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Almond Decline Caused by *Phytophthora megasperma* in Southeastern Anatolian Region of Turkey

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ABSTRACT

Decline symptoms were observed on young almond trees in commercial orchards of Adıyaman province of Turkey in 2013. Symptoms in affected trees included yellowing of foliage, cankers on roots, crowns and stem base, and dieback. An isolate of *Phytophthora* sp. was consistently isolated from necrotic tissues from taproots and crowns of symptomatic trees. The pathogen was identified as *Phytophthora megasperma* based on morphological features and DNA sequence of the internal transcribed spacer (ITS) region. Pathogenicity of the isolates was tested by shoot and stem inoculation, as well as soil infestation on almond saplings. Typical canker lesions developed on excised shoots after two weeks. Lesions also occurred on stems of scion and rootstock four weeks later. At the end of the soil infestation test, cankers covered the whole roots four months after inoculation. Eleven isolates of *P. megasperma* were assayed for sensitivity to mefenoxam. All isolates were found to be sensitive to mefenoxam with EC_{50} values less than 1 μg mL^{-1}.

Keywords: *Prunus dulcis*, almond decline, pathogenicity, mefenoxam sensitivity

ÖZET

Türkiye'nin Güneydoğu Anadolu Bölgesinde *Phytophthora megasperma*'nın Neden Olduğu Badem Ölümleri

Adıyaman ilindeki ticari badem bahçelerinde 2013 yılında genç badem ağaçlarında kurumalar gözlenmiştir. Hastalık belirtileri, yapraklarda sararma, kök, kökboğazı ve gövdenin alt kısımlarında kanserler ve geriye doğru ölüm şeklindeydi. Hastalıklı ağaçların ana kök ve kökboğazındaki nekrotik dokulardan bir *Phytophthora* türü izole edilmiştir. Patojen, morfolojik özelliklerine ve ITS bölgesinin DNA dizisine dayanılarak *Phytophthora megasperma* olarak teşhis edilmiştir. Patojenisite testleri, sürgün ve gövde inokulasyonları ile birlikte toprak inokulasyonu şeklinde yapılmıştır. Çeşit ve anaçların gövdeleri üzerinde dört hafta sonra sırasıyla ortalama 0.73 cm ve 0.32 cm uzunluğunda nekrozlar oluşurken, sürgünlerde nekroz uzunluğu ortalama 2.3 cm olmuştur. Toprak inokulasyon testi sonucunda ise kanserli dokular dört ay sonunda tüm kökleri kaplamıştır. Onbir adet *Phytophthora megasperma* izolatı mefenoxam'a hassasiyet yönüyle teste tabi tutulmuştur. Tüm izolatların EC_{50} değerleri 1 μg mL^{-1}'nin altında bulunmuş ve mefenoxam'a karşı duyarlı olarak karakterize edilmiştir.

Anahtar Sözcükler: *Prunus dulcis*, bademlerde kuruma, patojenisite, mefenoxam, duyarlılık

INTRODUCTION

Almond (*Prunus dulcis*) is grown on approximately 30.000 ha in Turkey with an annual production of 80.000 tons (Anonymous 2015). *Phytophthora* spp. are among the most serious soilborne pathogens of almond, causing death and decline in commercial orchards and nurseries (Browne and Doster 2002). More than 10% trees may die in severely affected orchards because almond seedling rootstock used commonly is highly susceptible to *Phytophthora* spp. (Browne and Doster 2002).

Several *Phytophthora* species affecting almonds have been reported worldwide. *P. cryptogea* and *Phytophthora* sp. infecting roots and trunks of almond trees were associated with yellowing of the leaves and gumming and cracking of the bark near soil level in Iran (Fatemi 1980). *P. syringae* infection on almond trees caused cankers and gumming, and it was associated with pruning wounds or injuries in California (Doster and Bostock 1988). In another study in California, *P. megasperma* was found causing mortality of primarily non fruit bearing trees in poorly drained soils due to crown rot of almond trees (Browne and Viveros 1998). In this study *P. cactorum* and *P. citricola* was also found causing crown rot and cankers of almonds. In Australia, *Phytophthora* species including *P. cambivora, P. citrophthora, P. cryptogea, P. megasperma, P. nicotianae* and *P. syringae* were isolated from crown cankers and soil around symptomatic almond trees, and *P. cactorum* from stem cankers of almonds (Wicks et al. 1997). Recently, *P. niederhauserii* was responsible for almond decline in Spain and Turkey (Perez-Sierra et al. 2010; Kurbetli and Değirmenci 2011a; Abad et al. 2014). Also in Turkey, *P. cactorum* and *P. citrophthora* caused serious losses associated with root and crown rot of almond seedlings in nurseries (Kurbetli and Değirmenci 2010).

