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Investigating the pathogenicity of *Alternaria alternata* on *Lonicera japonica*

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ABSTRACT

The present study was carried out to investigate the pathogenicity of *Alternaria alternata* and the effect of its metabolites on *L. japonica* from 2015- 2016 in Birjand plain area in eastern Iran. *A. alternata* isolates were inoculated on *L. japonica* detached leaves in laboratory conditions, on plants by spore suspension in the greenhouse and young branches by fungal mycelia. The effect of *A. alternata* metabolites was examined by injection of extracted metabolites from a 10-day culture of the fungus in a Czapek broth media into the leaves of the plant. Inoculated detached leaves, after 3 to 7 days of inoculation, exhibited different ranges of chlorosis and necrosis, with or without a yellow halo around some of this spot. Leaves of inoculated plants in the greenhouse showed chlorosis, necrosis and leaf spots with (Mo8 isolate) or without (H44 isolate) a yellow halo. Inoculated stems demonstrated rotting and death in the inoculation site and wilting of stems. Metabolites of some isolates particularly isolate with a yellow halo (Mo8) in inoculated detached leaves, caused necrotic leaf tissues five days after injection. The results showed that *A. alternata* could be a cause of leaf spot, chlorosis, and necrosis, and the metabolites of some isolates can cause the death of leaf cells of *L. japonica*. This is the first report of the *A. alternata* pathogenicity on this plant in the eastern part of Iran.

INTRODUCTION

Lonicera japonica L (known as golden-and-silver honeysuckle, in Persian known as Piche Aminoldulleh) from the family of Caprifoliaceae is a shrub, evergreen and rising, with a height of 2.5-5.5 m, very appropriate for creating vegetation or gardening (Shang et al. 2011). The leaves of this plant are reciprocal and simple, with flowering periods in small flowers and scented with cream colour, in the middle of winter to early spring. In another species of this plant, which has red-faced pink flowers, the flowers appear in the middle of spring and continue until summer and are appropriate for cold to warm areas, while it requires regular irrigation at the beginning of its growth, the bushes are able to grow over a period of prolonged drought (Peng et al. 2000). The propagation of this plant occurs via semicircular cuttings in late summer and autumn. *L. japonica* is a seasoned plant that tolerates cold winters, and a wide range of soils including poor drainage and salt and heavy metals (Williams et al. 2001).

Thus far, several fungal pathogens have been reported from *L. japonica* plants, especially as leaf spot disease in some parts of the world (Waipara et al. 2007). The species of *Insolibasidium deformans* cause the death of the plant in New Zealand (Waipara et al. 2007) and the United Kingdom (Beales et al. 2004). Species of *Pseudocercospora lonicerae* (de Miranda et al. 2014), *Colletotrichum gloeosporioides*, *Microsphaeropsis* sp., and *Gyoerffyella rotula* infect *L. japonica* and cause leaf spot and necrosis (Waipara et al. 2007). Some species of *Fusarium, Phomopsis*, *Phoma* and *A. alternata* cause brown spots or rotting of flowers or act as a saprophyte and secondary pathogen on this plant (Bi et al. 2012). Basidiocarps of *Chondrostereum purpureum* were collected from the basal stem of a Japanese honeysuckle vine at Waipu Gorge, Northland, but there were no visible signs that infection by this pathogen had reduced the growth or affected the health of this plant (Waipara et al. 2007). *C. purpureum* species infected wooden tissue and caused the death of aerial parts. *Erysiphe lonicerae* var. *lonicerae* caused powdery mildew on Japanese honeysuckle in Korea and Italy which is a limiting factor for the

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use of this plant (Lee et al. 2016). Several viral diseases are also influencing the growth of *L. japonica* in some parts of the world (Kitamura et al. 2004). *Tobacco streak virus* from the United States and *Tobacco leaf curl virus* from Japan have been introduced as an important viral pathogen for this plant (Ali et al. 2014). Thus far, no significant pathogens have been reported in Iran. Given that one of the factors of leaf spot of ornamental plants is *A. alternata* isolates, in this research, pathogenicity of various isolates of *A. alternata*, which had previously been collected from the soil and tissues in Birjand area, were studied on *L. japonica*.

MATERIALS AND METHODS

A. alternata isolates were obtained from the plant pathology laboratory of the University of Birjand, which was isolated and identified by the authors in 2015-2016 from the soil in Birjand area.

