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Severe decline of wax apple trees caused by *Fusarium solani* in northern Taiwan

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ABSTRACT. Wax apple (*Syzygium samarangense*) is an important fruit crop in Taiwan. Severe decline of wax apple trees was noticed in 2003 in northern suburban Taiwan. A fungus consistently isolated from diseased twigs of declining wax apple trees, was identified as *Fusarium solani* based on morphological characteristics. *Fusarium solani* from wax apple shared 92.0 to 98.6% and 93.0 to 99.6% intraspecific sequence similarity of ITS and 28S, respectively, with those available in GenBank. Upon inoculation, the isolated *F. solani* caused twig blight on healthy wax apple trees, and *F. solani* was reisolated from the diseased twigs, thus fulfilling Koch's postulates. All the control trees remained healthy throughout the experiment. Numerous microconidia of *F. solani* produced on the cut surfaces of diseased twigs under moist conditions were considered to be the main inoculum source for secondary infection of diseased trees and primary infection of healthy trees.

Keywords: *Fusarium solani*; ITS; 28S; Sequence similarity; Tree decline.

INTRODUCTION

Wax apple (*Syzygium samarangense* Merr. et Perry) which is native to Southeast Asia, is an important fruit crop in Taiwan. The main goal of commercial cultivation is the production of fresh fruit for local consumption. Pingtung County in southern Taiwan is the primary production area with more than 6000 ha of wax apple orchards. In this area, flowering and fruiting of wax apple trees are carefully regulated and managed (Wang and Hung, 2005). In contrast, there are about 80 ha of wax apple orchards with unregulated flowering and fruiting and minimum care in Taipei's northern suburbs. Several of these orchards are used for agricultural tourism. Additionally, wax apple trees have been planted around private homes because of their handsome dark evergreen foliage and the production of bright red fruit during the summer.

In 2003, many wax apple trees in suburban Taipei suffered from an unknown ailment. Affected trees displayed different stages of decline (Figure 1). Since then, an increasing number of wax apple trees around private homes, along the road sides, and in the orchards have died. Some of the dead trees were 20 to 30 years old. Several wax apple orchards were abandoned because of the disease. The possibility of the disease being spread to the main wax apple production area in southern Taiwan has become a major concern.

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At the beginning, *Phellinus noxius* (Corner) G. H. Cunn. was suspected to be the causal agent of wax apple tree decline because the pathogen is widespread (Ann et al., 2002) and has caused brown root rot and death among such trees before in Taiwan (Ann et al., 1999). However, no brown root rot or any other root abnormality typical of Phellinus rot was observed when the roots of diseased wax apple trees were exposed and inspected. Field observation also did not show evidence of insect damage (Wen, 2004). The disease appeared to originate from twigs. Most of the twigs of affected trees defoliated and died (Figure 2). The objectives of this study were to identify the causal agent of the wax apple tree decline and to determine the possible source of inoculum for infection in the field.

MATERIALS AND METHODS

Isolation and morphology of pathogen

To isolate the pathogen, sections (ca 5 mm diam., 10 mm long) of diseased twigs showing dark brown discoloration on the scraped surface were surface-sterilized with 0.6% NaOCl for 3 min. After rinsing with sterile distilled water, these tissues were blotted with sterilized paper towels, plated on 2% water agar, V-8 agar containing 10% V-8 juice, 0.02% $CaCO_3$ and 2% agar or selective medium for *Acremonium* spp. consisting of 10% V-8 juice, 0.02% $CaCO_3$, 50 ppm nystatin, 100 ppm ampicillin and 2% agar (Ko and Kunimoto, 1999). Single-microconidium isolates LW1-1 and LW1-2 obtained from diseased twigs collected from different locations were maintained on potato dextrose agar (PDA) at 24°C under

Figure 1. Wax apple trees at intermediate (left), severe (middle) or deceased (right) stage of decline.

