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Investigation of the wood destroying activity of *Armillaria mellea* on horticultural and forest plants species

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Abstract

Nineteen isolates of *Armillaria* spp. were collected from East Azerbaijan, Isfahan and Mazandaran provinces of Iran. The isolates were identified by using pairing tests and restriction analysis of Internal Transcribed Spacer 1 (ITS1) region. In this study, the wood destroying activity of four *A. mellea* isolates were investigated on twelve horticultural and forest plants species. The analysis of wood destroying activity data showed that the isolates M1 and E1 caused the highest and the lowest level of wood destroying activity with 8.782 and 6.719 % wood weight loss, respectively. The results indicated that the resistance of the plant species was very different. 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 respectively 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*. The tolerant plants species have potential to be used for the management of the disease and might potentially reduce the damages caused by *A. mellea*.

Key words: *Armillaria mellea*, paring tests, ITS1, wood destroying activity, Iran.

Introduction

The genus *Armillaria* has a world wide distribution and includes some of the most important root fungal pathogens of forest trees and fruit crops (Pegler, 2000). It comprises a group of fungi that causes an important disease known as Armillaria root rot. For most plant pathologists, this is a well-known disease due to the substantial losses that it can cause in natural forests, commercial forest plantations and horticultural crops (Hood *et al.,* 1991; Kile *et al.,* 1991). *Armillaria* spp. have been regarded as primary pathogens, stress-induced secondary invaders and saprophytes (Wargo and Shaw, 1985; Shaw and Kile, 1991).

Armillaria root disease was first reported on *Castanea crenata* in 1903 (Nomura, 1903). It has been recorded on a variety of hosts such as *Eucalyptus* and *Pinus* spp. (Wingfield and Knox-Davies, 1980) and on pines and other woody hosts (Coetzee *et al.,* 2000) from the northern parts of South Africa. It was also reported in Wyoming in the United States. Three *Armillaria* species were identified as *A. sinapina*, *A. gallica* and *A. ostoyae* (Blodgett and Lundquist, 2006). In Kansas, *A. tabescens* was found in windbreak plantings with elm trees. *Armillaria ostoyae* was found in several root disease centers in South Dakota and Wyoming. Harris (2004) reported that it was not a major problem in most of these areas, but it is likely contributing to mortality. Incidences of Armillaria root rot has also been reported on various planted and natural hosts in South America and Indo-Malaysia (Hood *et al.,* 1991). Many *Armillaria* species linked to outbreaks of the disease in South America were thought to be restricted to this area (Kile *et al.,* 1994).

Armillaria root disease is an important disease of fruit, nut, and vine crops in California and pears have been considered among the least susceptible to infection by *Armillaria* (Ogawa and English, 1991). French pear, Bartlett rooted cuttings, Old Home × Farmingdale (all *Pyrus communis*), *P. betulaefolia,* and *P. calleryana* have all been listed as immune or highly resistant (Raabe, 1979). Based on this premise, pear has been recommended as an alternative crop to replant sites in which *Armillaria* has been demonstrated to be a potential probl-

Table 1. Species and geographic location of haploid test strains of *Armillaria* spp.

Taxonomic Name	Isolate code	Collectors Name	Determined By	geographic locality
Armillaria borealis Marxm. & Korhonen	99 68\4	K. Korhonen	K. Korhonen	Finland
Armillaria borealis Marxm. & Korhonen	n, 2n	M.R. Asef	M.R. Asef	Iran
Armillaria cepistipes Velen.	MB 79.23.1	J.J. Guillaumin	K. Korhonen	Finland
Armillaria cepistipes Velen.	MB 79.24.1	J.J. Guillaumin	K. Korhonen	Finland
Armillaria gallica Marmx. & Romagn.	ME 70.1.2	J.J. Guillaumin	J.J.Guillaumin	France
Armillaria gallica Marmx. & Romagn.	n, 2n	M.R. Asef	M.R. Asef	Iran
Armillaria mellea (Vahl) P. Kumm.	87 085\10	K. Korhonen	Grillo	Italy
Armillaria mellea (Vahl) P. Kumm.	90 254\3	K. Korhonen	Grillo	Italy
Armillaria mellea (Vahl) P. Kumm.	90260\1	K. Korhonen	Munda	Yugoslavia
Armillaria ostoyae (Romagn.) Herink	99 088\3	K. Korhonen	K. Korhonen	Finland
Armillaria ostoyae (Romagn.) Herink	MC 79.27.1	J.J. Guillaumin	K. Korhonen	Finland
Armillaria sinapina Bérubé & Dessur.	96-7-1	Yuko Ota	Yuko Ota	Japan
Armillaria sinapina Bérubé & Dessur.	96-7-2	Yuko Ota	Yuko Ota	Japan
Armillaria tabescens (Scop.) Emel	NT 1-9	Yuko Ota	Yuko Ota	Japan
Armillaria tabescens (Scop.) Emel	NT 1-10	Yuko Ota	Yuko Ota	Japan

