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Identification of the Most Important Fungal Pathogens in *Euonymus* spp.

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***Euonymus* spp. (L.) are of the most important ornamental plants. Fungal plant pathogens of ornamental plants are the most important factors causing damage. Two pathogenic fungal species were isolated from naturally infected *Euonymus* species and identified. In order to isolate the fungus from disease tissues, the obtained samples were cultured on potato dextrose agar medium. Isolates were cultured due to sporulation on water agar medium. Morphological characters of isolates were studied in order to identify the taxonomy. According to the results, isolates were belonged to *Alternaria alternata* (Fries) Keissler, and *A. tennuissima* (Kunze ex Pers.) Wiltshire. Pathogenicity test of isolates was done in desiccators, and revealed the pathogenicity level of the species and their ability to cause leaf blight on *Euonymus* spp.. This reaction occurred as complete random design (CRD) with 8 treatment and 3 replications. Based on the variance analysis table of the evaluation of disease rating, the studied *Euonymus* spp. showed no significant reactions to *Alternaria alternata* and *A. tenuissima*. But based on the sizes and types of the spots appeared on the *Euonymus* spp. and Horsfall-Barratt system, plants were more affected by the *Alternaria alternata* compared with *A. tenuissima*, and its disease rating was higher and plants showed less tolerance.**

Key words: fungi; *Euonymus* spp.; Pathogenicity.

Euonymus spp. (L.) are of the most important ornamental plants having 175 species of deciduous, semi- evergreen, and evergreen shrubs, trees, and climbers found mostly in woodland and thickets, mainly in Asia (BRICKELL 2008). Especially fungal plant pathogens of ornamental plants are the most important factors causing damage (BRICKELL 2008). Powdery mildew fungi, infect almost all ornamental plants. They are commonly seen only on plants more naturally susceptible to the disease, including woody plants susceptible can be refer to *Eounymus* plants (NAMETH & CHATFIELD 2011). *Cercospora detructiva* and *C. euonymi* Ellis cause irregularly shaped brown spots on the leaves. The spots vary in size from pinpoints to half an inch across. The centers of large spots become grayish tan and the causal fungus produces tiny, black fruiting bodies on the upper surface of the spots (KLUEPFEL & MCLEOD 2011).

Anthracnose is caused by the fungus *Colletotrichum* species on *Euonymus.* Symptoms consist of small, brownish spots with light-colored centers on the leaves and twigs. Tiny cracks in the leaf spots indicate reproductive structures of the fungus. The disease is a problem during cool, wet springs. Variegated varieties are more susceptible (KLUEPFEL & MCLEOD 2011). Scab, caused by

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the *Sphaceloma* spp. or *Elsinoe euonymi-japonici* Jenkins & Bitanc. disfigures Japanese euonymus. Spots develop on both surfaces of leaves but are most common on the upper one. The spots are very small, grayish white with a raised orange-cinnamon, waxy-appearing margin and, in the larger spots, a raised, dark center. When they are numerous, the spots may merge together. The centers of the leaf spots sometimes fall out. On the stems, spots are similar to those on the leaves, but they are often darker in color and more likely to merge together (KLUEPFEL & MCLEOD 2011).

There are also different reports about fungal diseases of various plants. *Fusarium moniliforme* J.Sheldon is the causal agent of *Fusarium* leaf spot disease in dracaena and its derivatives. This disease was first explained on *Sansevieria* spp. in 1940, and it was also reported in many species of dracaena (JONES 1940). *Colletotrichum trichellum* (Fries) Duke was reported as the causal agent of anthracnose disease on *Hedera helix* L. (GARREN 1946). Brown leaf spot of *Dieffenbachia* spp. caused by *Leptosphaeria* spp. was first reported in 1966 (MARLATT 1966).

Aechmea fasciata (Lind) Baker leaf spot disease caused by the fungus *Exserohilum rostratum* (Drechsler) K.J. Leonard & Suggs was reported in 1974. This disease is reported to be severe on small plants when they are moved and wounded (MARLATT & KNAUSS 1974). Dieback disease of *Cissus rhombifolia* Vahl was reported in Ontario and Canada in 1982, the causal agent of which was reported to be *Pestalotiopsis menezesiana* (Bresadola & Torrend) Bissett (BISSETT 1982). Stem, leave, and root rot disease of *Dieffenbachia maculata* Forest & Kim Starr was diagnosed with the pathogen known as *Fusarium solani* (Martius) Saccardo (CHASE & EL-GHOL 1982). Leaf spot disease of *Calathea* and *Maranta* caused by *Drechslera setariae* (Sawada) S. Ito was identified in 1983 (SIMONE & BRUNK 1983). *Pestalozzia guepinii* (Savov) Desm. was introduced as the causal agent of grey blight disease of *Camellia*, which caused considerable damage in Japan (CHASE 1988). *Puccinia antirrhini* Dietel & Holway was introduced as the causal agent of snapdragon rust disease, and a comprehensive study was conducted on it in the U.S. (CHASE 1988).