Pathogenicity tests on detached leaves

The pathogenicity of *Alternaria* isolates was performed *in vitro* conditions by inoculation of *L. japonica* detached leaves (Gilchrist and Grogan 1976; Sharma et al. 2004). Young leaves without any symptoms of disease were selected, petiolate was removed, and the surface was disinfected by sodium hypochlorite. Small scars were created under the leaves surface with a thin and sterile scalpel, and 5 × 5 mm pieces of the 7-day-old fungal culture were placed on it. PCA media were used as a control for leaf tissue inoculation. Inoculated leaves were incubated on sterile and wet filter paper in a sterile Petri dish to maintain moisture, at 25°C incubator. After seven days, the symptoms on the leaves were studied. *Alternaria* isolation from inoculated leaf tissue was carried out by PCA media (Sharma et al. 2004).

Pathogenicity of *A. alternata* on *L. japonica* stem

Healthy branches without any apparent signs and symptoms of the plant disease were cut and transferred to the laboratory. These grassy and greenery branches have been placed in a container of water to keep their moisture and viability safe and dry throughout the inoculum period until the symptoms appear. Several internodes were selected at the lower end of the stem, and the surface was disinfected with 70% ethanol. A small scar was created on the stem with a sterile needle and a 2 × 2 mm piece of the 7-day colony of the fungi and was placed on it and covered with parafilm (Liu et al. 2017). After five days, the effect of fungus on the tissue of the branches was investigated, and the fungus was re-isolated from the inoculated branches (Garibaldi et al. 2016; Gilchrist and Grogan 1976).

Pathogenicity of *A. alternata* on *L. japonica* under greenhouse conditions

Pathogenicity of *A. alternata* isolates on *L. japonica* leaf in greenhouse conditions was performed by spraying 10^6 suspensions of fungal spores from 7-day-old colonies in PCA on healthy plant leaves without apparent contamination (Edwards et al. 2017; Gilchrist and Grogan 1976). Sterile water was used as a control. Inoculated plants were kept under 90% relative humidity and 25°C for 48 h and then transferred to a greenhouse with 70% moisture content (Edwards et al. 2017, Gilchrist and Grogan 1976). The results were evaluated after one week, and from leaves with leaf spot symptoms, the fungus was recovered.

Effect of *A. alternata* metabolites on *L. japonica*

Fungal isolates were cultured in Czapek liquid culture medium (Siciliano et al. 2015) for ten days at a temperature of 25 ° C (Wei et al. 2017). After ten days, 2 mg/l of the chlorothalonil (daconyl) fungicide was added to kill fungal cells and were centrifuged at 10000 rpm. The supernatant which contained fungal metabolites and chlorothalonil was injected into plant leaves. As a control, a liquid Czapek medium containing 2 mg/l of the chlorothalonil and sterile distilled water was used. Changes in injected tissues were investigated after a week (Meena et al. 2017; Siciliano et al. 2015).

RESULTS AND DISCUSSIONS

The results of the pathogenicity test on detached leaves

Five days after detached leaves inoculation, two types of reaction were observed at the inoculation site, and the remaining areas of the tissues were healthy and succulent. In the control samples and some of the inoculated leaves with *A. alternata* isolates, there was no change in colour, chlorosis or necrosis (Figure 1A). The other group was the isolates in which leaf tissue first showed chlorosis and then necrosis (Figure 1B-D). The amount of necrosis and its spread, as well as the yellow halo around chlorosis, differed in various isolates (Figure 1C). In some isolates, the rate of necrosis was very rapid, and within seven days, it covered the entire leaf area, the leaves became black and completely rotten (Figure 1D). The second group was isolates that slowed the spread of infection, and after seven days, the radius of the necrotic region was about 1 cm (Figure 1C). In the third group, the decay was very limited and only under the inoculation site. In some isolates that had symptoms of chlorosis and necrosis on the leaf, a yellow halo was visible around the necrosis area. The results revealed that the *A. alternata* isolates from Iran cause chlorosis and necrosis on the *L. japonica* leave *in vitro* conditions. The inoculation of detached leaves is an accepted method for the

study of *Alternaria* pathogenicity on plant tissue (Sharma et al. 2004). This method has already been used to investigate the reaction of different varieties of tomatoes to *Alternaria* (Foolad et al. 2000). This research confirms the application of the above method to investigate the pathogenicity of *Alternaria* on *L. japonicas* and shows the effectiveness of detached leaves inoculation method in detecting the pathogens of Japanese honeysuckle plant. Detached leaves inoculation method can be used as a primary test for the study of pathogenicity of *Alternaria* species on *L. japonicas* leaf tissue.

