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Identification of New Fungi Isolated from *Euonymus* spp. in Guilan Province

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Abstract

Euonymus spp. (L.) are some of the most important ornamental plants. Fungal plant pathogens of ornamental plants are the most important factors causing damage. In this research two pathogenic fungal species were isolated from naturally infected *Euonymus* species and identified. In order to isolation of fungi 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 *Phoma* sp. and *Colletotricum gloeosporioides*. Pathogenicity test of isolates was done in desiccators, and revealed the pathogenicity levels of these fungi and their ability to cause leaf blight on *Euonymus* spp. This reaction occurred as complete random design (CRD) with eight treatment and three replications. Based on the variance analysis, there was not any significant difference in the disease rating of studying fungi. 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 *Phoma* sp. compared with *C. gloeosporioides*., and its disease rating was higher and plants showed less tolerance.

Keywords: *Colletotrichum* spp., *Euonymus* spp., Fungi, Pathogens, *Phoma* sp.

INTRODUCTION

Ornamental plants play an important role at home, office, in out-doors and the promenades. (Mir Hosseini Moghaddam, 1996). *Euonymus* spp. (L.) are some 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). The fungi are the most important pathogenic factors which affect the quality and quantity of many ornamental plants like the *Euonymus* (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 referred to *Euonymus* plant (Nameth and Chatfield, 2011).

Cercospora leaf spot disease on *Euonymus* created by *Cercospora detructiva* and *Cercospora euonymi* that the spots vary in size from pinpoints to half an inch across. The centers of large spots become grayish tan and the causal fungi produce tiny, black fruiting bodies on the upper surface of the spots (Kluepfel and McLeod, 2011). Anthracnose is caused by *Colletotrichum* sp. on *Euonymus* spp. Symptoms consist of small, brownish spots with light-colored centers on the leaves and twigs. Tiny cracks in the leaf spots indicate fruiting structures of the fungus. Considerable defoliation can result. The disease is a problem during cool, wet springs. Variegated varieties are more susceptible (Kluepfel and McLeod, 2011).

Disease scab, caused by *Sphaceloma* spp. or *Elsinoe euonymi-japonici*, 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. 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 and McLeod, 2011).

In one of the first reports of anthracnose on ornamental plants as *Hedera helix* L. *Colletotrichum trichellum* (Fr.) Duke was identified as the pathogen (Garren, 1946). *Saintpaulia ionantha* Wedland was introduced as the first host plant of *Botrytis cinerea* (De Bary) Whetzel and in 1949 another report of gray mold of rubber tree *Ficus elastica* Roxb. was published (Beck and Vaughn, 1949).

In 1966 was reported brown leaf spot on *Dieffenbachia* spp. that causal agent was *Lepotosphaeria* sp. (Marlatt, 1966). Aechmea fasciata leaf spot disease caused by the *Exserohilum rostartum* was reported in 1974. This disease was severe on small plants when they were moved and wounded (Marlatt and Knauss, 1974).

Stem rot, leaf and root of *Dieffenbachia maculata* (Mart.) Sacc. (*Fusarium solani*) (Chase and El-Gholl, 1982) and *Kalathea* and *Maranta* leaf spot disease (*Drechslera setariae* (Sawada) S. Ito) (Simone and Brunk, 1983) are of the important diseases of ornamental plants.

In 1984, a kind of leaf spot on Dieffenbachia maculata was reported in New Zealand and Plectosphaerella cucumerina (Lindf.) W. Gams was introduced as pathogenic agent (Semer *et al.,* 1983).

In the United States, *Erysiphe cichoracearum*, *Puccinia chrysanthemi* and *Botrytis cinerea* introduced as the most important pathogenic factor of *Chrysanthemum* sp. (Chase, 1988). In 1953, *Fusarium oxysporum* f. sp. *gladioli* and *Fusarium bulbigenum* were introduced as the important pathogenic factors of *Gladiolus* sp. and *Narcissus tazetta* crown rot, respectively (Chase, 1988).

