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Decline of Two *Cinnamomum* Species Associated with *Phytophthora cinnamomi* in Taiwan

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ABSTRACT

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Yellowing and wilting followed by death of trees were found on *Cinnamomum osmophloeum* in plantations located at Chia-yi and Hwa-ling farms. Similar symptoms were also observed on trees of *C. camphora* in one plantation in Hua-lien county. All observed diseased trees were associated with *Phytophthora cinnamomi* causing root rot. All isolates of *P. cinnamomi* obtained from trees and rhizosphere soils of *C. osmophloeum* at Chia-yi farm were of A^1 mating type, whereas A^1 and A^2 mating types were isolated from trees and rhizosphere soils at Hwa-ling farm. Two isolates of *P. cinnamomi* obtained from roots and rhizosphere soils of *C. camphora* were A^1. Isolates of *P. cinnamomi* isolated from *C. osmophloeum* or *C. camphora* were able to cause the disease on tested plants of both *C. osmophloeum* and *C. camphora.* The fungus was reisolated from all inoculated plants. *P. cinnamomi* on *C. osmophloeum* was first documented in the world. *P. cinnamomi* on *C. camphora* was first reported in Taiwan.

Key words: *Cinnamomum osmophloeum, C. camphora, Phytophthora cinnamomi,* decline.

INTRODUCTION

Cinnamomum osmophloeum Keneh. is native only to Taiwan and originally grows in broad-leaved forests at altitudes between 400 and 1200 m in central and southern Taiwan (7). Leaves and barks of stems and roots of *C. osmophloeum* contain a high percentage of essential oils in certain clones (10) which are used for food flavor and medicine. It is potentially considered a candidate species for agroforest plantations due to the high quality and quantity of essential oils from its leaves. However, there are no large scale plantations of *C. osmophloeum* in Taiwan yet. During the disease survey of *C. osmophloeum* in 1991 in five experimental trials which were grown about seven to eight years ago at five locations including Tung-ho farm (東和農場，退輔會) at Taitung, Chia-yi farm (嘉義農場，退輔會) at Chia-yi, Pu-li farm (埔里農場，台糖公司) at Nantou, Chow-may farm (鄒梅農場，土地銀行) and Hwa-ling farm (華林農場，文化大學) in Taipei, declining and dead trees have been observed at Chia-yi and Hwa-ling farms. Meanwhile, decline of *C. camphora* (L.) Presl. trees were also observed in one plantation at Hua-lien county. In this paper, the author reports the identity of *Phytophthora cinnamomi* Rands, its association with tree decline and its pathogenicity to *C. osmophloeum* and *C. camphora.*

MATERIALS AND METHODS

Isolation

From tissues Roots and basal stem barks of *C. osmophloeum* were obtained from decline trees at Chia-yi and Hwa-ling farms, and from healthy trees at Pu-li, Tung-ho and Chow-may farms. Samples were collected from 25 trees from each area except Hwa-ling farm at which only 12 trees were sampled. Twenty roots were randomly cut from each tree within one meter around the truck base of the tree and within a depth 20 cm from the soil surface. They were washed under running tap water for one to two hours followed by surface sterilization with 0.5% NaClO for one min and dried with paper towels. As most rootlets were dark brown and necrotic lesions were not able to distinguishable from healthy tissue, seven fragments (1-2 cm long with diameters less than 5 mm) were randomly cut from each root. They were placed on a selctive medium (5) at 25 C for two to seven days. The selective medium consisting of 10% V-8 juice agar

(10% V-8 juice, 0.02% $CaCO_3$ and 2% Bacto agar) was supplemented with 100 ppm ampicillin, 50 ppm mycostatin and 10 ppm pentachloronitrobenzene. Seven pieces (*ca.* 0.5 × 0.5 × 0.3 cm) of surface-sterilized basal stem barks were also placed on a plate with selective medium. After incubation at 25 C for 2–4 days, mycelia of *P. cinnamomi* growing from tissue pieces were transferred to 10% V-8 juice agar. Roots of diseased *C. camphora* trees were collected and used to isolate *P. cinnamomi* using the method described above.