Figure 2. Branches of a declining wax apple tree with may diseased or dead twigs.

light, and used for further study.

The isolated organisms produced abundant microconidia on PDA. Macroconidia were produced after a piece (ca 10 × 10 × 3 mm) of PDA culture was transferred to water agar and incubated at 24°C under light. Chlamydospores were produced by growing the fungus in celery juice as previously described (Huang et al., 1983).

Pathogenicity tests

For pathogenicity tests, 5-year-old wax apple plants (90-120 cm high) growing in pots were inoculated. The fungus was grown in a wheat-oat medium (10 ml whole wheat grains, 10 ml whole oat grains and 10 ml distilled water) for 2 weeks at 24°C (Ko et al., 1986). Wax apple twigs, approximately 5-7 mm in diameter, were scraped gently with a surgical scalpel to remove the epidermis from bark tissue. Four grams of colonized grains were placed on the scraped portion of the twig, wrapped with Parafilm, and secured with vinyl tape. Grains were left on until the end of the experiment. Inoculated plants were checked every two days for the first sign of infection. Twigs similarly inoculated with autoclaved grains were used as controls.

Source of inoculum

To determine if diseased twigs may serve as a source of inoculum for secondary or primary infection, sections of disease twigs approximately 5 mm in diameter by 10 mm long were surface-sterilized as described above, and cut into two halves longitudinally under aseptical conditions. One portion of halfed sections were dipped in sterile distilled water for 10 sec, and then placed on sterilized moistened paper towel in a Petri plate. The other section halves were placed in empty sterile Petri plates and used as the control. Ten diseased and 10 healthy twigs were used for each location.

DNA extraction and polymerase chain reaction (PCR)

The DNA of *F. solani* isolate LW1-1 from wax apple was extracted from 0.1 g of 3-day-old mycelia grown on cellophane placed on PDA by the plant genomic DNA extraction kit (GeneMark Technology Co., Taichung, Taiwan). The ITS region was amplified with primers ITS1 and ITS4 (White et al., 1990). PCR was performed in a 50 µl volume reaction containing 2 µl DNA, 1 *p*mole of upstream and downstream primers and 2.5 units of SuperTaq polymerase (Protech Technology Enterprise Co., Ltd, Taipei, Taiwan) with buffer system recommended by the manufacturer. Cycling conditions of PCR were: initial denaturation at 94°C for 2 min, 30 cycles at 94°C for 30 sec, 55°C for 30 sec, 72°C for 1 min, and a final elongation at 72°C for 6 min. The PCR product was analyzed by electrophoresis in a 1.2% agarose gel. In the same manner the large ribosomal subunit, 28S, was analyzed with primer pairs LROR and LR7 (Vilgalys and Hester, 1990). The annealing temperature was changed to 50°C.

Cloning and sequence analysis

PCR amplified DNA products were cloned into pCRII-TOPO vector (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction. Plasmid clones with expected size DNA inserts were screened and used for sequencing analysis. Sequencing of the target DNA insert was done by an automatic DNA sequencer (ABI PRISM 377, Perkin-Elmer, CA, USA) with the BigDye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer Applied Biosystems, CA, USA). Sequence data were analyzed by Lasergene 7 Software (DNASTAR, Inc., USA).

RESULTS

Symptoms

On naturally infected wax apple trees, initial symptoms were leaves on the apical portion of infected twigs that turned gray and lost vigor. The color of leaves attached to twig sections that were brown changed to reddish brown and eventually fell off. The interior of the infected twig turned brown. As the disease progressed, increasing number of leaves were browning and abscising (Figure 2). The severity of decline increased with the number of increasing infected twigs and was reflected in the amount of fallen leaves (Figure 1). Eventually some branches were also infected and the whole tree died.