In Louisiana, *Alternaria alternata* (Fr.) Keissl was reported as the cause agent of leaf spot in Aloe vera (da Silva and Singh, 2012). In another study, rose stem wilt disease was studied in commercial farms of Brazil and *F. oxysporum* was reported as a pathogenic agent (Barguil *et al.*, 2009).

In the United States, anthracnose disease of white orchid flowers studied and *Colletotrichum karstii* was identified as the causal agent of disease (Jadrane *et al.*, 2012). In another study, Mir Abolfathi (2002) reported that Phytophthora nicotianae 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.

In another evaluation, Zadehdabagh *et al.* (2006) identified and introduced *Podosphaera pannosa, Peronospora sparasa*, as the causal agents of rose powdery mildew, rose downy mildew, rose grey rot, and rose *Cercospora* leaf spot in north of Khouzestan province, respectively.

Minasian *et al.* (2006) reported that *Physoderma narcissi* is the causal agent of disease on the ornamental plant narcissus of Khuzestan. In another study, collection, diagnosis and illustrated description of the ornamental plant pathogenic fungi flora were performed on 18 species of ornamental plants like: *Rosa, Sycas, Scindapsus, Euonymus, Dieffenbachia, Camlellia japonica, Cordyline, Nerium, Aglaonema, Dianthus*, and fungi as *Marssonia rosae* Briosi & Cavara (Bonord), *Oidium euonymi-japonici* Arcangeli, *Colletotrichum* sp. were introduced as the major pathogenic factors (Mir Hosseini Moghaddam, 1996).

The purpose of this study was isolation and identification of the most important fungi pathogenic factors which damage the *Euonymus* spp. on its different growth cycle in Guilan province.

MATERIALS AND METHODS

Collection and culture of fungal isolates

Diseased leaves of *Euonymus* spp. were sampled from cultivation areas *Euonymus* of in Guilan province of Iran at 2012. Leaves were transferred to the plant protection laboratory of Islamic Azad University of Rasht Branch, 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 25ºC for 2-3 days. PDA (potato dextrose agar) and WA (water agar) media were used for sporulation. Then Petri dishes containing media were incubated at 25ºC in the dark or artificial light supplied by fluorescent light on a 12 h light/dark photoperiod for 15-30 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 25ºC and 12 h photoperiod. Afterward, morphological observations were taken based on colony, conidium and conidiophore morphology and other characters morphological (Rai and Rajak,1993; De Hoog *et al*., 2000; Cannon *et al*., 2008).

Pathogenicity tests

The pathogenicity tests occurred in a complete random design (CRD) with 3 replications. The treatments including eight treatments. Pathogenicity tests were carried out in desiccators. *Euonymus* spp. were planted in plastic pots with 2.5 cm in diameter containing loam. 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°C, 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 and 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 was analyzed by SAS software and the means of treatments was compared with Tukey method.

RESULTS AND DISCUSSION

After the elementary diagnosis of all the obtained fungi isolates in genus, three fungi isolates of each species were chosen. They were assessed and diagnosed in species. Based on morphological features, two groups of fungi were diagnosed as:

1. Phoma sp. (Sacchardo)

The colonies were flat, released, and powdery to velvet like and expanded quickly (Fig. 1). In close-up, their color ranges from white- in the beginning- then olivaceous, gray and sometimes pink. The hyphae were wall-hanging, transparent to brown (Fig. 2). Pycnidium, if to be made, was large, spherical and 70-100 µm in diameters. Conidia were unicellular, colorless and ovoid (Fig. 3) and 1-3.5×2.5-10 µm. The features of this group of isolates corresponded with Phoma sp. (Sacchardo) (De Hoog *et al*., 2000; Rai and Rajak, 1993).