From soils When roots of the trees were selected for pathogen isolation, soils around the same trees were also collected. Four soil subsamples were collected at random from each tree. Soils were assayed for the presence of *P. cinnamomi* using baiting with azalea leaves (9). About 200 ml of soil samples was placed in 1000-ml beakers to which distilled water was added up to a total volume 300–400 ml. Five to seven leaf pieces (*ca.* 2 × 4 cm) as baits were floated in each beaker for 2–5 days at room temperature. Lesion baits were washed under running tap water for 1–2 hours and blotted dry. Pieces of lesion baits (*ca.* 0.5 × 0.5 cm) were placed on the selective medium. After incubation at 25 C for 2–4 days, mycelia of *P. cinnamomi* growing from leaf pieces were transferred to 10% V-8 juice agar.

Determination of mating types

The mating type of *P. cinnamomi* isolated was determined by pairing a small piece of culture block (*ca.* 3 × 3 × 3 mm) with the same size of A^1 tester (F29) or A^2 tester (F3) on a piece of 10% V-8 juice agar block (*ca.* 15 × 10 × 3 mm) in a Petri dish (9 cm diam.). The mating type of isolates F29 and F3 was determined by pairing with two known isolates P991 (A^1) and P731 (A^2) of *Phytophthora parasitica* Dastur supplied by G. A. Zentmyer. Ten pairings were placed in a Petri dish at equal distances along the edge. After incubation at 25 C in darkness for five days, agar blocks were examined microscopically. Those isolates forming oospores with A^1 tester were A^2, and those forming oospores with A^2 tester were A^1.

Production of sporangia and zoospores

The methods of Hwang and Ko (3) were used for production of sporangia and zoospores. Seven to ten young mycelial blocks (*ca.* 0.2 × 0.2 × 0.3 cm) were equally placed on a sterilized cellophane membrane covering a 10% V-8 juice agar plate (9 cm diam.). After incubation for one day at 25 C, the cellophane membrane with colonies was moved into a Petri dish with 20 ml 5% V-8 juice broth (5% V-8 juice and 0.02% $CaCO_3$). After incubation for one day at 25 C, the 5% V-8 juice broth was poured out and mycelial mats were washed with a mineral solution three times at an interval of 30 min. The mineral solution consisted of 0.236% $Ca(NO_3)_2$, 0.05% KNO_3, 0.0985% $MgSO_4$ and 0.046% FeEDTA (added after autoclaving). After incubation for 9 hours under light, the mineral solution was poured out and mycelial mats were reincubated in sterile distilled water for 15 hours for sporangial formation. Cultures were put at 15 C for three hours and moved back to 25 C for zoospore release.

Production of oospores

The polycarbonate membrane method described by Ko (4) was used to produce selfed oospores of *P. cinnamomi*. Each isolate was grown on 10% V-8 juice agar block (15 × 10 × 3 mm) in a small Petri dish and kept in a moist chamber. After incubation at 25 C in darkness for six days, the culture block was transferred into the center of a sterile Petri dish and coverd with a sterile polycarbonate membrane (0.2 μm, 90 mm diam.; Nucleopore, Pleasanton, CA 94566, USA). The mycelial surface of an one-day-old culture block of a standard isolate with opposite mating type and with the same size to serve as the hormone producer was placed inverted onto the polycarbonate membrane and superimposed onto the lower agar block. After incubation for six days in the same environmental conditions, the presence of oospores on the surface of the lower agar blocks was examined microscopically.

Pathogenicity tests

Isolates F29 and F3 of *P. cinnamomi* isolated from roots of *C. osmophloeum* at Chia-yi and Hwa-ling farms, respectively, were used for pathogenicity tests. Zoospore suspension used as an inoculum was adjusted to 10^4 or 10^5 zoospores/ml with a microliter syringe method (6). Roots of seedlings and cuttings (4–8 months old) were soaked into zoospore suspension for 30 min, one hour or two hours. Ten seedlings and cuttings were inoculated for each treatment and tests were twice repeated. After inoculation, seedlings or cuttings were replanted into the same pots (15 cm diam.) and placed in the greenhouse. After incubation for five days, wilting seedlings or cuttings were counted every five days. Roots of wilting plants were removed from soil, disinfested with 0.5% NaClO for three min and plated on the selective medium described above for reisolation of the organism. Seedlings or cuttings inoculated with sterile distilled water were used as controls.

