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Association of *Alternaria alternata* and *Cladosporium cladosporioides* with Leaf Spot in *Cissus quadrangularis* and *Ficus sycomorus*

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ABSTRACT
Cissus quadrangularis and *Ficus sycomorus* are two important wild plants in the Arabian Peninsula and other parts of the world. Leaf spot symptoms were observed on both plants in Oman, 50 km to the north-west of the Capital area, Muscat. Incidence of leaf spot disease in *C. quadrangularis* and *F. sycomorus* was 90-100% and 20-40%, respectively. Isolations were established in potato dextrose agar and the isolated fungi were identified based on sequences of the internal transcribed spacer region of the ribosomal DNA (ITS rDNA). *Cladosporium cladosporioides* and *Alternaria alternata* were found associated with the two plants. Pathogenicity test showed that *A. alternata* was pathogenic on *C. quadrangularis* while *C. cladosporioides* was pathogenic on *F. sycomorus*. This is the first report of association of *A. alternata* with *C. quadrangularis* and *C. cladosporioides* with *F. sycomorus*.

Key words: ITS-rDNA, molecular, Oman

INTRODUCTION

Oman is a home for about 1200 plant species within 124 plant families. Many of these plants are subject to extinction because of diseases, natural phenomena or human related activities (Al-Sadi *et al*., 2012a). *Cissus quadrangularis* Linn. is a widespread species distributed through tropical Africa, Sothern Arabia and India (Pickering and Patzelt, 2008). It is also found in Sri Lanka, Myanmar and Philippines. In Arabia, it is found in Oman, Yemen and Saudi Arabia (Ghazanfar, 2007). *Cissus quadrangularis* is a medicinal plant that has traditionally been used for treating skin infections and mastitis in livestock (Ghazanfar, 2007).

Cissus plants can be affected by leaf spots and root rot diseases. In Texas and Louisiana, three fungi cause leaf spot diseases on *Cissus* which are *Cercospora viticola*, *C. arboreae* and *Phyllosticta cissicola*. Other diseases of *Cissus* are rust caused by *Aecidium mexicanum*, root rot caused by *Phymatotrichum omnivorum* and smut caused by *Mycosyrinx cissi* (Dodge and Rickett, 1943).

Ficus sycomorus L. belongs to the family Moraceae. The genus *Ficus* consists of about 2000 species occurring throughout the Old and New World tropics and it is considered the largest genus of trees in Arabia after *Acacia* (Miller *et al*., 1988). *Ficus* is derived from the Persian 'fica', the Latin for fig. In Greek 'syka' means fig (Galil, 1968). Mature fruits of *Ficus* are eaten fresh or dried and stored for later use. Also, fruits are used as fodder for livestock, wild animals and birds.

Ficus trees are susceptible to various fungal, bacterial and viral problems. The genus *Ficus* is attacked by at least 30 species of fungi which cause leaf rust, branch and foliage blights, branch wilt and canker, root rots, fruit surface mold and spot rot, internal fruit rot and fruit souring. In Iran, *Ficus elastica* suffers from leaf spot disease caused by *Phoma glomerata* (Aghapour *et al*., 2009). In addition, *Xanthomonas campestris* pv. *fici* was reported associated with angular leaf spot and leaf drop on ornamental *Ficus* spp. (Duff, 1991).

Leaf spot symptoms were observed on *C. quadrangularis* and *F. sycomorus* in 2009 and 2010 in Oman. The symptoms were moderate on both plants in 2009 but severe on *C. quadrangularis* in 2010. There is a lack of information concerning pathogens associated with leaf spots in these plants. This study was carried out to characterize the

main fungal pathogens associated with leaf spots in *C. quadrangularis* and *F. sycomorus*. Knowledge in these areas will establish a basis for management of diseases affecting these plants.

MATERIALS AND METHODS

Collection of samples: Leaf samples were collected from *C. quadrangularis* and *F. sycomorus* showing leaf spot symptoms. Samples were collected from each plant species at two different times: February to March, 2009 and June to July 2010. The samples were collected from Al Khoud, Muscat, Oman.

Isolation: Leaf disks were surface sterilized in 1% NaOCl for 1 min, washed in sterile distilled water for 1 min and then dried on sterile filter paper. The leaf discs were cut into 2 mm pieces and then plated on 2.5% Potato Dextrose Agar (PDA), 4 pieces in each Petri dish. The plates were incubated at room temperature (22±1°C) for 7 days.

Identification of fungi: Fungal isolates were identified using sequences of the internal transcribed spacer region of the ribosomal DNA (ITS rDNA). DNA was extracted from mycelium following a modified protocol of Lee and Taylor (1990) as described by Al-Sadi *et al.* (2007).

