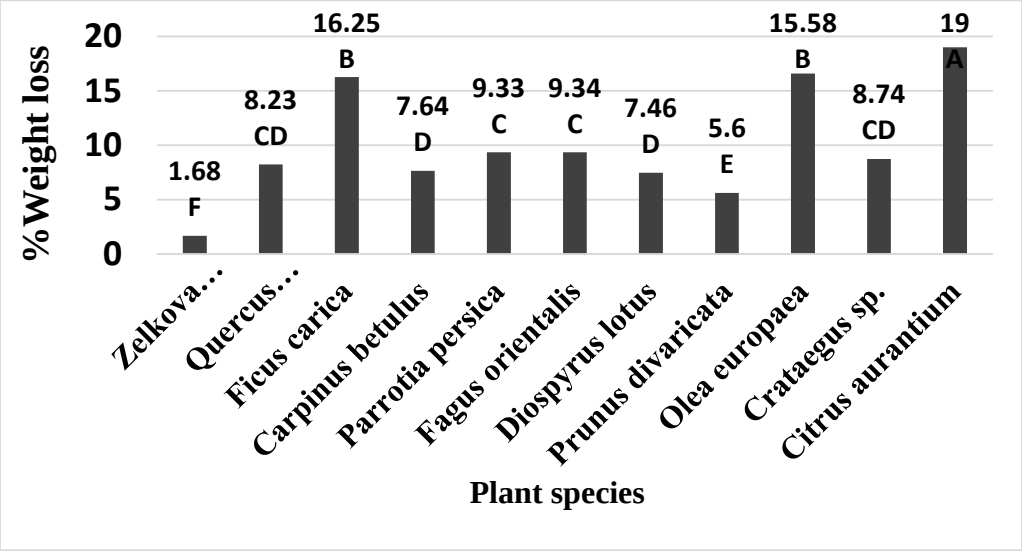

Fig 3. The mean comparisons of wood weight loss (%) of different plants species by *A. mellea* based on Duncan test at P<0.01.

aurantium, *Ficus carica* and O. *europaea* showed susceptibility to the most of *A. mellea* isolates while *Z. carpinifolia*. and *P. divaricata* exhibited tolerance reaction to most of *A. mellea* isolates. The published results revealed *Citrus* spp. and some species of the genus *Prunus* such as *Prunus caroliniana*, *Prunus ilicifolia* and *Prunus lyonii* were resistant to *A. mellea* (http://www.chasehorticulturalresearch. com/pdfs/ *Armillaria*_root.pdf). Donovan (2007) suggested all commonly grown citrus cultivars are susceptible to *A. mellea* in coastal NSW citrus orchards of Australia. Guillaumin et al. (2003) investigated the level of resistance to *A. mellea* within plum species (*Prunus domestica*, *Prunus insititia*, *Prunus cerasifera*) and created rootstock resistant to *Armillaria* spp.. Papachatzis et al. (2008) indicated *A. mellea* was observed as the most aggressive rot pathogens of *Ficus carica* cultivar (Smyrna) in central Greece. Infections are common in fig cultivars near the forest. The wood destroying activity of different isolates of *A. mellea* was investigated on twelve horticultural and forest plants species. The results exhibited the isolates M1 and E1 caused the highest and lowest level of wood destroying activity with 8.782 and 6.719 % wood weight loss, respectively. The results revealed the resistance of the plant species was very different and among the tested plant species. *Citrus aurantium*, *Juglans regia*, *Carpinus betulus* and *Acer* sp. with 10.430, 7.879, 7.401 and 7.342 % wood weight loss respectively, showed susceptible reactions to *A. mellea*. However, *Prunus devaricata*, *Amygdalus communis*, *Armeniaca vulgaris* and *Pyrus communis* with 3.491, 3.506, 4.648 and 5.337 % wood weight loss respectively were regarded as tolerant species to *A. mellea* (Dalili et al., 2010).

Materials and Methods

Sampling and fungal isolation

The infected plants with suspicious symptoms of *Armillaria* infection, with mycelial fans, rhizomorph signs or basidiocarps, were collected from various regions of Mazandaran provinces in north of Iran. Samplings were made from the 12 different host species during 2011-2012. For isolating haploid strains, the suspension of basidiospore was spread on water agar and incubated at 22±1ºC. After germination, the germinated basidiospores were separated and placed on malt extract agar (20 g/l malt extract, 16 g/l agar). In order to derive the dikaryotic isolates, the infected tissues or basidiocarps were sterilized in 96% ethanol for 1 min, and washed with sterilized water. The small pieces of the tissues were excised and placed on the Petri dishes containing malt extract agar amended with benomyl WP 50 (4 µg a.i./ml) and streptomycin sulfate (100 µg/ml) added after autoclaving. The Petri dishes were incubated at 22±1ºC (Worrall, 1991).