em (Smith, 1941). Martin (2007) tested suscepti- bility of different horticultural and forest plants species to *Armillaria*. In Iran, *A. mellea* is widely distributed throughout the country and it has been reported as the pathogen of fruit and forest tree species (Saber 1974; Asef *et al.,* 2003; Dalili *et al.,* 2008; Ershad, 1995). The objective of this study was to investigate the wood destroying activity of *A. mellea* on forest and horticultural plant species and to find resistant sources to the pathogen.

Materials and methods

Sampling and fungal isolation

Nineteen samples were collected from 11 horticultural and forest plant species showing symptom of *Armillaria* infection in Mazandaran, East Azerbaijan and Isfahan provinces of Iran from 2006-2008. The infected tissues, rhizomorph or basidiocarps were sterilized in 96 % ethanol for 1 min, and small pieces were excised and placed on plates containing malt extract agar (20 g/l malt extract, 16 g/l agar) amended with benomyl WP 50 (8 mg/l) and streptomycin sulfate (100 mg/l) after auto-claving. The plates were incubated at 22±1ºC (Worrall, 1991).

Identification of *Armillaria* spp.

Pairing tests

Pairing tests of the Iranian diploid and haploid isolates were done by using the known haploid test strains of *Armillaria* spp. The seven biological species were used and each isolate was paired with two or three different test strains of the known biological species. Haploid test strains of *Armillaria* spp. and the geographic sources are presented in Table 1. The inoculums, which consisted of undifferentiated mycelium without crust or rhizomorph. Mycelial plugs (3 mm diam.) were derived from the margin of a growing culture. These plugs were placed side by side. Two different pairs of isolate were settled in each Petri dish and each pairing was repeated twice. The Petri dishes with diploid-haploid or haploid-haploid pairings were incubated at 22±1ºC and the evaluation was done after 6-8 weeks (Korhonen, 1978).

Restriction fragment length polymorphism (RFLP)

DNA extraction

The isolates were grown in liquid MYE (2% Malt extract and 0.3% Yeast extract) at 22ºC in the dark for 4 weeks. Mycelium was harvested by centrifugation (15300 g for 20 min) and washed in sterile distilled water (Coetzee *et al*., 2000). The freeze dried mycelia were mechanically disrupted by grinding them into fine powder under liquid nitrogen using a mortar and pestle. The DNA was extracted using cetyltrimethylammonium bromide (CT-AB) method and resuspended in 50 µL of TE (10 mM Tris-Base, 1 mM EDTA, pH 8.0) and stored at 4ºC for later use (Zolan and Pukkila, 1986).

Amplification of ITS1

The ITS1 region, located between the 18 S and the 5.8 S ribosomal DNA genes, was amplified by PCR 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, 200 µM of each dNTP, 1 x PCR buffer supplied with the enzyme, and 4mM $MgCl_2$, 50 pmol of each primer. The final reaction volume was adjusted to 50 µL with H_2O (Bragança *et al*., 2004). Amplification was carried out using initial denaturation 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 TAE at 100 V for 90 min, using 100 bp DNA Ladder as molecular size marker (Gezahgne *et al*., 2004).