In 2013, an increased level of young almond tree mortality has been observed in commercial orchards in Adıyaman, one of the largest almond growing province of Turkey. A *Phytophthora* sp. was consistently isolated from necrotic roots and crown tissues of symptomatic trees. The aims of this study were to identify and assess the pathogenicity of the causal agent of almond tree mortality in Turkey and to study sensitivity to phenylamide fungicide mefenoxam of *P. megasperma* isolates.

MATERIALS AND METHODS

Sampling and Isolation

Almond saplings (2 to 3 years old) from nine commercial orchards in Adıyaman province (located in Southeastern Anatolian Region of Turkey) showing decline symptoms were surveyed from April to July 2013. Symptoms in affected trees included chlorosis and dieback resulted in tree death. Examination of the lower stem, revealed in the presence of a reddish brown cankers expanding from root collar to stem; frequently, lesions girdled the stem leading to severe aerial symptoms and feeder roots were decayed. In the orchards, losses in various degrees up to 15% were observed. Symptomatic trees were uprooted; roots and lower stem sections were brought to the laboratory in order to isolate the pathogen.

Diseased almond roots were first washed in tap water and then air-dried. Small sections of 3 to 5 mm diameter were cut from the margin of the lesions and a minimum of 10 sections per agar plate were plated on 1.7 % corn meal agar (CMA) amended with PARP (per liter: pimaricin, 4 mg; ampicillin, 250 mg; rifampicin, 10 mg and pentachloronitrobenzene [PCNB], 75 mg) without surface sterilization. Plates were incubated at 22°C in the darkness and growing colonies were examined after three days under the light microscope. Emerging *Phytophthora* colonies were initially identified by presence of coenocytic hyphae with wide branching angles. Pure cultures were obtained by transferring single hyphal tips from the edge of the colonies onto carrot agar (CA) (200 ml boiled carrot juice, 800 ml distilled water and 20 g agar) containing 20 mg/l β-sitosterol for identification.

Morphological and Cultural Characteristics

Isolates were studied in different growth media including CA, CMA, malt extract agar (MEA) and potato dextrose agar (PDA). The presence of sporangia, hyphal swellings, or chlamydospores was examined. For this purpose, 5-mm-diameter disks were cut from the growing edge of 5-day-old cultures grown on CA at 24°C in the dark, and placed in 6-cm-diameter Petri dishes previously filled with 7 ml of 1.5% non-sterile soil extract solution to

stimulate sporangial production. The dishes were kept under continuous light for 12 to 48 h at 24°C. *Phytophthora* sp. was identified using identification keys of Erwin and Ribeiro (1996) and Gallegly and Hong (2008).

PCR, Sequencing and Phylogenetic Analysis

Internal transcribed spacer (ITS) regions of ribosomal DNA (rDNA) of five isolates were amplified using the universal primer pairs ITS-1 (5' TCC GTA GGT GAA CCT GCGG 3') and ITS-2 (5' GCT GCG TTC TTC ATC GATGC 3') (White et al. 1990). The PCR conditions and cycling program were performed according to Perez-Sierra et al. (2010). PCR products were separated in 1.5% agarose gels, stained with ethidium bromide and visualized under UV light. Sequence analysis was done. Nucleotide sequences were subjected to an NCBI BLAST search (http://www.ncbi.nlm.nih.gov/BLAST/), and isolates were identified according to the homology with sequences already present in GenBank. The nucleotide sequences of five isolates from almond were submitted to GenBank.

