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Identification and detection of agent of loquat leaf spot and fruit rot in north of Iran

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ABSTRACT

Alternaria alternata is a fungus which has been recorded causing leaf spot and other diseases on over 380 host species of plant. It is an opportunistic pathogen on numerous hosts causing leaf spots, rots and blights on many plant parts. A leaf spot and fruit rot on loquat, caused by *A. alternata*, was found in the plantations and entertainment venues for the first time in Iran in Sari city, Mazandaran province. The fungus was isolated from leaves and fruits, and its pathogenicity was confirmed. Pathogenicity tests showed that *A. alternata* could infect loquat, which developed the same symptoms after inoculation as those observed naturally in the field. The fungus was identified based on morphological characteristics and confirmed using comparisons of DNA sequences of Alt α 1 locus. This report is the first on loquat leaf spot and fruit rot disease caused by *A. alternata* in Iran.

Key Words: *Eriobotrya japonica*, Mazandaran, Molecular analysis, Alt α 1 gene.

INTRODUCTION

Loquat (*Eriobotrya japonica* (Thunb.) Lindl.) is an Asian fruit and a member of the Rosaceae family. The species is native to southeastern China and mainly grow on subtropical and mild temperate regions. Currently it is also cultivated in other areas namely in the South Africa, South America, Australia and California (Cha *et al.* 2011). Loquat is an evergreen large shrub or small fruit tree with a rounded crown and short trunk. Now, it is commonly to be found in plant gardens and as an ornamental plant along the sides of road or river and parks in Iran. Fruits, leaves and pyrene of the plant are useful as traditional herbal medicine to reduce various diseases such as lung and stomach discomfort, cough and wound healing (Nagy & Shaw 1980). Tosun *et al.* (2009) reported that the loquat fruits showed a significant antioxidant activity. Brown rot, decline of trees and Entomosporium and Spilocea leaf spots diseases of *E. japonica* have been reported in Iran caused by *Monilinia fructigena*, *Fusicoccum dimidiatum* and *Entomosporium maculatum* and *Spilocea pyracanthae*, respectively (Ershad 2009). During regular observations from 2012- 2015, severe infection was observed on loquat leaves and fruits in all the plants growing at different sites in the gardens and entertainment venues of central of Mazandaran province, Iran. Approximately 30-55% of plants were affected with disease. Therefore, the present study was carried out for isolation and identification of pathogen associated with diseased leaves and fruits of loquat.

METHODOLOGY

Isolation and Identification of pathogen

Diseased leaves and fruits with typical symptoms of dark brown necrotic lesions were collected.

Samples were cut into small pieces and surface disinfected with 0.6% sodium hypochlorite solution for 1 min, rinsed thoroughly with sterile distilled water, the moisture was removed with sterilized filter paper, and the pieces were cultured on potato dextrose agar (PDA) plates and incubated in the dark at 28 ◦C. Microscopic observations were conducted by mounting fungal tissues of 10-day-old colonies in water and morphological characteristics of conidia were described.

Pathogenicity test

Pathogenicity of the fungus was confirmed twice (two experiments) by inoculating healthy leaves and fruits loquat plants grown in an experimental field. Separately, ten leaves and fruits per experiment were sprayed with ~5 mL of conidial suspension (1×10^5 conidia mL^{-1}) obtained from 15-day-old culture plates. Before inoculation, all plants inoculated and water-sprayed controls, were disinfected with 0.1% sodium hypochlorite solution for 1 min and rinsed thoroughly with sterile distilled water. After inoculations, control and inoculated leaves and fruits were covered with polythene bags for 8 days.