Restriction analysis of ITS1

To perform restriction analysis of ITS1 region, 5 µL sample of each PCR product was digested with 3 U of HinfI restriction enzyme, in a final volume of 10 µL, following the manufacturer's instructions. After incubating overnight at 37°C, 1.5 µL of bromophenol blue solution (0.25% bromo-phenol blue, 0.25 % xylene cyanol, 10 mM EDTA, 15 % Ficoll in water) were added to each sample to stop the reaction. Each reaction sample was run on 3 % w/v agarose gel, in 0.5 x TAE 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 using UV light (Gezahgne *et al*., 2004).

Wood destroying activity

In order to investigate the wood destroying activity of *A. mellea*, the 12 plants species: *Citrus aurantium, Juglans regia, Carpinus betulus, Acer sp., Populus nigra, Malus domestica, Parrotia persica, Platanus orientalis, Pyrus communis, Armeniaca vulgaris, Amygdalus communis* and *Prunus devaricata* were evaluated. Pieces of the bar were prepared in 10×10×100 mm size. The samples were dried in an oven at 105ºC for 24 hours until obtaining absolute dry conditions was ensured and were weighed with ±0.001g accuracy. The bar samples were put in test tubes with malt extract culture medium and sterilized. The samples were inoculated with mycelial plug (10 mm diam.) of the isolates. Four isolates from Mazandaran (M1 and M4), East Azerbaijan (A6) and Isfahan (E1) provinces were used. The control treatment was not inoculated with the mycelium of fungus. The tubes were incubated at 24±1 ºC. After 60 days, the bars were taken out of the tubes and the outer mycelia peeled off. The samples were dried at 105 ºC for 24 hours then weighed. The experiment was conducted following a completely randomized design with three replications. To evaluate the wood destroying activity of the isolates, the percentage weight loss of each plant species was calculated using the formula:

$$C(\%) = [(P - P_1) / P] \times 100$$

C- Percentage of weight loss
P- The weight of tree bars under absolutely dry conditions before inoculation
P1- The weight of tree bars under absolutely dry conditions after inoculation (Nanagulyan, 1997).

Results and discussion

Identification of *Armillaria* spp.

Mating analysis

To identify the Iranian isolates, a total of 1710 pairings were performed using a set of two or three test strains from the each of seven *Armillaria* species. The majority of isolates were paired with two or three of the known test strains but most of the isolate s showed unclear reaction (Fig.1).

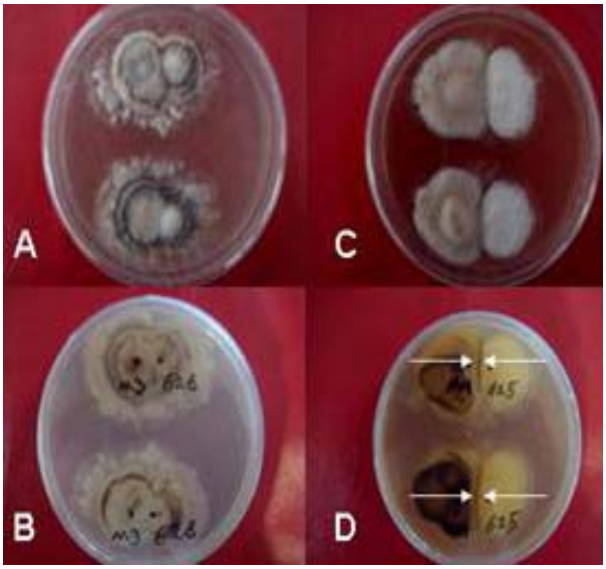

Fig 1. Compatible (a & b) and incompatible (c & d) reactions and formation of black line in contact locality of two incompatible colonies

The results showed that the method allowed the identification of 17 isolates (89 %) in haploid- haploid and diploid-haploid pairings. Fifteen isolates were identified as follows: *A. mellea* from *Fagus orientalis*, *Parrotia persica*, *Citrus aurantium*, *Carpinus betulus*, *Crataegus pentagyna*, *Platanus orientalis*, *Armeniaca vulgaris*, *Amygdalus communis, Juglans regia* and *Pyrus communis*. *Armillaria gallica* was isolated from *Fagus orientalis*, and *Diospyros lotus*. The remaining isolates displayed unclear reactions with all the test strains (Table 2).