Isolation and identification

When diseased wax apple twigs collected from two different locations on June 5, 2007 were used for isolation, distinctive conidiophores with microconidia in false heads (Figure 3A) were found on cut surface of every section placed on water agar or selective medium (Table 1). However, on V-8 agar the same kind of fruiting bodies were found on only two of four sections from location 1. Water agar and selective medium, therefore, were chosen for isolation of the fungus from diseased wax apple twigs collected from four other locations on June 24, 2007. Again, the same kind of fruiting bodies were found on the cut surfaces of all the diseased twig sections tested (Table 1).

On PDA, the fungus formed white colony with dense aerial mycelium and yellowish pigments beneath the colony. Microconidia developed abundantly in spherical false heads on tips of conidiophores which were long and sturdy monophialids. They were oval, ellipsoid, reniform, and fusiform in shape, had none to 1-2 septa and measured 3-16 × 3-5 μm. Macroconidia developed when a PDA culture block (ca 5 × 5 × 3 mm) was transferred to water agar and incubated at 24°C with light for 7 days. They were fusoid with a well-marked foot cell, and 5 to 7 septate measuring 14-46 × 3-5 μm. Chlamydospores developed abundantly in celery juice after 1-month incubation at 24°C in darkness. They were globose to oval, 6-9 × 7- 10 μm, and terminal or intercalary. They also formed chains. The fungus fits the description of *Fusarium solani*

Table 1. Isolation of the fungus producing distinctive conidiophores with microconidia in moist heads temporarily designated as Fx from diseased twigs of wax apple trees at different locations in suburban Taipei (Beitou) of northern Taiwan.

Locations	Collection date	No. of twigs with Fx/No. of twigs tested		
		Water agar	Selective medium	V-8 agar
1	6/5/07	4/4	4/4	2/4
2	6/5/07	2/2	2/2	2/2
3	6/24/07	5/5	5/5	NT[a]
4	6/24/07	1/1	1/1	NT
5	6/24/07	2/2	2/2	NT
6	6/24/07	4/4	4/4	NT

[a] NT = not tested.

Figure 3. Conidiophores of *Fusarium solani* with microconidia in false heads (100X magnification) produced on the cut surface of diseased twig section placed on 2% water agar (A) or moistened paper towel (B).

(Mart.) Sacc. (Booth, 1971; Huang and Sun, 1997). Isolate LW1-1 of *F. solani* used in this study was deposited in the Culture Collection Center of Food Industry Research and Development Institute, Hsinchu, Taiwan with the accession no. BCRCNO 34274.

Sequence comparison of ITS and 28S

There were hundreds of ITS sequences of *F. solani* in GenBank. Therefore, only nine randomly selected plant pathogenic isolates were used for comparison with wax apple isolate. The ITS sequence of wax apple isolate shared more than 98% similarity with that of *F. solani* f. sp. *robiniae* or *F. solani* f. sp. *pisi*, but only 92% similarity with that of *F. solani* f. sp. *glycines* (Table 2). Since there were only 10 full length 28S sequences of *F. solani* in GenBank, all of them were used for comparison with that of wax apple isolate. The 28S sequence of wax apple isolate shared 99.6% similarity with that of isolate FRC#S1027 or LCP9.2 of *F. solani*, but only 93.0% similarity with that of *F. solani* from tropical forest (Table 3).

Pathogenicity tests

Two weeks after inoculation, some inoculated twigs showed disease symptoms similar to those observed on naturally infected plants. Leaves above inoculation site turned gray initially, became brown to reddish brown, and fell off eventually. Both isolates of LW1-1 and LW1-2 were pathogenic causing disease incidence ranging from 60 to 100% on healthy wax apple twigs in three separate tests (Table 4). *Fusarium solani* was recovered from all the diseased twigs thus fulfilling Koch's postulates for proving pathogenicity. All the control twigs remained healthy throughout the experiment.