DNA extraction, gene amplification and sequencing

Fungal DNA extractions were performed according to the modified method reported by (Groppe & Boller 1997). Fresh mycelium (200 mg) was transferred to a sterilized mortar and grinded with liquid nitrogen, then transferred in .5-mL Eppendorf tube containing 500 ml of CTAB (hexacetyldecyltrimethylammonium bromide), extraction buffer (0.7 M NaCl, 10 mM EDTA, 50 mM Tris-HCl [pH 8.0], 1% 2-mercaptoethanol, 1% CTAB) and incubated for 30 to 60 min at 65ûC. An equal volume of SEVAG (chloroform-isoamyl alcohol [24:1, vol/vol]) was added, and samples were gently mixed for 30 min on a rocking platform. The samples were spun in a micro centrifuge for 10 min (15,000 × g), and the aqueous upper phase was transferred to a fresh tube. DNA was precipitated with an equal volume of isopropanol, and the tubes were spun in a micro centrifuge for 10 min. The resultant pellets were washed with 70 and 100% ethyl alcohol, dried, and re-suspended in TE (10 mM Tris HCl [pH 8.0], 1 mM EDTA). The DNA was re-precipitated with 0.3 M sodium acetate and 2 volumes of ethanol. Samples were again spun, and the pellets were washed, dried, and re-suspended in TE (200 ml) and stored at −20 °C until use for amplification reactions. One housekeeping gene, namely Alt α 1 encoding major allergen of Alternaria spp. was used. Small modifications of the PCR protocol were made, e.g. increasing the annealing temperature by 3°C for the Alt α 1 gene (Hong *et al.* 2005). Amplifications were performed in a total volume of 25 µl. Reaction mixture consisted of 1 µl of DNA, 1.5 U of Taq DNA polymerase (Sinagen, Tehran, Iran), Buffer at the final 1x concentration, 0.2 mM concentrations of each dNTP and 0.5 µM of primers for each gene. Amplifications were carried out in the Biorad thermal cycler under the following conditions: initial step at 94ºC for 1 min, 35 cycles of denaturation at 94ºC for 30 sec, annealing at 54ºC for 30 sec and extension at 72ºC for 1 min. The final step was at 72ºC for 10 min. DNA sequencing was by Bioneer Co. (Daejeon, South Korea). Dendrograms were reviewed and edited using the Bioinformatics software for sequence alignment, BioEdit software (Hall 1999). A maximum-likelihood tree was constructed using the MEGA6 program (Tamura *et al.* 2013).

RESULTS AND DISCUSSION

A leaf spot and fruit rot disease of loquat was observed during 2012–2015 in Sari, Mazandaran, Iran. The symptoms recorded were small, circular, brown necrotic spots all over the foliage. The spots gradually enlarged in size and later became irregular in shape, or remained circular with concentric rings or zones. In the later stage of infection, the spots coalesced resulting in withering, extensive drying and shedding of leaves. On fruit, the symptoms started as small, concentric, dark brown, sunken spots, which coalesced and covered the entire surface of the fruit. Affected fruit rotted completely, causing severe yield losses. “Fig. 1a and 1b”. The same fungus was consistently isolated on PDA from all infected samples and no other fungi were isolated from the infected leaves and fruits. These cultures gave rise to initially white colonies which turned to grayish-black later due to abundant sporulation. Conidia were produced in long chains, pale to light brown, obpyriform, with a beak one to seven transverse and up to three longitudinal septa, and measured 10 to 45 µm long × 7 to 22.5 µm wide. Conidiophores were straight, septate, light to olive golden brown with conidial scar, and measured 35 to 100 µm long × 2 to 5 µm wide. The morphological descriptions and measurements of the fungus are similar to *Alternaria alternata* (Simmons 2007). Pathogenicity tests conducted on healthy leaves and fruits of loquat resulted in the development of typical symptoms of the disease on leaves and fruits after 6-8 days, whereas no symptoms were observed on control plants. The same pathogen was consistently re-isolated and identified from all inoculated plants, confirming Koch’s postulates. The Alt α 1 gene sequences (GenBank accession numbers KJ396786 and KR912174) showed high similarity (99-100%) of identity not only among all isolates from loquat but also to other *A. alternata* isolates deposited in GenBank. The Alt α 1 region showed that all isolates of *A. alternata* clustered together in a monophyletic group with 100% similarity “Fig. 2”. In contrast, *A. dauci*, *A. tenuissima*, *A. porri* and *A. solani* clustered in a monophyletic group, correlating with the fact that these species produce larger-size conidia (Simmons 2007). In this study, the incidence of leaf spot and

Fig. 1. A& b. Natural symptoms of loquat leaf spot (left) and fruit rot (right) caused by *Alternaria alternata*.