The evolutionary history of the isolates was inferred using the Neighbor-Joining method (Saitou and Nei 1987). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al. 2004) and are in the units of the number of base substitutions per site. Phylogenetic analyses were performed in the computer program MEGA version 6.0 (Tamura et al. 2013).

Pathogenicity Tests

Two isolates were tested for pathogenicity using excised, 1-year-old shoots of almonds cv. Ferraduel collected in dormancy in the first experiment (Jeffers et al. 1981; Scott et al. 1992). For each isolate, ten shoots washed, surface sterilized in 10% NaOCl for 15 min, rinsed three times in sterile distilled water, then cut to 15 cm in length and trimmed to a slant at the base. Shoots were placed upright 1 cm deep in a two-week-old cultures containing CMA supplemented with pimaricin (10 mg/l). Control shoots were buried in sterile CMA. Shoots were incubated for two weeks at 22°C in the dark. The outer bark was removed and lesions were measured. Symptomatic tissue was excised from the margin of the canker and plated onto CMA-PARP medium to re-isolate the pathogen.

Pathogenicity of two isolates was tested by stem inoculation technique on almond saplings cv. Ferraduel grafted on wild rootstocks in the second experiment (Scott et al. 1992; Perez-Sierra et al. 2010). Stems of both rootstocks and scions of five three-year-old almond saplings for each isolate were inoculated with a 5-mm plug from five-day-old culture grown on CA. Five plants inoculated with sterile agar plugs were used as control. The wounds were covered with moist, autoclaved cotton wool and wrapped with aluminum paper to prevent drying. Plants were kept in a controlled glasshouse at 25±1°C and watered as needed. Four weeks after inoculation, the outer bark was removed and lesions were measured. Measurements were averaged and the diameter of the disk was subtracted. Re-isolations were made as described above. Excised twig assay and stem inoculation test were performed twice. Results of canker length on twigs and stems were subjected to analysis of variance according to a completely randomized design, and means separated according to the Duncan's Multiple Range Test.

In the third experiment, two isolates of *P. megasperma* were tested for pathogenicity on 3-year-old plants of almond rootstock grown from seed. For each isolate five rootstocks were used. Also five non-inoculated saplings were used as control. Each of the rootstock plants was transplanted to a 5-liter pot containing soil mixture (1:1, vol:vol, mixture of orchard soil:sand) inoculated with inoculum at a rate of 5% produced by growing isolates for two weeks at 22°C in the dark on twice autoclaved wheat grains moistened with distilled water. Plants were incubated in a growth chamber for 4 months at 25±1°C and were kept constantly wet by placing them in trays filled with 2 to 3 cm of distilled water 4 days per week. At the end of the experiment, roots were washed free of the soil mix; the outer bark was removed and pathogenicity was evaluated. Re-isolations were made as described above.

Mefenoxam Sensitivity Assay

Eleven isolates of *P. megasperma* were assayed for sensitivity to mefenoxam (APRIN XL 350 ES; Syngenta Agro). Stock cultures from the isolates were grown on CA for five days. The fungicide was diluted in sterile distilled water, and then added to the autoclaved media at 50°C in order to prepare 0.1, 1, 5, 10, and 100 μg mL^{-1} concentrations. Media without any fungicide served as controls. Agar discs (5 mm in diameter) were removed from actively growing margins of the fungal cultures and transferred to the center of a 9-cm-diameter Petri dishes. Three replicates were prepared per fungicide concentration. The experiment was conducted twice. Dishes were incubated

at 24°C for 7 days in the dark. Colony diameters were measured in two directions for each individual dish and mean diameters were found. Growth percentage of each replicates for each concentration of fungicide was calculated as follows: (colony diameter in fungicide amended petri dish / colony diameter in control petri dish) x100.

Regression equations were determined between fungicide concentrations and percent mycelia growth by means of Minitab Package Program (Minitab Inc., State College, PA, USA). EC_{50} values of the fungicides were calculated via the equations. Isolates were characterized as sensitive if colony growth on media amended with 5 µg mL^{-1} of fungicide was less than 40% of the isolate growth on non-amended media. Intermediate isolates exhibited growth on media amended with 5 µg mL^{-1} greater than 40% of that on non-amended media, but growth on media amended with 100 µg mL^{-1} fungicide less than 40% of that on non-amended media. Resistant isolates exhibited growth on media amended with 100 µg mL^{-1} greater than 40% of that on non-amended media (Parra and Ristaino 2001; Perez-Sierra et al. 2010).