2. Colletotrichum gloeosporioides (Penz.)

The colonies were brown to gray-black; their surfaces were rough with radical growth (Fig. 4). Conidia were colorless unicellular (without transverse walls), oval to fusiform, rarely were curve or dumbbell-shape with round end, 10-15 µm in length and 5-7 µm in diameter (Figs. 5 and 6). Conidiophores were straight, simple and short, 20-30 × 2-3 µm (Fig. 7). The features of this group of fungi conformed to Colletotrichum gloeosporioides (Penz.) (Cannon *et al.*, 2008).

The first symptoms of isolates of Phoma sp. appeared 24 h after inoculation on *Euonymus*

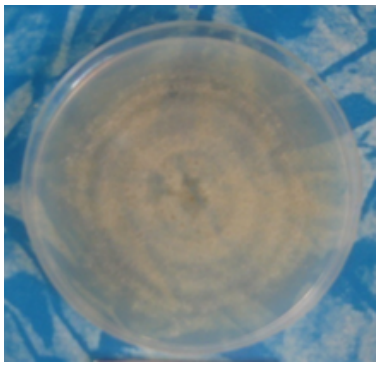

Fig. 1. Colony of *Phoma* sp. on PDA.

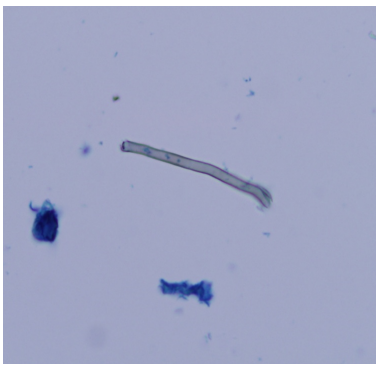

Fig. 2. Hyphae of *Phoma* sp. (×1200).

Fig. 3. Conidia of *Phoma* sp. (×1200).

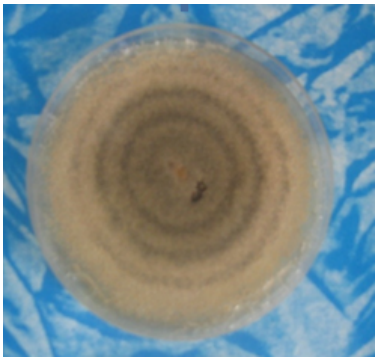

Fig. 4. Colony of *Colletotrichum gloeosporioides* on PDA.

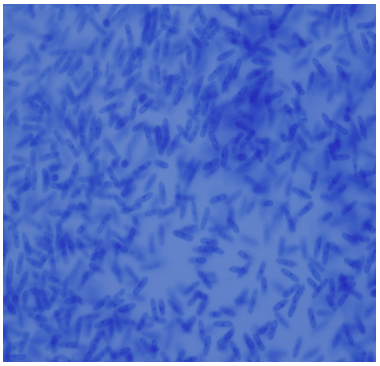

Fig. 5. Conidia of *C. gloeosporioides* (×460).

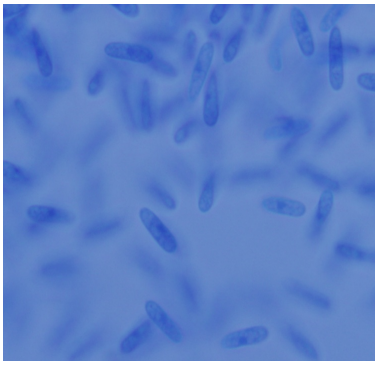

Fig. 6. Conidia of *C. gloeosporioides* (×1200).