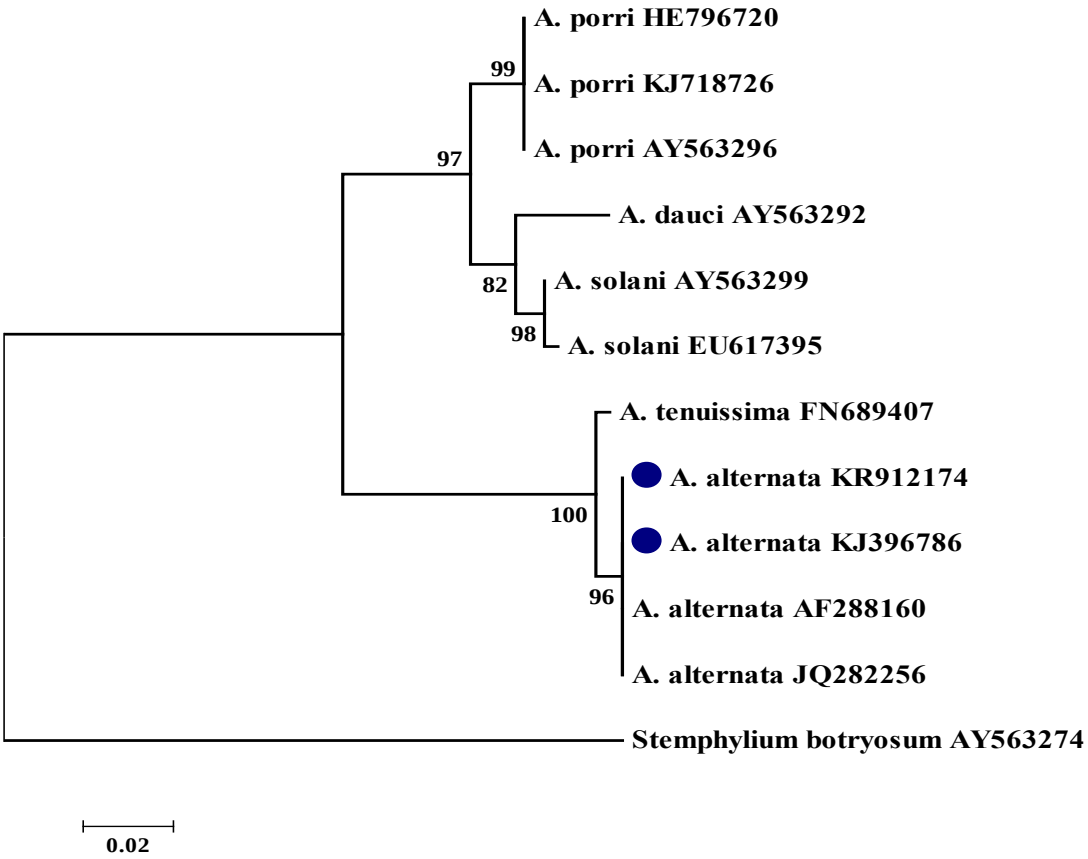

Fig. 2. Fig. 2. Maximum-likelihood tree of the Alt α 1 regions for 12 sequences. *Stemphylium botryosum* was used as the out-group. The bootstrap value (C50 %) at each branch calculated from 1000 replications, and the bar represents 0.02 substitutions per nucleotide position. The bold blue circular symbols (●) represent isolates of this study.

fruit rot caused by *A. alternata* was 30–55% in the plantations of loquat, which significantly reduced fruit production. *Alternaria* spp. cause common diseases on leaves, stems, fruits and flowers of a wide range of plant species (Agrios 2005). There are reports of *Alternaria* sp. causing leaf spot on loquat in Florida, Japan, Mexico, Taiwan and Venezuela (Farr & Rossman 2013) and fruit rot on the same plant species in Taiwan (Ko *et al.* 2010). Furthermore, *A. alternata* has been reported as the causal agent of a spot disease on leaves and fruits of loquat in Palestine (Batta 2005) and Greece (Tziros 2013). Nevertheless, to our knowledge, this is the first report of *A. alternata* on loquat in Iran.

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