RESULTS

Field Symptoms, Isolation and Identification

Symptoms in affected trees included chlorosis, a reddish brown cankers progressing to stem from the roots, and dieback. Necrotic tissues had completely girdled the trees showing severe aerial symptoms. Losses in various degrees up to 15 % occurred in the orchards. An isolate of *Phytophthora* sp. was consistently isolated from necrotic tissues taken from the margin of the lesions on taproots and crowns of symptomatic almond trees collected. The same pathogen was found in all nine orchards surveyed.

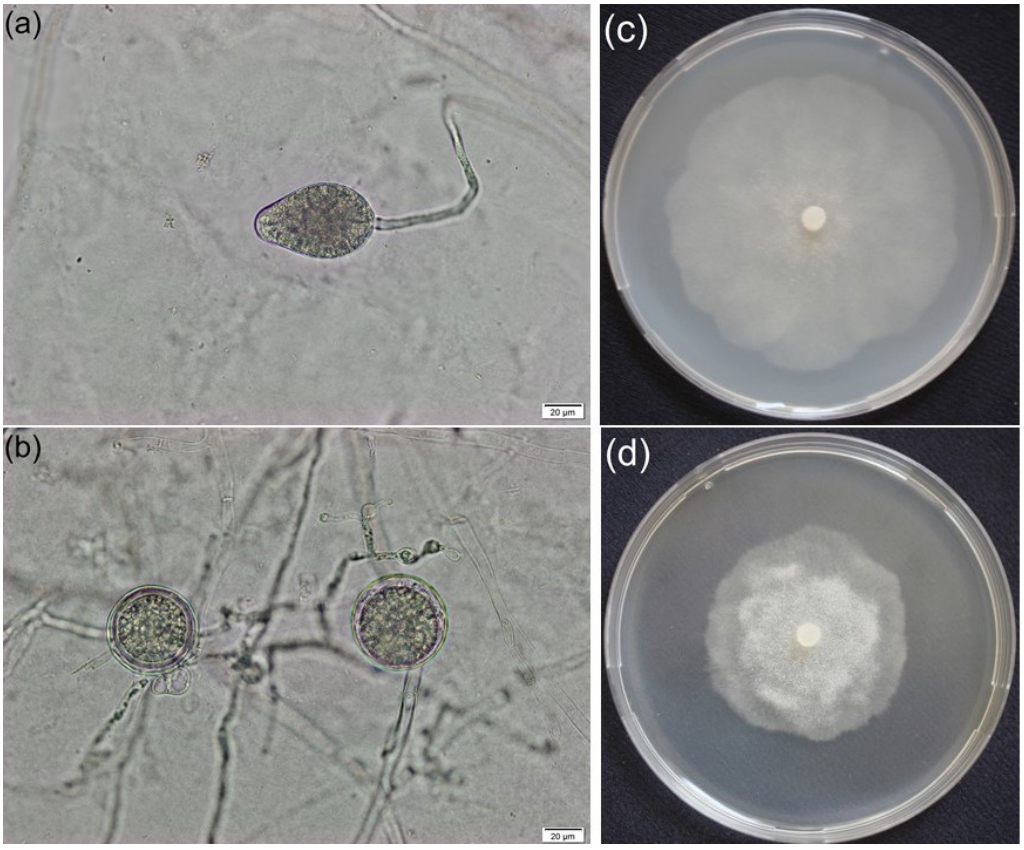

Fig. 1. Sporangium (a) and oospores (b); seven-day-old cultures on CA (c) and MEA (d) of *Phytophthora megasperma*.