Seedlings of *C. camphora* (about one year old) were also used to test pathogenicity to *P. cinnamomi*. Roots of tested plants were soaked into zoospore suspension (isolates F25 A^1 isolated from the decline tree of *C. camphora*) at a concentration of 10^4 zoospores per ml for one hour. Ten plants were inoculated for each treatment and tests were repeated

once. Seedlings and cuttings inoculated with sterile distilled water were used as controls.

For cross inoculation, seedlings and cuttings of *C. osmophloeum* and seedlings of *C. camphora* were soaked into zoospore suspension of isolate F25 A^1 or isolate F29 A^1 at a concentration of 10^4 zoospores per ml for one hour. Ten plants were inoculated for each treatment and tests were repeated once. Seedlings and cuttings inoculated with sterile distilled water were used as controls.

Growth of *Phytophthora cinnamomi*

To determine the effect of temperature on linear growth of *P. cinnamomi*, culture discs (5 mm diam.) cut with a cork borer from the advancing margin of a colony were placed on petri dishes (9 cm diam.) containing 10% V-8 juice agar. Cultures were incubated at 5, 10, 15, 20, 25, 30 and 35 C in darkness. Five plates were used for each treatment and the experiment was repeated once.

RESULTS

Symptoms and isolations

The initial symptoms of diseased *C. osmophloeum* trees were yellowing, wilting and decline of the foliage (Figs. 1 and 2). The infected plants eventually died due to severe stem rot and root rot. *P. cinnamomi* killed *C. osmophloeum* at every growing stage from young seedlings or cuttings to established plants in the fields. Similar symptoms were also observed on *C. camphora* trees in one plantation at Hwa-lien county. Phytophthora wilt of *C. osmophloeum* was serious during the wet seasons at the locations Chia-yi and Hwa-ling farms that had a high percentage of clayish soils.

A total of 239 isolates of *P. cinnamomi* were obtained from 32 trees and their adjacent soils at decline areas including Chia-yi farm and Hwa-ling farm (Table 1). The fungus was not isolated from three healthy areas including Pu-li, Tung-ho and Chow-may farms. *P. cinnamomi* was isolated from 20 of 25 trees and their adjacent soils at Chia-yi farm and all 12 tested trees and their adjacent soils at Hwa-ling farm. All isolates of *P. cinnamomi* obtained from trees and soils at Chia-yi farm were A^1 (Table 1). The mating type distributions of isolates of *P. cinnamomi* obtained from trees and rhizosphere soils at Hwa-ling farm had ratios 36 A^1: 20 A^2 and 23 A^1: 15 A^2, respectively. Two isolates of *P. cinnamomi* obtained from roots and rhizosphere soils of *C. camphora* were A^1.