Analysis of ITS1 region

The amplification of ITS1 region with primers ITS1 and ITS2 resulted in a single fragment in the test strains and all Iranian isolates. The length of the amplicon was estimated at 360 bp for *Armillaria* species (Fig. 2). When HinfI was used to digest the ITS1 amplicon, two clearly distinct patterns in the test strains were obtained, one specific for all *A. mellea* isolates (mellea pattern) and the other common to the remaining *Armillaria* spp. (non-mellea pattern). Both patterns consisted of two fragments as follows: fragments with 230 bp and 130 bp in length (mellea pattern; ME1, ME2) and fragments with 290 bp and 70 bp in length (non-mellea pattern; B1, B2, O1, C1, G1, G2, S1, S2, T1, T2 in Fig. 3).

Seven isolates from Mazandaran were identified as *A. mellea*. *Fagus orientalis*, *Parrotia persica*, *Citrus aurantium*, *Carpinus betulus* and *Crataegus pentagyna* were the hosts of *A. mellea*. Two isolates (M8 and M9)

Table 2. Summary characteristics and results of the mating analysis of the *Armillaria* isolates used in this study

Isolate No.	Derivation	Host	Localities	Pairing-test results	ITS RFLP patterns
M1	Wood fragment	*Fagus orientalis* Lipsky	Mazandaran	*A. mellea*	I
M2	Basidiocarp	*Parrotia persica* C.A. Mey	Mazandaran	*A .mellea*	I
M3	Basidiocarp	*Parrotia persica* C.A. Mey	Mazandaran	*A. mellea*	I
M4	Basidiocarp	*Citrus aurantium* L.	Mazandaran	*A. mellea*	I
M5	Basidiocarp	*Citrus aurantium* L.	Mazandaran	*A. mellea*	I
M6	Wood fragment	*Carpinus betulus* L.	Mazandaran	*Armillaria sp.*	I
M7	Wood fragment	*Crataegus pentagyna* Walds. et Kit.	Mazandaran	*Armillaria sp.*	I
M8	Rhizomorph	*Fagus orientalis* Lipsky	Mazandaran	*A. gallica*	II
M9	Rhizomorph	*Diospyros lotus* L.	Mazandaran	*A. gallica*	II
A1	Wood fragment	*Armeniaca vulgaris* Lam.	East Azerbaijan	*A. mellea*	I
A2	Wood fragment	*Amygdalus communis* L.	East Azerbaijan	*A. mellea*	I
A3	Wood fragment	*Juglans regia* L.	East Azerbaijan	*A. mellea*	I
A4	Wood fragment	*Pyrus communis* L.	East-Azerbaijan	*A. mellea*	I
A5	Wood fragment	*Armeniaca vulgaris* Lam.	East Azerbaijan	*A. mellea*	I
A6	Mycelium	*Juglans regia* L.	East Azerbaijan	*A. mellea*	I
E1	Wood fragment	*Platanus orientalis* L.	Isfahan	*A. mellea*	I
E2	Wood fragment	*Amygdalus communis* L.	Isfahan	*A. mellea*	I
E3	Wood fragment	*Amygdalus communis* L.	Isfahan	*A. mellea*	I
E4	Wood fragment	*Juglans regia* L.	Isfahan	*A. mellea*	I

showed pattern non-mellea species which were identified by pairing tests as *A. gallica* (Fig. 4).

Six East Azerbaijan's isolates (A1-A6) were identified as *A. mellea* sampled from *Amygdalus communis, Juglans regia, Pyrus communis* and *Armeniaca vulgaris*. Similarly, *A. mellea* was detected on *Platanus orientalis, Amygdalus communis* and *Juglans regia* in Isfahan province (Fig. 5). All of the isolates that were identified by the pairing and ITS1 RFLP-PCR analysis have been shown in table 2.