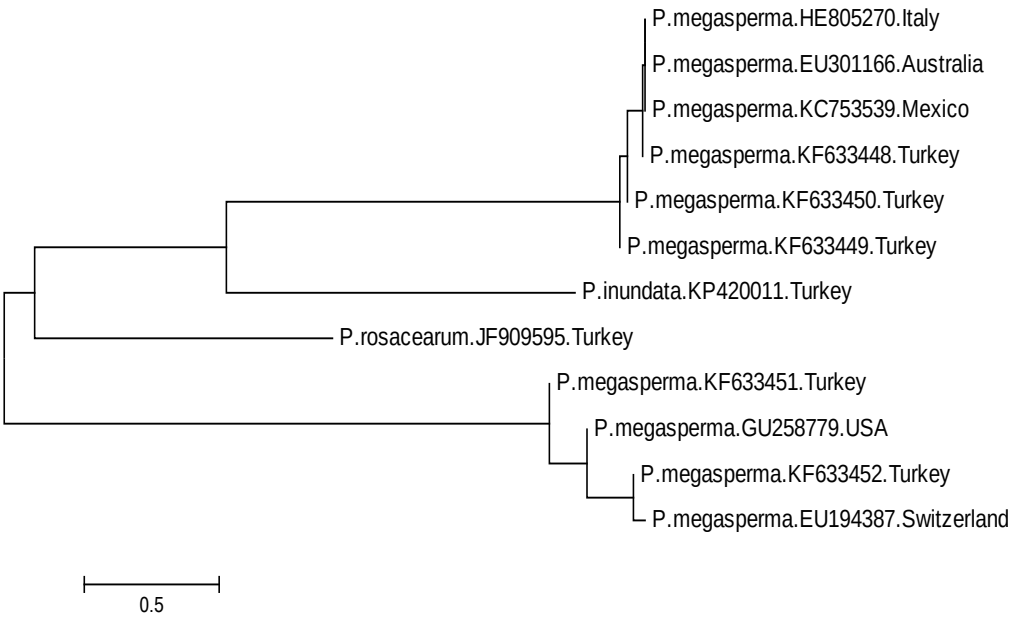

Fig. 2. Dendrogram showing genetic relatedness of 5 isolates of *Phytophthora megasperma* isolated from almonds in southeastern Turkey, and 5 isolates of *Phytophthora megasperma*, one isolate of *Phytophthora inundata* and one isolate of *Phytophthora rosacearum* obtained from GenBank.

Based on morphological characteristics (Fig. 1) and sequencing the ITS, the isolates identified as *Phytophthora megasperma* Drechsler. The genetic relatedness between ITS sequences obtained from the present study and GenBank deposited sequences, selected as representative of *Phytophthora* spp., is shown in dendrogram of Fig. 2.

Pathogenicity Tests

In all three experiments, isolates of *P. megasperma* were proved to be pathogenic and capable to cause lesions. Reddish to brown lesions, from 1.4 to 3.8 cm in length (mean 2.3 cm), were produced by the isolates on excised 1-year-old shoots of almond, while no cankers developed in control shoots except for a slight, superficial oxidation developed on the cut surface. The pathogen was re-isolated from necrotic tissues excised from the margin of the canker and plated on the semi-selective medium. In the second experiment, four weeks after inoculation, cankers on stem of scions extended from 0 to 2.2 cm in length (mean 0.73 cm), whereas no cankers were seen in the control plants. Lesions on stem of rootstocks developed from 0.1 to 0.5 cm in length (mean 0.32 cm), while collenchyma callus of healthy appearance was produced over the wound point over the same time period on rootstock control plants. *P. megasperma* was re-isolated from symptomatic tissues of both scions and rootstocks. Lesion lengths on twig and stem of rootstock of almond inoculated with *P. megasperma* were significantly different from those of controls ($P<0.05$), while there was no significant differences between lesion lengths on stem of scion of inoculated and non-inoculated plants (Table 1). The roots of inoculated rootstock plants were invaded by the pathogen and canker lesions covered the whole root at the end of the experiment after four months. When the outer bark of the roots was removed, reddish brown canker lesions which are typical for *Phytophthora* crown and root rot revealed, while no cankers developed in the roots of non-inoculated plants. *P. megasperma* was re-isolated from necrotic tissues.