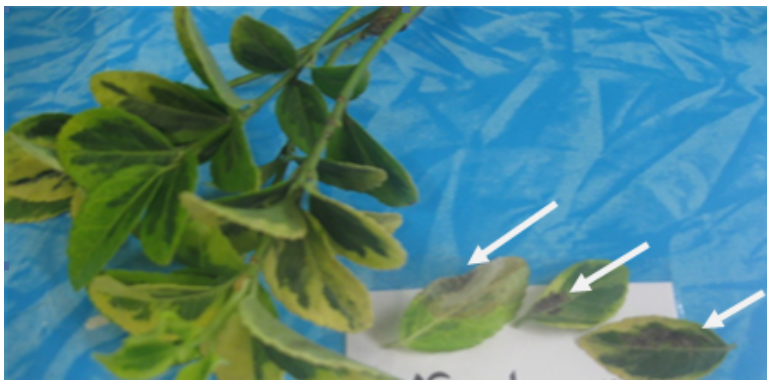

Fig. 8. Symptoms of *Phoma* sp. on *Euonymus* spp.

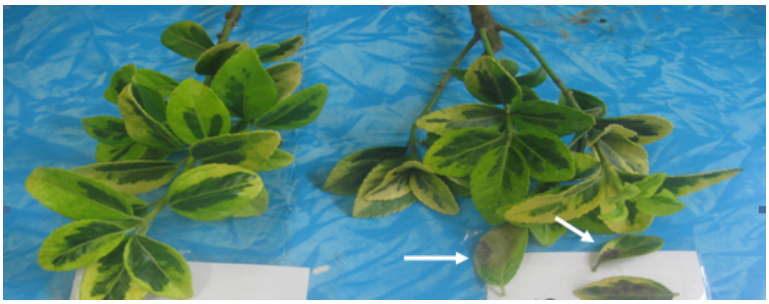

Fig. 9. The comparison between symptoms of *Phoma* sp. on *Euonymus* spp., left (control), right (treatment).

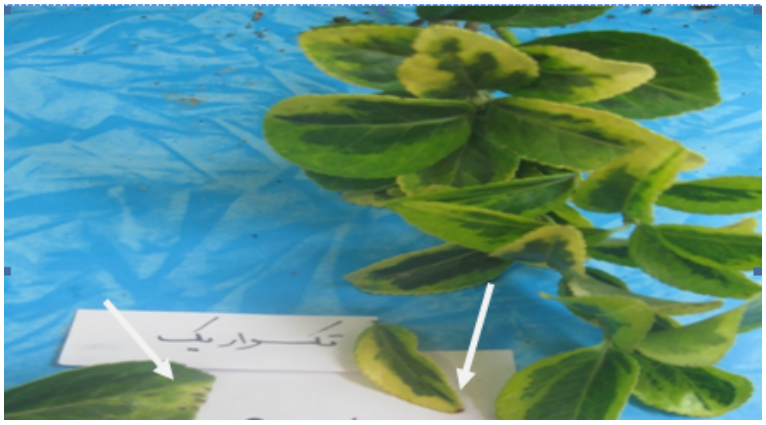

Fig. 10. Symptoms of *C. gloeosporioides* on *Euonymus* spp.

spp. Symptoms were gray-black spots, small and ovoid that in the middle of leaves were observed and gradually increased in the top of the leaves and veins. Finally, in the last day, spots were interconnected and produced necrotic lesions (Figs. 8 and 9).

The first symptoms of isolates of Colletotrichum gloeosporioides appeared 72 h after inoculation as brown spots, small and rounded on the edges of the leaf tip and in the days after the spots were developed and were expanded toward leaf middle parts. Finally, in the last day, the spots were further developed and produced necrotic lesions on the leaves (Figs. 10 and 11).

Analysis of variance indicated that there was not any significant difference in the disease rating of studying fungi (Table 1).

Also based on comparing mean-squares, there was no significant difference between the studying fungi on the *Euonymus* spp. (Table 2). But according to the observations based on Horsfall-Barratt system, the made disease rating by Phoma sp. was more than the disease rating of Colletotrichum gloeosporioides on the *Euonymus* spp.