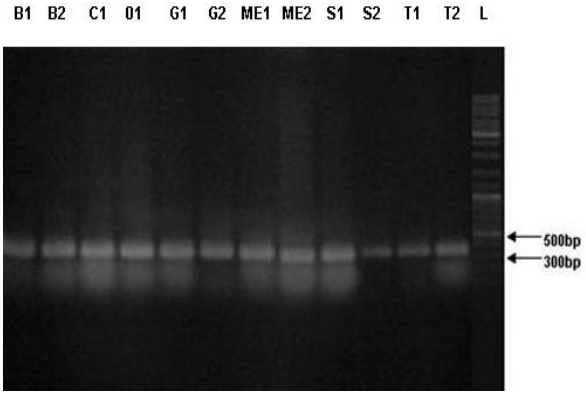

Fig 2. ITS1 amplicons of *Armillaria* test strains; B = *A. borealis*, C = *A. cepistipes*, O = *A. ostoyae*, G = *A. gallica*, ME = *A. mellea*, T = *A. tabescens*, (L: 100 bp DNA ladder)

Armillaria mellea was isolated from different conifers and hardwoods from Japan and *A. gallica* was the prevalent species of the genus *Armillaria* (Ota *et al*., 1998). *A. mellea* was introduced as the main species in Kenya (Otieno, 2003). The fungus was mainly a pathogen of broadleaved trees in ornamental parklands, natural woodlands, fruit orchards, etc at Malawi, but it can kill young coniferous trees (pines, spruce, *etc*.) planted in sites where the broadleaved species were felled (FAO, 2007).

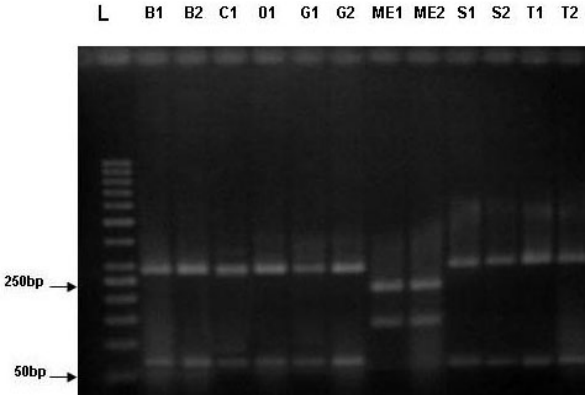

Fig 3. Restriction profiles of ITS1 region of test strains with HinfI. Lanes L 50 bp DNA ladder; B = *A. borealis*, C = *A. cepistipes*, O = *A. ostoyae*, G = *A. gallica*, ME = *A. mellea*, T = *A. Tabescens*

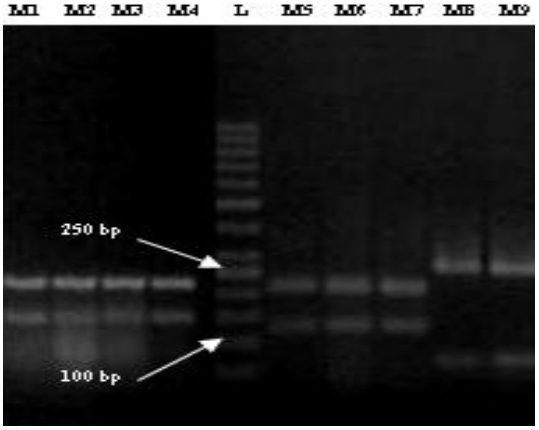

Fig 4. Restriction profiles of ITS1 region with HinfI. Lanes L 50 bp DNA ladder; M1, M2, M3, M4, M5, M6, M7 (mellea pattern), M8, M9 (non-mellea pattern) belong to Mazandaran province