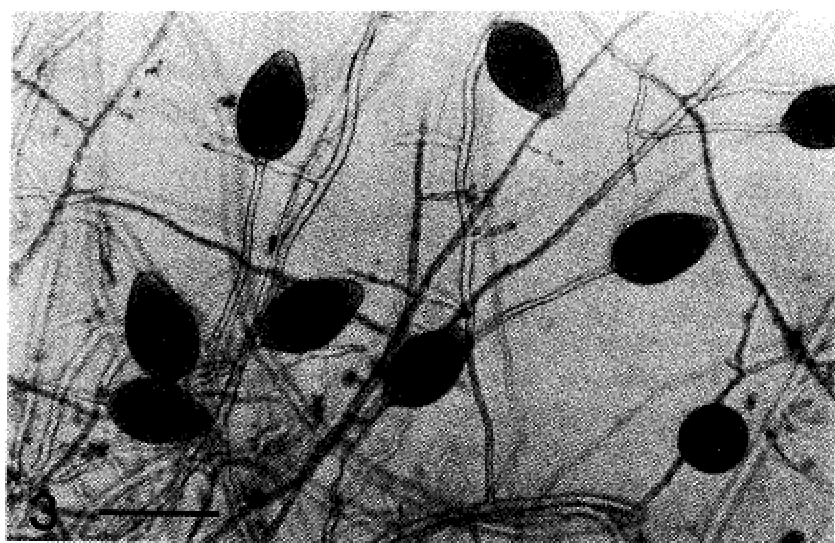
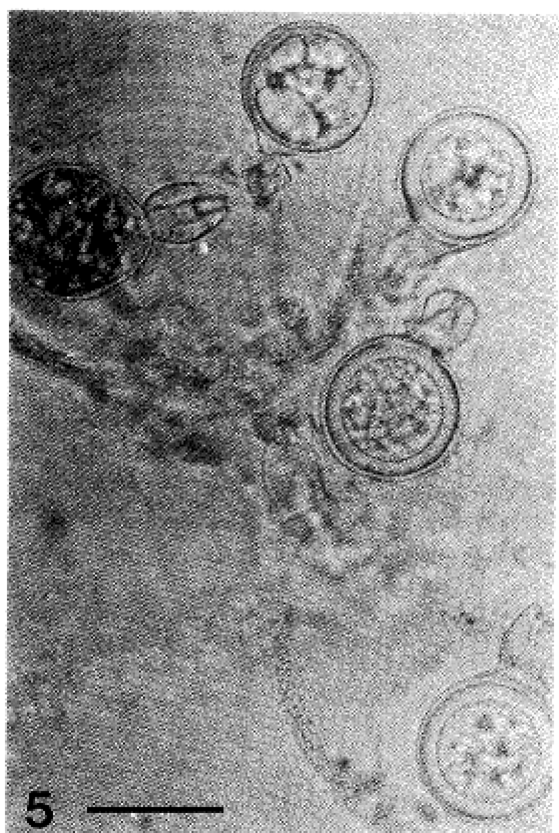
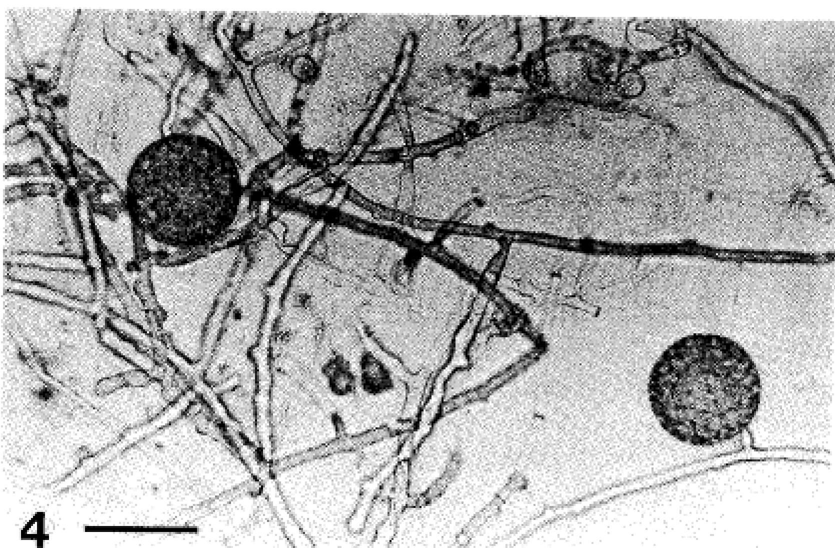
Characteristics of *Phytophthora cinnamomi*

Mycelium of *P. cinnamomi* on 10% V-8 juice agar was hyaline and slender with diameter 5–7 μm. Large amount of sporangia were produced on mycelial mats using the method developed by Hwang and Ko (3). These sporangia were broadly ellipsoid or ovoid, without papilla and nondeciduous, ranging from 61.5–72.0 × 35–40 μm (Fig. 3). Chlamydospores were globose to pyriform with size from 34.5 μm to 51.5 μm, terminal or on short lateral branch, abundant in clusters (Fig. 4). Hyphal swellings were coralloid with frequent round nodules then become broad and tough. Selfed oogonia were of diameter 38.5–51.0 μm, Oogonial walls were smooth. Antheridia were amphigynous, long, 20–24 × 16 μm (Fig. 5). Oospores nearly filled the

TABLE 1. Isolation of *Phytophthora cinnamomi* from rhizosphere soils and roots of *Cinnamomum osmophloeum* trees in Taiwan