Source of inoculum for secondary and primary infections

When diseased twigs obtained from the declining wax apple trees in the field were cut into sections and kept under moist conditions, the pathogen *F. solani* produced microconidia on cut surfaces of most sections tested (Table 5, Figure 3B). Most of the diseased twigs collected

Table 2. The ITS sequence similarity between *Fusarium solani* isolated from wax apple and nine plant pathogenic isolates of *F. solani* available in GenBank.

Taxon	Isolate	Associated habitat	GenBank accession no.	Similarity (%)
Fusarium solani f. sp. *mori*	MAFF 840046	Unknown	AF 129105	97.7
F. solani f. sp. *pisi*	MAFF 840047	*Pisum sativum*	AF 130142	98.6
F. solani f. sp. *robiniae*	NRRL 22161	Unknown	AF 178395	98.5
F. solani f. sp. *batata*	NRRL 22402	Unknown	AF 178408	94.0
F. solani f. sp. *cucurbitae*	NRRL 22142	*Cucurbita* sp.	AF 178411	97.6
F. solani f. sp. *glycines*	NRRL 22825	*Glycine max*	AF 178419	92.0
F. solani f. sp. *piperis*	NRRL 22570	*Piper nigrum*	AF 178422	94.7
F. solani f. sp. *eumartii*	Fs 122	Tomato	DQ 164845	94.8
Nectria haematococca	NRRL 22141	Soybean	L 36619	98.0

Table 3. The 28S sequence similarity between *Fusarium solani* isolated from wax apple and isolates of *F. solani* available in GenBank.

Taxon	Isolate	Associated habitat	GenBank accession no.	Similarity (%)
Fusarium solani	Unknown	*Lotus japonicus*	AB 258994	99.0
F. solani	BOL STR 060803	Tropical forest	DQ 139962	93.0
F. solani	NRRL 34123	Unknown	DQ 236687	97.3
F. solani	FRC#s 1027	Unknown	DQ 236813	99.6
F. solani	LCP 9.2	Unknown	EF 579657	99.6
F. solani f. sp. *batatas*	NRRL 22402	Unknown	AF 178377	98.1
F. solani f. sp. *glycines*	NRRL 22823	*Glycine max*	AF 178387	96.6
F. solani f. sp. *glycines*	NRRL 22825	*Glycine max*	AF 178388	96.6
F. solani f. sp. *piperis*	NRRL 22570	*Piper nigram*	AF 178391	96.4
F. solani f. sp. *radicicola*	Unknown	Unknown	AY 819046	99.2

Table 4. Incidence of twig blight on wax apple trees after inoculation with isolates LW1-1 and LW1-2 of *Fusarium solani*.

Isolate	No. diseased / No. inoculated[a]		
	Exp. 1	Exp. 2	Exp. 3
LW1-1	4/5	3/5	5/5
LW1-2	5/5	5/5	5/5
Control	0/5	0/5	0/5

[a]Data were recorded one month after inoculation.

Table 5. Formation of conidiophores with microconidia in moist heads of *Fusarium solani* on sections of diseased wax apple twigs collected from various locations.

Location	Treatment	No. of sections with *F. solani* microconidia /No. of sections tested	
		4	8 (days after treatment)
1	Moistened	10/10	10/10
	Control	0/10	0/10
2	Moistened	5/10	7/10
	Control	0/10	0/10
3	Moistened	5/10	6/10
	Control	0/10	0/10
4	Moistened	8/10	9/10
	Control	0/10	0/10
5	Moistened	8/10	10/10
	Control	0/10	0/10
6	Moistened	4/10	9/10
	Control	0/10	0/10
7	Moistened	3/10	6/10
	Control	0/10	0/10

from seven different locations surveyed produced *F. solani* microconidia on cut surfaces under moist conditions but not under dry conditions. The amounts of diseased twigs showing *F. solani* microconidia under moist conditions ranged from 60% at locations 3 and 7 to 100% at locations 1 and 5.