Alternaria alternata (Fries) Keissler as casual agent leaf spot on *Aloe vera* in Louisiana (DA SILVA & SINGH 2012) and in Eastern Croatia, *Alternaria helianthiinficiens* E.G. Simmons, Walcz & R.G. Roberts as casual agent foliar and stem blight on sunflower were reported (VRANDECIC *et al*. 2012). BARGUIL *et al.* (2009) studied the Brazilian farms rose stem wilt disease and *Fusarium oxysporum* Schlechtendal as pathogen was reported for the first time.

Colletotrichum karstii Y.L. Yang, Zuo Y. Liu, K.D. Hyde & L. Cai was reported as causal agent of anthracnose disease in white Orchids flowers in the United States (JADRANE *et al*. 2012).

In Iran, was reported *Phytophthora citrophthora* (R.E. Smith & E.H. Smith) Leonian on *Acacia* trees and *P. drechsleri* Tucker on *Cedrus* trees as pathogens (MIR ABOLFATHI & ERSHAD 1995). In Isfahan, isolated and identified *Phytophthora megasperma* Drechsler as the pathogen on inverted tulips (SHAFIZADEH & BORDBAR 2002). In other study, reported that *Phytophthora nicotianae* (Dastur) G.M. Waterhouse is the causal agent of the diseases such as *Lilium* bulb rot, rhizome-like tubers of *Alstroemeria*, stem and root rot of the roses imported from Holland, and *Dieffenbachia* and *Peperomia* produced in ornamental plant breeding centers of Markazi and Tehran provinces (MIR ABOLFATHI 2002). Also, *Podosphaera pannosa* (Wallroth) de Bary, *Peronospora sparsa* Berkeley, *Botrytis cinerea* J.F.H. Beyma, and *Cercospora* sp. identified and introduced as the causal agents of rose powdery mildew, rose downy mildew, rose grey rot, and rose *Cercospora* leaf spot in northern Khuzestan province, respectively (ZADEHDABAGH *et al.* 2003).

MINASIAN *et al*. (2004) reported that *Physoderma narcissi* (G. Poirault) Saccardo & Trotter is the causal agent of disease on the ornamental plant *Narcissus* from Khuzestan. MIR ABOLFATHI (2004) reported that *Collectotrichum gloeosporioides* (Penzig) Penzig & Saccardo is the causal agent of anthracnose disease on azalea in the nurseries of Mazandaran province. In Iran, *Euonymus* powdery mildew was reported by ESFANDIARY in 1948 and it has spread all over the country, especially in northern Iran, causing considerable damage (BEHDAD 1987). One of the main objectives of the present study is to identify

the most important fungal pathogens damaging at their different growth stages of *Euonymus* spp. in Guilan province in Iran.

MATERIALS AND METHODS

Collection and culture of fungal isolates

Diseased leaves of *Euonymus* spp. were sampled from five locations in each field from Guilan province in Iran. Leaves were transferred to the laboratory and then isolated the fungi from disease samples. Leaf pieces with lesions were surface sterilized with 0.5% sodium hypochlorite solution, washed by sterile distilled water and placed on potato dextrose agar in Petri dishes at 25°C for 2-3 days. PDA and WA media were used for sporulation. Then Petri dishes containing media were incubated at 25°C in the dark or artificial light supplied by fluorescent light on a 12 h light/dark photoperiod for 15-25 days (ZHANG *et al*. 1996). For avoid of bacterial contamination, sulfate streptomycin antibiotic was used (SAFARI MOTLAGH 2010). Conidia were single- sporulated. Monoconidial isolates of the recovered fungi were maintained on half- strength potato dextrose agar slants in test tubes as stock cultures (SAFARI MOTLAGH 2010).

Study and identification of fungi

Morphological studies were carried out on potato dextrose agar and water agar media. Cuts of colonies were placed onto potato dextrose agar medium for 2-3 days. Then, section of colonies was transferred to water agar medium for 7-10 days in incubator at 25°C and 12 h photoperiod. Afterward, morphological observations were taken based on colony, conidium and conidiophore morphology and other characters morphological (ELLIS 1971; SIMMONS 2007; RAI *et al*. 1993; DE HOOG *et al*. 2000; CANNON *et al*. 2008).