Location[1]	No. of trees investigated	No. of trees associated with *P. cinnamomi*	No. of isolates obtained from roots		No. of soils samples with *P. cinnamomi*	No. of isolates obtained from soils	
			A^1	A^2		A^1	A^2
Chia-yi							
Chia-yi farm	25	20	108	0	20	37	0
Taipei							
Hwa-ling farm	12	12	36	20	12	23	15
Chow-may farm	25	0	0	0	0	0	0
Nan tou							
Pu-li farm	25	0	0	0	0	0	0
Taitung							
Tung-ho farm	25	0	0	0	0	0	0

[1] Declining and dead trees were observed at Chia-yi and Hwa-ling farms.

Figs. 1–5. 1. Healthy *Cinnamomum osmophloeum* trees. 2. Decline *Cinnamomum osmophloeum* trees. 3. Sporangia of *Phytophthora cinnamomi.* Bar = 70 μm. 4. Chlamydospores of *P. cinnamomi.* Bar = 40 μm. 5. Oogonia, oospores and antheridia of *P. cinnamomi.* Bar = 45 μm.

oogonium. Oospores wall were colourless and about 2 μm thick.

Isolates F29 and F3 showed similar growth responses to temperature, with optimum temperatures 20–30 C and minimum temperature 10 C. Neither isolate grew at 5 and 35 C (Fig. 6).

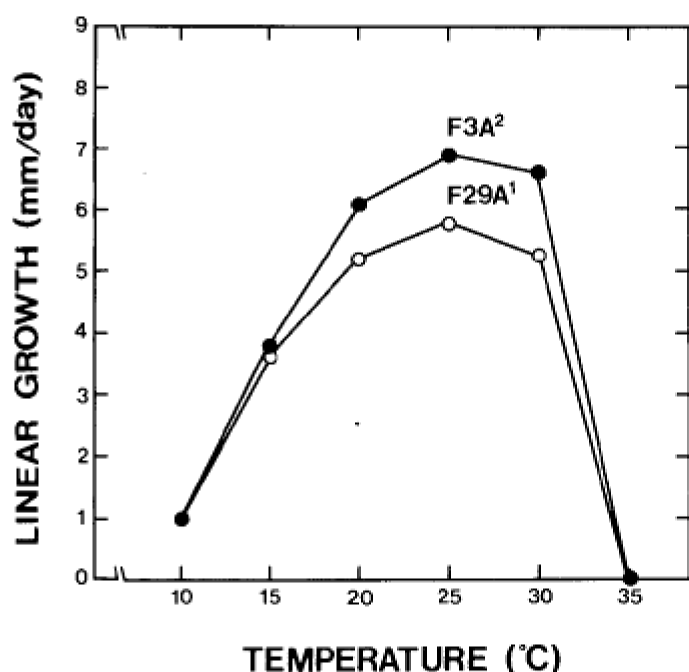


Fig. 6. Growth rate of isolates F3 A[2] and F29 A[1] of *Phytophthora cinnamomi* on 10% V-8 juice agar.

TABLE 2. Pathogenicity of isolated *Phytophthora cinnamomi* on *Cinnamomum osmophloeum* and *C. camphora*

Plants tested	Isolates used[2]	Disease incidence (%)[1]	
		Exp. I	Exp. II
Cinnamomum osmophloeum			
Seedlings	F3 A[2]	100	90
	F19 A[1]	90	80
	F25 A[1]	90	90
Cuttings	F3 A[2]	100	100
	F29 A[1]	90	90
	F25 A[1]	80	100
Cinnamomum camphora			
Seedlings	F29 A[1]	90	100
	F25 A[1]	90	90

[1] Percentage of plants killed was based on a total of 10 plants tested.

[2] Isolates F3 A[2] and F19 A[1] were isolated from *C. osmophloeum*; isolate F25 A[1] was isolated from *C. camphora*.