DISCUSSION

The results indicate that wax apple tree decline in the northern suburbs of Taipei is actually twig blight caused by *F. solani* which also invades branches during the later stage of disease development. To our knowledge, this is the first report of a wax apple disease caused by *F. solani*, and is also the first observation of an epiphytotic disease of wax apple trees. Measures for preventing the spread of the disease to the major wax apple production area in southern Taiwan are currently under investigation. How the disease started and where this pathogen came from deserve further study. Considerable specialization in pathogenicity has been demonstrated in *F. solani* and a number of formae speciales have been proposed in this species (Booth, 1971). Two formae speciales, *F. solani* f. sp. *cucurbitae* and *F. solani* f. sp. *pisi*, have been reported previously from Taiwan (Huang and Sun, 1997). It is not known if the *F. solani* pathogenic to wax apple trees is host specific.

Although *F. solani* is generally considered a soil-borne plant pathogen (Booth, 1971), the twig blight of wax apple trees caused by *F. solani* documented in the present report is apparently an air-borne disease. Die-back of American holly (*Ilex opaca* Ait) caused by *Fusarium martii* Appel & Wollenw, currently *Haematonectria haematococca* (Berk. & Broome) Samuels & Rossman 1999 was reported as early as 1941 (Bender, 1941). Recently, die-back of Indian rosewood (*Dalbergia sissoo* Roxb. ex DC) caused by *F. solani* has also been reported from Nepal (Shakya and Lakhey, 2007). Root infection of trees usually results in slow decline, while trunk infection frequently causes quick decline (Ko, 2009). Tree decline caused by twig infection as shown in this study is gradual and is similar to the decline resulting from root infection. However, twig blight usually spreads faster than root rot.

This study also revealed the production of abundant microconidia on the cut surfaces of diseased wax apple twigs under moist conditions. There were many diseased twigs remaining on the affected wax apple trees in the field. These diseased tissues are likely to serve as the main inoculum source for secondary infection of the diseased trees and for primary infection of the healthy trees. Therefore, removal of diseased twigs and branches appears to be very important in the control of the disease.

The ITS sequence of *F. solani* from wax apple shared 92.0 to 98.6% similarity with those of the same species available in GenBank (Table 2). Although *Phytophthora palmivora* (Butler) displayed high intraspecific ITS sequence similarity ranging from 97.8 to 100%, variable intraspecific ITS sequence similarity has also been reported for *Phytophthora capsici* Leonian ranging from 92.2 to 100% and *Peronospora parasitica* (Pers. Ex Fr.) Fr. ranging from 75.4 to 99.6% (Zhang et al., 2007). The 28S sequence of *F. solani* from wax apple shared 93.0 to 99.6% similarity with those of the same species available in GenBank (Table 3). This is in conformity with the report of *Bremia lactucae* Regel which showed intraspecific 28S sequence similarity of 92.1 to 99.4% (Zhang et al., 2007).

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台灣北部由 *Fusarium solani* 所引起的蓮霧樹嚴重衰亡

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蓮霧是台灣重要果樹之一，2003 年在台灣北部郊區發現蓮霧樹有嚴重衰亡現象。由罹病樹枝常分離到的真菌，根據其型態鑑定爲 *Fusarium solani*。此菌的 ITS 序列與基因庫同種菌的序列有 92.0 到 98.6% 的相似度，其 28S 序列則有 93.0 到 99.6% 的相似度，此菌接種到健康蓮霧樹枝，可使之產生與自然界一樣的病徵。由得病的樹枝又可分離到相同的菌，因而通過柯霍氏法則證明蓮霧樹枝死亡是由 *F. solani* 所引起，因此而造成蓮霧樹的衰亡。罹病樹枝剖開，放在潮濕的環境會產生許多微分生孢子，因此罹病樹枝是病樹第二次感染的主要病源，也是健康樹第一次感染的主要病源。

關鍵詞： 蓮霧 ； 樹枝枯萎 ； 茄鐮孢菌 ； 樹衰亡。