Identification of Armillaria mellea based on Polymerase Chain Reaction- Restriction Fragment Length Polymorphism (PCR- RFLP)

The freeze dried mycelia (100 mg) were disrupted by grinding to a fine powder under liquid nitrogen using a mortar and pestle. DNA was extracted by CTAB (hexadecyltrimethylammonium bromide) method (Zolan and Pukkila, 1986). The extracted DNA was resuspended in 50 µL of TE (10 mMTris-Base, 1 mM EDTA, pH 8.0).

Amplification of ITS1 region, located between the 18S and the 5.8S ribosomal DNA genes, was conducted by using primers ITS1 and ITS2 (White et al., 1990). The PCR reaction mixture (50µL) included 80 ng of template DNA, 2 U of Taq DNA polymerase, total dNTP 800 µM, 1 x PCR buffer supplied with the enzyme, and 4mM $MgCl_2$ (Fermentas Inc., USA), 50 pmol of each primer. The final reaction volume was adjusted to 50 µL with H_2O (Bragança et al., 2004). The mixture was denatured at 95°C for 2 min followed by 35 cycles at 95°C for 30 sec, 58°C for 30 sec, for 2 min at 72°C and final cycle at 72°C for 10 min, run on 1.2% w/v agarose gel (Fermentas Inc., USA), in 0.5 X TBE at 100 V for 90 min, using 100 bp DNA Ladder as molecular size marker (Gezahgne et al., 2004). Restriction analysis of ITS1 region was carried out by manufacturers' recommended (Fermentas Inc., USA), 5 µL of PCR product was digested with 3 U of *Hinf*I restriction enzyme, in a final volume of 10 µL. After overnight incubation at 37°C, the reaction was stopped by adding 1.5 µL of bromo-phenol blue solution (0.25% bromo-phenol blue, 0.25% xylene cyanol, 10 mM EDTA, 15% Ficoll in water) and run on 3% w/v agarose gel, in 0.5 X TBE at 100 V for 2 h and 30 min, using 50 bp or 100 bp standard DNA ladder (Fermentas Inc., USA). The gels were stained with ethidium bromide solution (0.5 mg/ml) and visualized under UV irradiation (Gezahgne et al., 2004).

Wood destroying activity in vitro

Resistance of 11 plants species including *Z. carpinifolia, Quercus castaneifolia, Carpinus betulus, Ficus carica, Parrotia persica, Fagus orientalis, D. lotus, O. europaea, Crataegus* sp., *Prunus divaricata* and *Citrus aurantium* was studied *in vitro*. In this study, pieces of the bar were prepared in 10x10x100 mm size. The samples were dried in oven in 105 ºC for 24 hours until absolute dry condition and weighed with ±0.001g accuracy. The bars were placed in test-tubes with malt extract culture medium and sterilized. The tested samples were inoculated with one inoculum disc (5mm in diameter on malt extract agar media). Three isolates of *A. mellea* were used including A1, A15, and A23 and the check treatment was inoculated with malt extract agar media. The test-tubes incubated in 24±1 ºC and after 60 days, the bars were taken out of test-tubes and peeled off. The samples were dried in 105 ºC during 24 hours and weighed. The experiment was conducted based on Completely Randomized Design (CRD) with three replications. For resistant evaluation of the plant species, the percent of weight-loss was calculated as follow:

$$C = [(P - P1) / P] \cdot 100\%$$

in which, C= Percent of weight-lost; P =The weight of tree bars in absolutely dry condition before inoculation P1- The weight of tree bars in absolutely dry condition after inoculation (Nanagulyan, 1997).

Conclusion

Twenty seven isolates of *Armillaria* spp. were obtained from 12 different hosts in Mazandaran province of Iran. The fungi isolated from *Fagus orientalis, Parrotia persica, Citrus aurantium, Abies alba, Carpinus betulus, Alnus subcordata, Acer* sp., *G. caspia, Diospyrus lotus, Picea abies, Q. castanefolia* and *Crataegus pentagyna*. The *A. mellea* species was isolated from *Fagus orientalis, Parrotia persica, Citrus aurantium, Abies alba, Carpinus betulus, Alnus subcordata, Acer* sp., *G. caspia,* and *Crataegus pentagyna*. *Armillaria mellea* was the most abundant species among the collected samples. Four isolates showed non-mellea pattern. By using of the method 85.18% of the isolates were identified as *A. mellea*. The wood destroying activity of *A. mellea* isolates evaluated on the different host plants species. In this study, reaction of three isolates of *A. mellea* against 11 plant species was investigated. Results showed that the isolates had significant difference on the rate of wood destroying activity and the isolates were placed into different groups. The isolateA23 with 11.82% weight loss had the highest level of wood destroying activity. The results exhibited, the reaction of plant species to wood destroying activity by *A. mellea* was significantly different and *Z. carpinifolia* indicated maximum level of tolerance. This investigation also revealed tested forest plant species more tolerant than horticultural plant species. In order to management of Armillaria root rot in the high risk locations, using of the tolerance species will be necessary to decrease the decline, mortality of plants and its production. Citrus cultivation is very important in Mazandaran province, by attention to susceptibility of *Citrus* sp. against *A. mellea*, it is necessary to avoid from planting of different cultivars of *Citrus* spp. in near of forest regions of Mazandaran.

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