Pathogenicity tests

Both isolates F29 A[1] and F3 A[2] which were isolated from *C. osmophloeum* caused death of inoculated *C. osmophloeum* plants (Table 2). Isolate F29 A[1] was also able to cause disease of inoculated *C. camphora* plants. Isolate F25 A[1] which was isolated from *C. camphora* caused death of both inoculated *C. camphora* and *C. osmophloeum* plants. Disease incidence of inoculated plants ranged from 80–100% among all treatments. Seedlings and cuttings of *C. osmophloeum* and *C. camphora* started to show foliage wilting one week after inoculation followed by death of seedlings and cuttings. Root lesions and necrosis were present on inoculated plants. Inoculated plants showed similar foliage symptoms at various concentrations of zoospore suspensions and various treatment periods. *P. cinnamomi* was reisolated from all inoculated plants. All control plants remained healthy during the experiments.

Two months after inoculation, seedlings and cuttings of *C. camphora* killed by inoculated fungus were 90% and 100%, respectively. *P. cinnamomi* was reisolated from all inoculated plants two months after inoculation. All control plants remained healthy during the experiments.

DISCUSSION

P. cinnamomi was first obtained from diseased *Cinnamomum burmannii* (Nees) Blume in Sumatra and described as a new species in 1922 by Rands (8). Since then the fungus has been reported from over 60 countries as the cause of diseases of more than 950 plant varieties and species (11). In addition to *C. burmannii*, the fungus also caused disease in other six *Cinnamomum* species including *C. camphora*, *C. cullilawan* Blume, *C. glanduliferum* (Wall.) Nees, *C. oliveri* Bail. and *C. sintok* Blume (11). The present results show that *P. cinnamomi* was isolated from decline areas of *C. osmophloeum* plantations but not healthy areas, and from declining and dead *C. camphora* trees indicating that decline of *C. osmophloeum* and *C. camphora* is related with *P. cinnamomi*. Both Phytophthora diseases on *Cinnamomum* species were first reported in Taiwan. In addition, *P. cinnamomi* appears not to have been previously described as a pathogen of *C. osmophloeum*.

P. cinnamomi is widely distributed in natural forests on the island of Taiwan (1,2,5). Meanwhile, the fungus appears to coexist with many indigenous plant species and to cause no disease problem to those plants (5). But, *P. cinnamomi* was able to cause a serious disease on many cultivated fruit trees such as citrus, pineapple and avocardo in Taiwan (1). *P. cinnamomi* has never isolated from *C. osmophloeum* trees which

live in natural forests (unpublished data). However, *P. cinnamomi* certainly caused a serious decline of *C. osmophloeum* in certain plantations, especially when *C. osmophloeum* trees were grown on clayish soils and in areas of poor soil drainage such as Chia-yi and Hwa-ling farms.

Only a single mating type of *P. cinnamomi* was isolated from *C. camphora* and *C. osmophloeum* grown at Hwa-lien plantation and Chia-yi farm, respectively. However, both A^1 and A^2 mating types of *P. cinnamomi* were simultaneously detected in the same soils and tree roots of *C. osmophloeum* grown at Hua-ling farm, indicating the possibility of sexual reproduction by this fungus in nature.

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摘 要

張東柱．1993．*Phytophthora cinnamomi* 與兩種樟屬植物萎凋病的相關性．植病會刊 2:1-6.
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在嘉義農場和華林農場之土肉桂 (*Cinnamomum osmophloeum*) 栽培區發現其地上部黃化和萎凋，繼而全株死亡。相類似的病徵也在花蓮地區樟樹 (*C. camphora*) 造林地發現。所有罹病植物均呈現根腐現象。從罹病植物的根部及其根圈土壤均可分離到 *Phytophthora cinnamomi*。分離自嘉義農場土肉桂根部及其根圈土壤的菌株都是 A^1 配對型；然而分離自華林農場土肉桂根部及土壤的菌株則有 A^1 和 A^2 配對型兩種。至於分離自樟樹的兩菌株是 A^1 配對型。將分離自土肉桂或樟樹之 *Phytophthora cinnamomi* 接種到土肉桂種子苗和扦插苗與樟樹種子苗時，可引起接種植株根腐，萎凋及死亡，且 *P. cinnamomi* 可在人工接種罹病組織再分離得到。樟樹之疫病在台灣爲新紀錄；土肉桂之疫病在世界其他地區尚未被報導。

關鍵詞：土肉桂、樟樹、*Phytophthora cinnamomi*、萎凋病。