Table 1. Canker development on excised shoots and stems of rootstock and scion inoculated with two isolates of *Phytophthora megasperma* obtained from almond trees in Turkey

	Mean lesion length (cm)		
Isolate	Excised twig assay[a]	Stem inoculation of scion[b]	Stem inoculation of rootstock[b]
1	2.48 ± 0.19 ab	0.86 ± 0.37 ab	0.34 ± 0.07 ab
2	2.11 ± 0.11 b	0.60 ± 0.19 bc	0.30 ± 0.07 b
Control	0.44 ± 0.04 c	0.00 ± 0.00 c	0.00 ± 0.00 c

[a] Two weeks after inoculation and 10 replicates
[b] Four weeks after inoculation and 5 replicates
Mean values (n=5) followed by the same small letters within the column are not significantly different according to Duncan's Multiple Range Test ($P \leq 0.05$).

Mefenoxam Sensitivity Assay

Radial growth on control plates (no mefenoxam amendment) ranged from 4.0 to 5.8 cm for *P. megasperma* isolates (mean for all isolates = 5.03 mm). The addition of mefenoxam to the culture medium significantly reduced growth rates in all isolates. EC_{50} values of the fungicide on eleven *P. megasperma* isolates were between 0.0219 and 0.5117 μg ml^{-1}. It was found that all isolates were sensitive to mefenoxam.

DISCUSSION

In the last decade almond plantations have been established in large agricultural lands in Southeastern Anatolian Region of Turkey, including Adıyaman province where young almond trees declined. This study showed that *Phytophthora megasperma* was associated with decline and mortality of these almonds. Some *Phytophthora* spp. such as *P. cactorum, P. citrophthora* and *P. niederhauserii* have previously been reported on almond (Kurbetli and Değirmenci 2010; 2011a) in Turkey. *P. megasperma* has been found on other hosts such as apple (Kurbetli and Değirmenci 2011b), kiwifruit (Kurbetli and Ozan 2013) and sour cherry (İ. Kurbetli, unpublished data), suggesting that it may potentially spread efficiently in fruit orchards of Turkey.

P. megasperma is a pathogen with a worldwide distribution and has a wide host range including many fruit trees such as almond, apple, apricot, avocado, cherry, citrus, kiwifruit, olive, peach, plum and walnut (Erwin and Ribeiro 1996). It causes root and crown rot of almond usually in non-fruit bearing trees and tree losses especially in poorly drained soils in Australia and USA (Wicks et al 1997; Browne and Viveros 1998). The isolates obtained from almonds and used in pathogenicity tests in this study were pathogenic on excised shoots, stems and the roots of almond saplings. On the other hand, in the first two experiments, necrosis lengths on excised shoots and stems of both rootstock and scion did not develop as expected. However, *P. cactorum, P. citrophthora* and *P. niederhauserii* isolated from almonds caused much longer lesions on stem of almond rootstocks in previous study (Kurbetli and Değirmenci 2012). It is considered that stem inoculation and excised twig assays for pathogenicity in almond are not appropriate for *P. megasperma* although they are used for many *Phytophthora* species.

Several *Phytophthora* species including *P. capsici, P. erythroseptica P. cryptogea, P. palmivora* and *P. nicotianae* associated with bell pepper, potato, floriculture crops and ornamental plants were screened for sensitivity to mefenoxam (Parra and Ristaino 2001; Taylor et al. 2002; Hwang and Benson 2005; Hu et al. 2008; Olson et al. 2013). *Phytophthora* isolates ranging from 6% to 69% were characterized as resistant or intermediately resistant in their studies. In addition, Olson et al. (2013) reported that 9 out of 28 *P. megasperma* isolates were found sensitive to mefenoxam, while 19 isolates exhibited intermediate or insensitive. On the other hand, all almond isolates of *P. niederhauserii* tested were found sensitive to mefenoxam (Perez-Sierra et al. 2010) as in this study. For now, it can be said it can continue to be used mefenoxam in the management of Phytophthora diseases affecting almonds.

Control of *Phytophthora* crown and root rot using the fungicides after appearing foliar symptoms is generally difficult. Preventing flooding or waterlogging, and establishing orchards on well-drained soils help to avoid the disease. Considering that almond seedling rootstock used commonly is highly susceptible to *Phytophthora* spp., the most effective and sustainable means of control is to grow resistant cultivars to the pathogen.

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