Pathogenicity tests

This reaction occurred as complete random design (CRD) with 8 treatment and 3 replications.

Pathogenicity tests were carried out in desiccators. *Euonymus* spp. were planted in plastic pots 2.5 cm in diameter containing farm soil. In each of two desiccators (one desiccator as control) two pots of *Euonymus* spp. at the 3-4 leaf stage were placed. Distilled water was added to pots. Pots were placed at 25^0C, 12 D: 12 L photoperiod and a relative humidity of more than 90%. Pots were inoculated with 8×10^4 conidia per ml. To increase the surface adsorption, 1% tween-20 was applied. This suspension was sprayed on the leaves using a sprayer. It should be mentioned that before inoculation, all pots were sprayed with distilled water. Evaluation was done 10 days after inoculation based on lesion type and size in reaction to inoculation: 1= lesions absent, 2= small, unexpanded lesions, 3= slightly to moderately expanded lesions, 4= large lesions (ZHANG *et al*. 1996). Therefore, standard evaluation system and Horsfall- Barratt system were applied for determine of disease rating of fungi (ZHANG *et al*. 1996; BERTRAND & GOTTWALD 1997).

$$\text{Disease rating} = \frac{(N_1 \times 1) + (N_2 \times 2) + ... + (Nt \times t)}{(N_1 + N_2 + ... + N_t)}$$

Where N is number of leaves in each of rate, t is number of treatments.

Data Analysis

Data analysis was done using SAS software. In order to compare average values, Tukey test was used.

RESULTS

The fungi isolates belonged to *Alternaria* spp. These isolates were divided into 2 groups based on morphological characters, as follows:

Characteristics of first group

The 4 cm diameter colony with four pairs of concentric rings of growth and sporulation on agar surface (Fig. 1). Surface sporulation in light-exposed rings was a dense turf of multiplebranched chains of conidia. The aerial portion of the colony (light- deprived rings) consisted of a less dense growth of abundant subarborescent hyphae that produce open, entangled heads of branched chains of conidia. The typical sporulation pattern comprised a single suberect conidiophores and an apical cluster of branching chains of small conidia separated by short secondary conidiophores (Fig. 2). In light exposed areas of a colony, the primary conidiophore was comparatively short, 40-70 × 3-4 µm; it remained simple or may become 1-3 branched or geniculate, with corresponding numbers of

Table 1. Variance analysis of disease rating in *Euonymus* spp. affected by *Alternaria alternata* and *A. tenuissima*

SOV	DF	SS	MS	F
Treatment	3	0.040291	0.013430	1.54ns
Error	8	0.0698	0.0872	
Total	11	0.11	-	-

SOV: sources of variations, DF: degree of freedom, SS: sum of squares, MS: mean of squares.
ns: not significant at p=5%

Table 2. Comparison of means of disease rating affected by *Alternaria alternata* and *A. tenuissima* in *Euonymus* spp..

Fungi	Disease rating
Alternaria alternata	1.176a
A. tenuissima	1.136a

Treatments having at least one similar letter do not show a significant difference at the probability level of 5%.

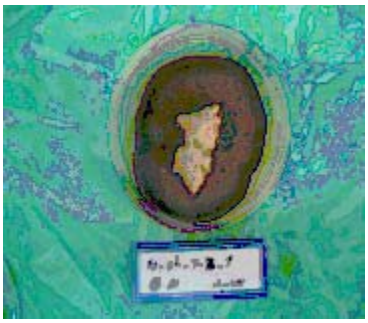

Fig. 1. Colony of *Alternaria alternata* on PDA

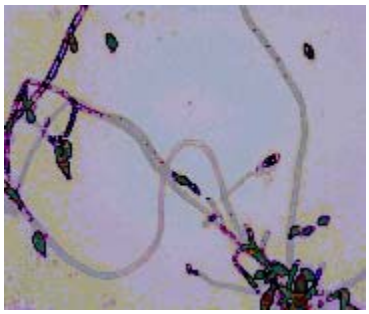

Fig. 2. Hyphae, conidiophore and conidia of *Alternaria alternata* (×460)

Fig. 3. Conidia of *Alternaria alternata* (×1200)

Fig. 4. Colony of *Alternaria tenuissima* on PDA

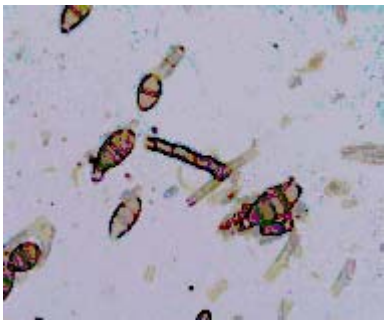

Fig. 5. Conidiophore and conidia of *Alternaria tenuissima* (×1200)