Two primers were used for amplification of the ITS rDNA region of fungal isolates: ITS1 and ITS4 (White *et al*., 1990). The PCR reaction mixture consisted of PuReTaq™ Ready-to-Go™ PCR beads (GE Healthcare, Dornstadt, Germany), 0.4 μM ITS1 primer, 0.4 μM ITS4 primer, 2 μL of diluted DNA (12 ng μL^{-1}) and Milli-Q water up to a final volume of 25 μL. Amplification was performed using an automated thermal cycler. The cycling parameters were initial denaturation at 95°C for 10 min and then 35 cycles of denaturation for 30 sec at 95°C, annealing at 55°C for 30 sec and extension at 72°C for 90 sec (Al-Sadi *et al*., 2013). The final extension was for 10 min at 72°C. The quality and quantity of the extracted DNA were checked by means of electrophoresis in 1.5% agarose gels in 0.5x Tris-borate-EDTA buffer (TBE) at 110 V for 60 min. Bands were visualized under UV light.

The PCR products were purified according to the manufactures protocol using QIA quick PCR Purification kits (Qiagen, Hilden, Germany). Samples were sequenced at Macrogen (Seoul, Korea) using ITS1 and ITS4 primers.

The forward and backward ITS sequences were first aligned using ChromasPro (Technelysium Pty Ltd., Tewantin, Queensland, Australia). The resulting ITS sequence for each isolate was then compared with sequences of fungal isolates deposited at the National Center for Biotechnology Information (NCBI) using BLAST search.

Pathogenicity test: In order to confirm that the isolated fungi are pathogenic on *Cissus quadrangularis* and *F. sycomorus*, pathogenicity test was conducted using a detached leaf method (Al-Sadi *et al*., 2012b). *Cissus* and *Ficus* leaves were surface sterilized and placed on moistened filter papers in Petri-dishes. Two treatments and a control were used for each plant species. The first treatment involved applying spores of the fungus without wounding while the second treatment involved applying spores of the fungus after making a 2 mm wound on the upper surface of the leaves. Then, 5 μL spore suspension of *Alternaria* (37 spores μL^{-1}) or *Cladosporium* (67 spores μL^{-1}) was applied on leaves, 3 drops on each leaf with at least 10 mm distance between one drop and the other. Control leaves (with and without wounds) were treated with sterile distilled water. Three replicate leaves were used for treatment/control and the leaves were incubated at 25±1°C for 10 days. Symptom development was recorded daily and the experiment was repeated 4 times. The inoculated fungus was re-isolated from leaves developing symptoms.

RESULTS AND DISCUSSION

Leaf spot of *Cissus quadrangularis*: Leaf spot symptoms which were observed on *C. quadrangularis* plants at Oman Botanic Garden (OBG) were numerous small (0.5-10 mm in diameter), circular and black spots which appeared on the lower surface of the leaves. As disease progressed, leaf spots expanded and turned dark to tan brown in color. At the same time, the upper surface of the leaf appeared yellow in color and as the severity increased the upper yellow spots enlarged and some changed to tan brown with gray concentric rings (Fig. 1). Disease incidence ranged from 90-100%, with 13-30% of the leaf area covered by leaf spots. Isolations yielded from *Cladosporium cladosporioides* and *Alternaria alternata* (Table 1).

Dark spots were seen after three days of inoculation on the surface of wounded and non-wounded *Cissus* leaves inoculated with *A. alternata* spores. No symptoms were observed on the control leaves or leaves inoculated with *C. cladosporioides*. Similar results were obtained when the experiment was repeated twice. *Alternaria* was re-isolated from the wounded and non-wounded leaves.

Cissus plants in other parts of the world are known to be affected by leaf spots which are caused by *Cercospora viticola*, *C. arboreae* and *Phyllosticta cissicola* (Dodge and Rickett, 1943). None of these pathogens was

Table 1: Frequency of isolation and pathogenicity of fungal pathogens associated with leaf spot symptoms in plants

		Pathogenicity test	
Species and isolated fungi	Isolation (%)	Wounded	Non-wounded
Cissus quadrangularis			
Cladosporium cladosporioides	33	-	-
Alternaria alternata	50	+	+
Ficus sycomorus			
Cladosporium cladosporioides	67	+	+
Alternaria alternata	50	-	-

Isolation and pathogenicity tests were conducted at least at two different times. A (+) sign indicates development of symptoms, while a (-) sign indicates that the pathogens caused no symptom