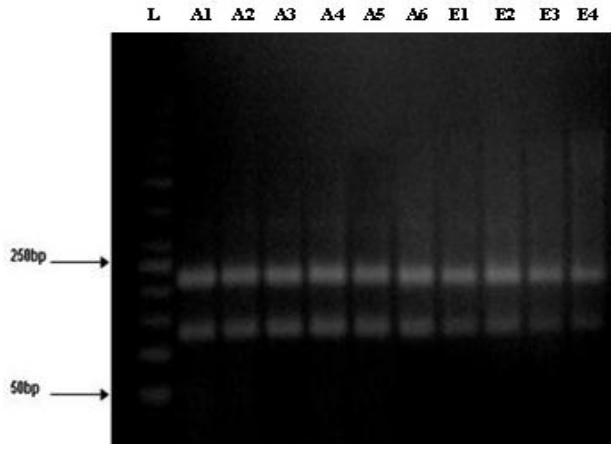

Fig 5. Restriction profiles of ITS1 region with HinfI. Lanes L 50 bp DNA ladder; A1, A2, A3, A4, A5, A6 (East Azerbaijan) and E1, E2, E3, E4 (Isfahan)

Armillaria mellea was reported in central and south Europe, but is common only in the southern and western parts of this area (Korhonen, 2004).

Armillaria gallica was common in coniferous and broad-leaved forests in the high altitudes of central and northern Greece, predominating in the beech forests. The fungus was a weak parasite or a saprophyte of forest trees and was occasionally found on cultivated plants (Tsopelas, 1999). *A. gallica* has been reported from North America (Anderson and Ullrich, 1979). The species was the second most commonly collected species in Wisconsin that was found on Angiosperms (Banik *et al*., 1995).

Armillaria mellea was identified by RFLP analysis on *Coprinus betulus* and *Qurcus petraea* in Serbia and Montenegro (Kečа *et al.,* 2006). *A. mellea* was reported on Quercus spp. by RFLP analysis of ITS region (Coetzee *et al*., 2001). ITS PCR-RFLP profiles of *A. mellea* was digested with *Alu* I, *Hinf* I and *Nde* II (Otieno *et al.,* 2003).The amplification of the ITS1 region with primers ITS5' and ITS2 resulted in a single fragment in all Portuguese and European reference isolates. The length of the amplicons was estimated as 370 bp for *A. mellea* and 360 bp for the remaining *Armillaria* species. This size difference 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 for all *A. mellea* isolates and the other common to the remaining *Armillaria* spp. Both patterns consisted of two fragments as follows: fragments with 245 bp and 125 bp in length (mellea pattern) and fragments with 290 bp and 70 bp in length (non-mellea pattern). By using of this method, different isolates of *A. mellea* from France, Itali and Greece were identified (Bragança *et al.,* 2004).

Armillaria mellea was previously reported from different regions and hosts in Iran (Saber, 1974., Ershad, 1995; Asef *et al*., 2003; Dalili *et al*., 2008). Asef *et al* (2003) reported *A. mellea* on *Ulmus minor, Rosa* sp., *Populus nigra, Amygdalus communis, Cerasus avium, Quercus macranthera, Platanus orientalis, P. persica*, *Acer* sp. and *F. orientalis* in different areas of Iran by Pairing tests. *A. gallica* was previously reported only from stumps in northern of Iran.

Wood destroying activity analysis

The data of weight loss were analyzed with MSTATC statistical program. The isolates showed that the ability of wood destroying activity were different. Analysis of variance of wood destroying activity of the isolates showed that there was significant difference ($P<0.01$) among isolates and plant species. The means comparison of different isolates indicated that isolate M4 with 8.782% weight loss had the highest level of wood destroying activity (class A), while isolates M1, A6, and E1 with 8.117, 7.086 and 6.719% weight loss, respectively were settled in the next class. The control treatment with 0.1804 % weight loss was placed in class E (Fig. 6).

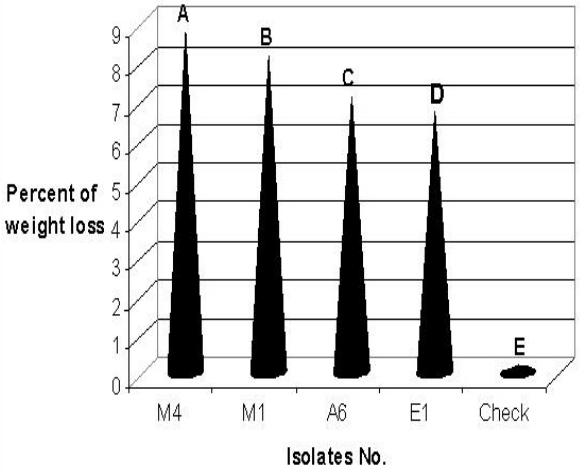

Fig 6. Comparisons of mean of wood weight loss (%) of different isolates of *A. mellea* based on LSD test