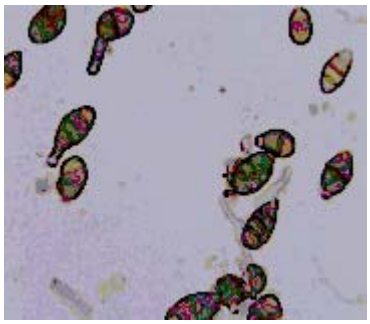

Fig. 6. Conidia of *Alternaria tenuissima* (×1200)

Fig. 7. Symptoms of *Alternaria alternata* on *Euonymus* spp.

Fig. 8. Symptoms of *Alternaria tenuissima* on *Euonymus* spp.

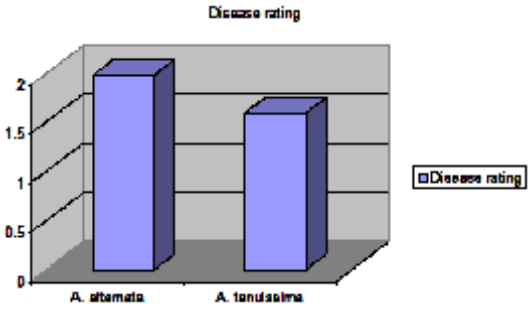

Fig. 9. Diagram of the comparison of *A. alternata* and *A. tenuissima* mean disease rating in *Euonymus* spp.

primary conidia chains. Branching within the primary chains was initiated when a few secondary conidiophores developed a series of conidiogenous loci or when secondary conidiophores arised from conidium body cells. In older portions of a colony, the erect conidiophores and their sporulation heads presented a picture of a solid turf of overlapping, interwoven conidial chains (Fig. 2). Single chains of conidia in the branching head may have up to 15- 20 conidia. The first 1-2 conidia in a chain usually remain long-elliptical as they mature; conidia produced later in the chain became ovoid, ellipsoid, or subsphaeroid (Fig. 3). Initial elliptical conidia were 25-30 (-40) × 5-9 µm, with 4-7 transverse septa and a few or no longisepta; subsequent spores were 7-25 (-40) × 5-12 µm, with 1-7 (very commonly 3) transepta and very few or no longisepta (Fig. 3).

The secondary conidiophores in chains may be obsolescent, but almost always was a single cell 2-3 × 3-5 µm; occasionally it became 2-celled and, in the case of lateral conidiophores produced from conidium cells, up to 30 × 3-4 µm. Conidia appeared olivaceous a dull grey- green- brown, when first mounted in lactic acid for microexamination; they lose the green tone after a few days and exhibited pale to moderate shades of yellowish to golden brown (Fig. 3). The wall ornamentation was densely punctulate in juvenile conidia, became granular to variously verrucose as conidia mature. The characteristics of this group corresponded with *Alternaria alternata* (Fries) Keissler, Beih (Simmons 2007).

Characteristics of second group

Colonies grey to dark blackish brown (Fig. 4). Conidiophores were solitary or in groups, simple or branched, straight or flexuous, more or less cylindrical, septate, pale or mid pale brown, smooth, with 1 or several conidial scares, up to 115 µm long, 4-6 µm thick (Fig. 5). Conidia were solitary or in short chains, straight or curved, obclavate or with the body of conidium ellipsoidal tapering gradually to the beak which is up to half the length of the conidium, pale to mid golden brown, usually smooth, sometimes minutely verruculose, generally with 4-7 transverse and several longitudinal or oblique septa, slightly or not constricted at the septa, overall length 22-95 µm, 8-19 µm thick in the broadest part, beak 2-4 µm thick, swollen apex 4-5 µm wide (Figs. 5,6). The characteristics of this group corresponded with *Alternaria tennuissima* (Kunze ex Pers.) Wiltshire (Ellis 1971).

Alternaria alternata and *A. tenuissima* were pathogen on *Euonymus* spp. The first symptoms of *Alternaria alternata* appeared 48 h after inoculation on *Euonymus* spp. Symptoms were elongated and brownish spots that gradually increased in the top of the leaves and a white layer appeared on some of the leaves and was produced leaf blight (Fig. 7).

The first symptoms of *Alternaria tenuissima* appeared 72 h after inoculation on *Euonymus* spp.. Symptoms were elongated and

brownish spots that gradually increased in the top of the leaves and veins and produced necrotic lesions (Fig. 8).