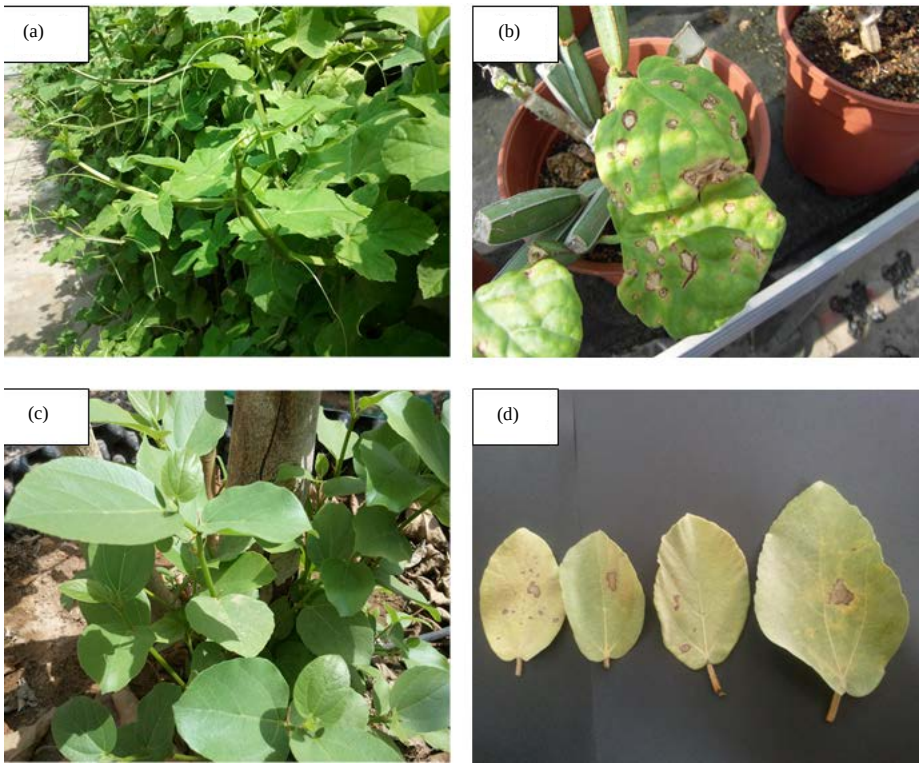

Fig. 1(a-d): Foliar symptoms in (a) *Cissus quadrangularis*, (b) *Ficus sycomorus*, (a-c) Healthy and (b-d) Symptomatic plants

isolated from *Cissus quadrangularis* in this study. On the other hand, leaf spot on *C. quadrangularis* was found to be caused by *Alternaria alternata*. The leaf spot on *C. quadrangularis* in Oman differs from leaf spot on other *Cissus* species by having circular and black spots which change to gray concentric rings as the severity increases.

Leaf spot of *Ficus sycomorus*: *Ficus sycomorus* plants at Oman Botanic Garden were found to be affected by leaf spot symptoms. At the initial stage of infection, small, dark brown spots appeared on the leaves which gradually become larger and irregular in shape with tan centers and surrounded by dark brown margins with a yellowish halo. On severely infected leaves several spots coalesced to form large areas of necrosis on leaves until whole leaves died (Fig. 1). Leaf spots varied from 1 to 15 mm in diameter. About 40 and 20% of *F. sycomorus* plants at OBG were found to be affected with leaf spot symptoms in November 2008 and in April, 2009, respectively. The leaf area covered with leaf spots in both years varied from 1-10%.

Isolations yielded *Alternaria alternata* (50%) and *Cladosporium cladosporioides* (Table 1). Five days after inoculation, small, brown spots were observed on the wounded and non-wounded leaves of *F. sycomorus* inoculated with spores of *C. cladosporioides*. The same pathogen was re-isolated from lesions developing on the inoculated leaves. No symptom was observed on the control leaves. No symptom was observed on the wounded and non-wounded leaves inoculated with spores of *Alternaria*.

Ficus sycomorus leaf spot was found to be caused by *Cladosporium cladosporioides*. In Florida, *F. carica* which is the common edible fig is affected by Cercospora leaf spot caused by *Cercospra fici* and leaf rust caused by *Cerotelium fici* (Chupp, 1953).

Molecular analysis showed that the ITS sequences of *Alternaria alternata* isolates from Oman share 100% similarity to *A. alternata* (AY154682) while the ITS sequences of *C. cladosporioides* share 99.2% similarity to *C. cladosporioides* (KJ767065). One representative ITS rDNA sequence each of *A. alternata* and *C. cladosporioides* was deposited in GenBank under the accession numbers KP256809 and KP256810, respectively.

This is the first record of association of *Cladosporium cladosporioides* with leaf spot of *F. sycomorus* and the first report of association of *Alternaria alternata* with leaf spot of *C. quadrangularis*. More studies are required to examine pathogens associated with diseases in wild plants in Oman.

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