The host plants had different reaction to wood destroying activity by *A. mellea*. Comparison of the means of different plants species showed that 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 10.430% weight loss had the highest level of wood destroying activity (class A) to *A. mellea*, and *Juglans regia*, *Carpinus betulus*, *Acer* sp. with 7.879, 7.401 and 7.342 % weight loss respectively were placed in the next group (class B). *Prunus devaricata*, *Amygdalus communis*, *Armeniaca vulgaris* and *Pyrus communis* with 3.491, 3.506, 4.648 and 5.337% weight loss were resistant to *A. mellea* (Fig. 7).

This study showed that the wood destroying activity of isolates were different while the reaction of hosts was constant. *Citrus aurantium*, *Carpinus betulus*, *Juglans regia* indicated susceptibility to the most of *A. mellea* isolates while *Amygdalus communis*, *Armeniaca vulgaris*, *Prunus devaricata* and *Pyrus communis* showed tolerance reaction to most of *A. mellea* isolates.

The published results showed that *Citrus* sp., *Carpinus* sp., *Juglans regia* were susceptible while *Pyrus communis* and *Prunus* spp. Were resistant to *A. mellea*

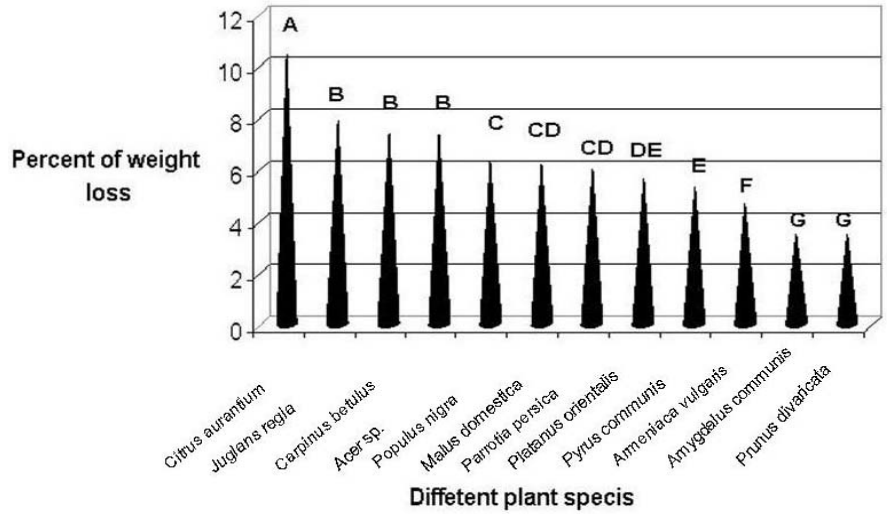

Fig 7. The mean comparisons of wood weight loss (%) of different plants species to *A. mellea* based on LSD test

(http://www.chasehorticulturalresearch.com/pdfs/*Armillaria*_root.pdf). Donovan (2007) reported citrus cultivars were susceptible to *A. mellea* in citrus orchards of Australia. Martin *(*2007) investigated the resistance of some plants species and introduced *Acer* sp., *Malus* sp. and *Juglans regia* as susceptible hosts to *A. mellea*. The level of resistance to *A. mellea* within plum species (*Prunus domestica, Prunus insititia, Prunus cerasifera*) was investigated and rootstock resistant to *Armillaria* spp. was created (Guillaumin *et al.,* 2003). Pears have traditionally been considered to be highly resistant to *Armillaria mellea* (Rizzo *et al.,* 1998). *Armenica vulgaris* cultivar Marianna was reported as resistant cultivar but was not immune to *A. mellea* (Adaskaveg *et al.*, 2007).

Therefore in order to management of Armillaria root rot in the high risk locations, using of the tolerant species will be necessary to decrease the damage by the fungus.

Acknowledgments

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