Based on the variance analysis table of the evaluation of disease rating, the studied *Euonymus* spp. showed no significant reactions to *Alternaria alternata* and *A. tenuissima* (Table 1). Also, based on the mean comparisons of traits there was no significant difference between *Alternaria alternata* and *A. tenuissima* (Table 2).

Based on the sizes and types of the spots appeared on the *Euonymus* spp. and Horsfall-Barratt system, plants were more affected by the *Alternaria alternata* compared with *A. tenuissima*, and its disease rating was higher and plants showed less tolerance (Fig. 9).

DISCUSSION

Da Silva & Singh (2012), several of the *Aloe vera* plant leaf spot infection investigated at the University of Louisiana. Large, necrotic, sunken, circular to oval, dark brown spots were present on both surfaces of the leaves. After isolation and analysis, *Alternaria alternata* was identified as the causative agent. In the pathogenicity test that seven days after inoculation, necrotic leaf spots were observed on the inoculated plants and no leaf spots were observed on control plants. This is the first report of *Alternaria alternata* was on the *Aloe vera* in Louisiana.

In another study, Barjuil *et al*. (2009) observed rose stem rot in the commercial farms of Sierra region in Brazil. The symptoms of the disease included several spots on the stem and wilting of rose plantlets. After isolation and analysis, the fungal pathogen known as *Fusarium oxysporum* was identified. In a pathogenicity test carried out at room temperature after 20 days, the tissue of the inoculated plantlet began to show the symptoms. No symptoms were observed on the control plantlets. This is the first report of root rot resulting from *Fusarium oxysporum* on rose in Brazil.

In a study conducted on bird of paradise plant in Goliran flower and plant complex, and after the symptoms on the infected leaves of the plant were observed, Moghaddam (1996) identified *Alternaria* sp. after isolating and studying the disease agent. During the pathogenicity test, the symptoms were observed after 5 days by the water-soaked spots appearing on the separated laminas inside the desiccator.

Jadrane *et al*. (2012) *Colletotrichum* sp. isolated from white *Phalaenopsis* flowers growing in a greenhouse in San Francisco. This *Phalaenopsis* was a common commercial orchid hybrid. The white petals showed anthracnose-like lesions where necrotic tissue is surrounded by a ring of green tissue. After inoculation observed that isolates possessed the same characteristics as previously described for *Colletotrichum karstii*. In the pathogenicity tests, fifteen to twenty days after inoculation, lesions were visible on the petals sprayed with *Colletotrichum* isolates and controls remained healthy. This is the first report of infection and green island formation caused by C. *karstii* on orchid flower in the United States.

Abolfathi & Ershad (1995) observed blight and wilt incidence of rose branches in greenhouses and centers for breeding different roses in the cities of Varamin, Karaj, and Mahallat, and Isfahan province. Next, they identified some isolates of the fungi *Botrytis cinerea, Phomopsis oblonga* (Desmazières) Traverso and *Pestalotiopsis* sp. through the cultivation of infected tissue in synthetic media. The results of the pathogenicity test on the plant showed that water-soaked spots resulting from inoculation with *B. cinerea* appeared after 7 days. *P. oblonga* pycnidia also appeared on the surface of sunken, light brown oval spots with distinct margins after 10-14 days. Moreover, the acerolas carrying the spores of *Pestalotia* sp. appeared on the surface of the wide and amorphous spots after 10 days. In another study conducted in the eastern regions of Guilan on *Camellia*, an isolation of the fungus *Colletotrichum gloeosporioides* (Penzig) Penzig & Saccardo was identified through culturing the tissue on the spots of infected leaves. Pathogenicity test showed that the symptoms appeared on the spore-inoculated leaves with a little delay in the form of small brown spots scattered on the surface of leaves. Moreover, the symptoms on the cut leaves inside the desiccator were observed after 6 days, and black pimples were formed on the spots (Moghaddam 1996).

In this study, we isolated *Alternaria alternata* and *A. tenuissima* from *Euonymus* spp. for first time, and then we compared disease ratings

of their. It was found that the disease rating of *Alternaria alternata* is more than *A. tenuissima* and plants showed less tolerance to it. This study could be preface for scrutiny diseases of *Euonymus* spp., that is one of the most important ornamental plants in Iran

CONCLUSION

Given that the present study might be one of the first studies on identifying *Euonymus*-infecting fungi, resource constraint both in Iran and abroad can be one of the difficulties of the study in identifying the pathogens of *Euonymus* spp..

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