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

Seyed Ali Reza Dalili*¹, Siranush G. Nanagulyan ², Seyed Vahid Alavi³, Mohammad Razavi⁴

¹,³Department of Plant Protection, Agricultural and Natural Resources Research Center of Mazandaran, P.O. Box 48175-556, Sari, Iran  
²Department of Botany, Faculty of Biology, Yerevan State University, 1 A. Manoogian St., 0025 Yerevan, Armenia  
⁴Iranian Research Institute of Plant Protection, P.O. Box 1454-19395, Tehran, Iran  

*Corresponding author: ar_dalili@yahoo.com

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-
em (Smith, 1941). Martin (2007) tested susceptibility 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 Azerbaijain 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 autoclaving. The plates were incubated at 22±1°C (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±1°C 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 22°C 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 (CTAB) method and resuspended in 50 μl of TE (10 mM Tris-Base, 1 mM EDTA, pH 8.0) and stored at 4°C 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 MgCl₂, 50 pmol of each primer. The final reaction volume was adjusted to 50 μl with H₂O (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 (Gezaigne et al., 2004).

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

\[ C(\%) = \left( \frac{P - P_1}{P} \right) \times 100 \]

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).

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

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

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).
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 mellea 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 Ulrich, 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 Quercus petraea in Serbia and Montenegro (Kča 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, Italy 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).

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

This project is financially supported by the grant of Agricultural and Natural Resources Research Center of Mazandaran.

References