In a study, *Alternaria alternata* was identified as the causative agent in *Aloe vera.* In the pathogenicity test, seven days after inoculation, necrotic leaf spots were observed on the inoculated

Fig. 11. The comparison between symptoms of *C. gloeosporioides* on *Euonymus* spp., left (control), right (treatment).

plants and no leaf spots were observed on control plants. This was the first report of *Alternaria alternata* on the *Aloe vera* in Louisiana (da Silva and Singh, 2012).

Mir Abaolfathi (2004) studied the outbreaks of leaf spot disease and Azalea leaf-falls in gardens of Mazandaran province which made a great damage and introduced *Colletotrichum gloeosporioides* as a pathogenic agent. The symptoms were the abundant small spots on the two surfaces of leaves and had a specific boundary in red-brown color; the spots in the young leaves were restricted to the surface layers and they could not be seen in the surface against the leaf. Of the tissues with symptoms in a few cases the *Pestalotiopsis* was isolated. The isolates pathogenicity of *Colletotrichum gloeosporioides* isolated from the infected plants was studied by inoculation of suspension of isolates on the leaf and the vase plant branches of *Azalea* in a greenhouse. The disease symptoms were just observed in the plants inoculated with *C. gloeosporioides*. The results revealed the anthracnose disease factor in gardens of Mazandaran province is *Colletotrichum gloeosporioides* (Mir Abolfthi, 2004).

In another study, in the Eastern regions of Guilan, *Colletotrichum gloeosporioides* isolated from *Camellia*. The results of pathogenicity tests showed that the symptoms on the cut leaves inside the desiccator were observed after six days, and black pimples were formed on the spots (Mir Hosseini Moghaddam, 1996) which was inconsistent with findings of the present study about appearance time of symptoms.

In another study, rose stem rot in the commercial farms of Sierra region in Brazil was evaluated and *Fusarium oxysporum* was identified as a pathogen. In a pathogenicity test carried out at

Table 1. Analysis of variance of disease rating in *Euonymus* spp. affected by *Phoma* sp. and *C. gloeosporioides*.

S.o.V	df	SS	MS	F
Treatment	7	0.040291	0.013430	1.54^{ns}
Error	16	0.0698	0.0872	
Total	23	0.11	-	-

ns: not significant at $p<0.05$.

Table 2. Comparison of means of disease rating affected by *Phoma* sp. and *C. gloeosporioides*.

Fungi	Disease rating
Phoma sp.	1.080 a
C. gloeosporioides	1.023 a

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

room temperature after 20 days, the tissue of the inoculated plantlet began to show the symptoms. No symptoms were observed on the control plantlets (Barguil *et al*., 2009).

Jadrane *et al*. (2012) isolated *Colletotrichum* sp. from white *Phalaenopsis* flowers growing in a greenhouse in San Francisco and observed that isolates possessed the same characteristics with *Colletotrichum karstii.*

In studying on the *Strelitzia reginae* was observed the disease symptom on the infected plant leaves, *Alternaria* sp. The results revealed the disease symptoms after five days in burned spots appear in the leaf laminas (Mir Hosseini Moghaddam, 1996).

In the present study in which the sampling was done in the spring and summer of 2011 and 2012 from the green environments of cities of Guilan province from the *Euonymus* spp., *Colletotrichum gloeosporioides* and *Phoma* sp. were detected and then the different isolates pathogenicity tests on the golden *Euonymus* in the desiccators were assessed through spore-pouring on the plants by providing the spore suspension of fungi. The results of the present study on the *Euonymus* spp. in the laboratory conditions based on Horsfal-Barat suggested that all the studied species on this plant were pathogenic but *Phoma* sp. showed a more disease intensity than the other tested fungus. This study could be prefaced for scrutiny diseases of *Euonymus* spp., that is one of the most important ornamental plants in Iran. This study was the first report of leaf blight caused by *Colletotrichum gloeosporioides* and *Phoma* sp. on *Euonymus* spp. from Iran.

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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