The results of pathogenicity test on *L. japonicas* stem

Five to seven days after inoculation of *L. japonica* stems, the tissue decay of the stem from the inoculum to the sides of the stem expanded (Figure 1F). Increasing the level of infection was resulted in the stiffness of the stem end and its death. As long as moisture conditions were provided, this infection spread to the bottom of the stem, and the area above the inoculum was wilted and dried (Figure 1F). There was no change in stem tissue in control treatment groups (Figure 1E). In the test until the tenth day, when the stem tissue was still not affected by the test conditions, the uninfected tissues were completely healthy and juicy and were well isolated from the contaminated tissue, but after this period, chlorosis and loss of some leaves occurred. This phenomenon was not related to the effects of the isolates and was related to the nutritional conditions of the tissue. Therefore, it is recommended that this test be reviewed at the same time, and the results should not be considered after this period. Some *Alternaria* isolates of this study, including control treatments, did not affect stem tissue and inoculated stems were healthy and succulent.

Results of pathogenicity test in greenhouse conditions

Three to five days after the spore spraying of the leaves, chlorosis was observed at the leaf surface (Figure 1H). This colour change began on most pathogenic isolates from the margins of the leaves and spread to the inside of the leaf. The stains created on the margin of leaves in chlorosis symptoms demonstrated that the tissues gradually became necrotic and dried (Figure 1H). Necrotic spots were evident in some yellow halo isolates. The spread of spots led to a lot of leaf chlorosis. With the increase in chlorosis and necrosis of the tissue surface, leaf loss occurred. The infected leaves that remained on the stem were dried and black (Figure 1I). Inoculated stem tissue also showed black spots due to the activity of the fungus. The results showed that *A. alternata* in greenhouse conditions cause necrotic leaf spots and maturation. Some *Alternaria* isolates and control treatments did not affect leaf tissue and inoculated leaves did not show any necrosis symptoms (Figure 1G).

Effect of *A. alternata* metabolites on Honeysuckle Leaves

Five days after metabolites injection, leaves showed chlorosis and necrosis in the injection area and but the controls and metabolites of some of the isolates did not affect leaf tissue (Figure 1Jc). The greatest effect of metabolites was on isolates that had a yellow halo in the previous experiments (Figure 1J). This test was carried out on young leaves that were in the plant shadow since leaves that were older or exposed to direct sunlight often contained thick tissue and the injection of metabolites into them was not easily possible. Extension of metabolites in the leaf area was not very wide, but more in a small area, about 5 mm, around the injection site. Injection of young leaves was easily done, and the injection site did not result in injuries. The lack of plant's reaction to the metabolites of some isolates, especially isolates that did not produce yellow halos, revealed that some of the isolates contained either mycotoxins or a toxic compound that resulted in tissue death.

CONCLUSIONS

This is the first report of *A. alternata* pathogenicity on Honeysuckle from the east of Iran. This plant has a dense canopy (McCusker et al. 2010), which increases the moisture content in the aerial parts and provides conditions for infection by *A. alternata* fungus. *A. alternata* isolates caused leaf spot or stem rotting under favourable conditions on the tissues of this plant (Chaerani and Voorrips 2006). Due to the perennial nature of the plant, the old-tissues that remain at the end of the growing season can be a good site to increase *Alternata* disease (Schulz et al. 1993). The results of this study showed that *A. alternata* species could develop on the leaf and stem tissue and some isolates are also able to destroy the tissues of this plant by producing toxins. The yellow halo created on the tissue was also due to the presence of toxin affecting the plant in the metabolites of these isolates (Pinto and Patriarca 2017). It is recommended that the spread of diseases caused by this pathogen in different parts of Iran should be investigated, and selective or non-selective mycotoxin production by pathogen and its effects on this plant should also be investigated. Honeysuckle is a perennial plant, and the old tissues are usually located in the plant shadow (Peng, Mei, Jiang, Zhou and Sun 2000), and the conditions for the spread of fungal contamination on the tissues of the lower and the inside of the canopy of the plant are provided. It is

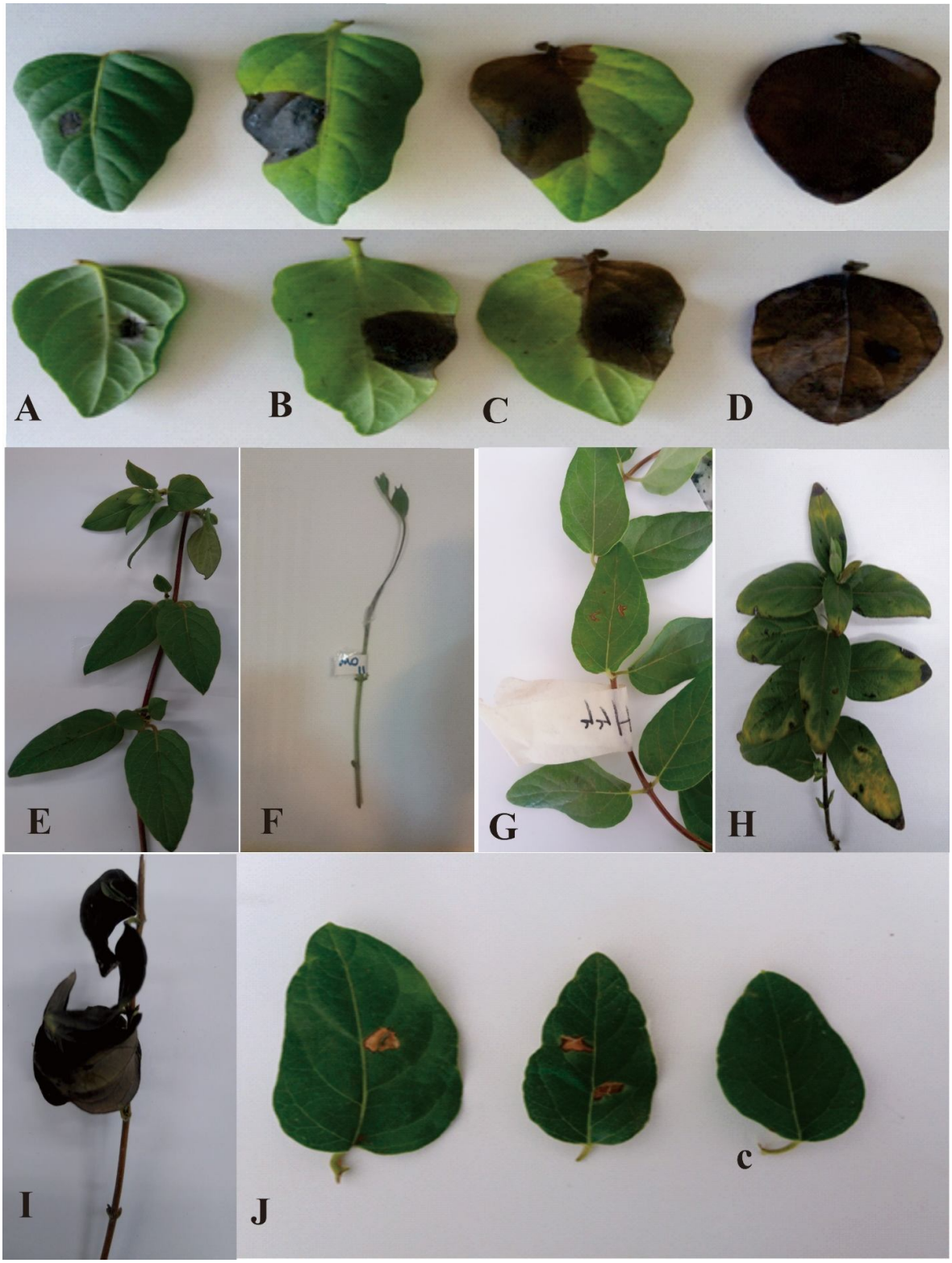

Figure 1. Pathogenicity of *Alternaria alternata* on Honeysuckle and reaction of host tissues to *Alternaria* metabolites. Inoculated detached leaves (A-D) and Young stems (E-F), Sporulation of leaf tissues (G-I), Metabolites injection (J)

recommended that the old and contaminated tissues be removed to reduce moisture content in the plant's canopy. Failure to spray the plant and prevent moisture increase can also prevent the spread of this pathogen.

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