RES. BULL. PL. PROT. JAPAN No. 29: 37~44 (1993)

Detection of *Phytophthora citrophthora* and *P. syringae* from Orange Fruit by Serological Methods

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Abstract: Brown rots of sweet orange fruit were found at plant quarantine inspection in Honmoku, Yokohama, in 1991. To diagnose the disease, *Phytophthora capsici* antiserum previously prepared was used in ELISA, DIBA and immunofluorescent assay. Four isolates obtained from the diseased fruit which showed positive reactions in the serological tests were identified as *Phytophthora citrophthora* and *P. syringae* on the basis of their morphological and physiological characteristics. Those isolates were pathogenic to sweet orange fruit and citrus leaves. Citrus brown rot "kassyoku-huhaibyo" in Japanese, is proposed for the disease caused by *P. syringae*.
Key words: Detection, Orange fruit, *Phytophthora citrophthora*, *Phytophthora syringae*.

INTRODUCTION

Brown rot caused by *Phytophthora* spp. is a pre- and post-harvest decay of citrus fruit. Sweet orange, *Citrus sinensis* Osbeck, fruit which showed brown rot were found at plant quarantine inspection in Honmoku, Yokohama in 1991. They were from USA. To diagnose the disease, rabbit antiserum prepared for *P. capsici* Leonian was used in serological tests. This paper describes the results of etiological studies including the symptoms, serological tests for detection of the causal organisms and their identification. A part of this work has been published elsewhere (Kimishima and Goto, 1992).

MATERIALS AND METHODS

Serological tests

Rabbit antiserum prepared for *P. capsici* was tested to detect the pathogen. The antiserum showed genus-specific reactivities in enzyme-linked immunosorbent assay (ELISA) (Kimishima, et al., 1989). Three serological techniques were used and they were ELISA, dot immunobinding assay (DIBA) (Hibi and Saito, 1985) and immunofluorescent assay (IFA) (Nishio et al,1983). Antigen samples for ELISA, DIBA and IFA were prepared by the following methods. Extracts were obtained by grinding and precipitating 1g of the rind of healthy or naturally infected orange fruit in 10−20ml of phosphate-buffered saline (PBS) −0.05% Tween20. Their supernatants were used as antigen samples for ELISA and DIBA. Rind tissues were picked from the infected fruit

* Import permit number issued by Ministry, Foresty and Fisheries is 3Y1882.

incubation at 15－20°C for 2-3 days in tap water and used for IFA. Plates for ELISA tests were coated with γ-globulin diluted to 2.5 μg/ml in carbonate buffer. Conjugate was used at 1/400 dilution. The absorbance was measured with a Microplate Reader (Corona) at 405nm. Antigen samples for DIBA were applied onto the nitrocellulose membrane (NCM) and dried on a filter paper. The NCM was saturated by incubation with γ-globulin diluted to 2 μg/ml and then treated with conjugate diluted at 1/5000. After color development, the reaction was stopped and then the NCM was washed in a distilled water and air-dried for visual observation. Rind tissues were saturated by incubation with γ-globulin diluted to 50 μg/ml in PBS for 1 hr in a humid chamber at 37°C. After washing in PBS for 5-10 min, the rind tissues were placed in a solution of goat anti-rabbit γ-globulin labelled with fluorescein isothiocyanate (FITC), diluted at 1/20, for 1hr at 37°C. Excess goat anti-rabbit γ-globulin labelled with FITC were removed by rinsing for 5-10 min in PBS. Preparations were mounted in distilled water and examined with the reflecting fluorescence microscope (Nikon XF-EF).

Isolation

Pieces of rinds taken from the margin of discolored parts were washed under running tap water for 1-2 min, air-dried and placed on a selective medium in petri dishes for the isolation of *Phytophthora* (Masago, et al, 1977). They were incubated in the dark at 15°C and 25°C, for 3-5 days, and margin of emerging colonies were transfered onto water-agar (WA) for single hyphal tipping. Representative isolates, UO-1, UO-9112, UO-9116 and UO-9201, were used for describing the morphology on V-8 agar (V-8 : 200ml Campbell V-8 juice, 2.5g calcium carbonate, 15g agar per liter) and on hemp seed flooded with destiled water.

Identification

Isolates were identified on the basis of colony morphology, mycelial characteristics, cardinal temperatures and production, morphology and size of sporangia, oogonia and antheridia (Stamps et al. 1990; Waterhouse and Waterston, 1964a; Waterhouse and Waterston, 1964b; Waterhouse, 1963).

For observation of sporangia, the autoclaved hemp seeds were placed in contact with V-8 agar where the isolate was pre-cultured, and incubated at 15°C or 25°C for 1－3 days in petri dishes. The infected seeds were transferred into sterile distilled water and incubated above condition. To study the production and morphology of sex organs, hemp seeds and hemp seed agar (HSA : 100g hemp seed, 15g agar per liter) were used. Oospores of UO-1 and UO-9201 were produced on the infected hemp seed flooded at 10-15°C for 2－3 weeks. UO-9112 and UO-9116 were paired with each A1 mating type isolate of *P. parasitica* Dastur* (=*P. nicotianae* var. *parasitica* (Dastur) Waterh.) (University of California, Riverside: UCR, Collection P1751) and *P. capsici* (IFO 30696), paired also with each A2 mating type isolate of *P. parasitica** (UCR, Collection P3118) and *P. capsici* (IFO 30698), or grown alone on HSA at 15°C for 3-4 weeks. Each isolate was placed on two V-8 agar plates and plates were incubated at various temperatures ranging 5 to 35°C. The radial growth of the mycelium was

measured after 4 or 7 days of incubation.

Pathogenicity

The pathogenicity of UO-1, UO-9112, UO-9116 and UO-9201 were tested several times to sweet orange fruit and following leaves: orange, Iyo (*Citrus iyo* hort. ex Tanaka), Yuzu (*C. junos* Sieb ex Tanaka), Natsudaidai (*C. natsudaidai* Hayata), Hassaku (*C. hassaku* hort. ex Tanaka), Kumquat (*Fortunella* sp.) and Trifoliate orange (*Poncirus trifoliate* (L.) Raf.). The wounded plant surface was prepared by injuring with a cork borer or a razor. Mycelial mats with an agar culture were placed onto the wounded parts of fruit and/or leaves of each plant. Inoculated plants were maintained in moist condition at 15 or 25°C for 4-12 days. Uninoculated plants were served as controls. The development of lesions was observed.

RESULTS

Symptoms of orange fruit found

Four samples of sweet orange fruit listed in Table 1 were found. Symptoms of them were characterized by a brown discoloration of the rind (Fig. 1). The affected area was firm and leathery, and it retained the same degree of firmness and elevation as the adjacent healthy rind. Infected fruit had characte ristic pungent and rancid odor.

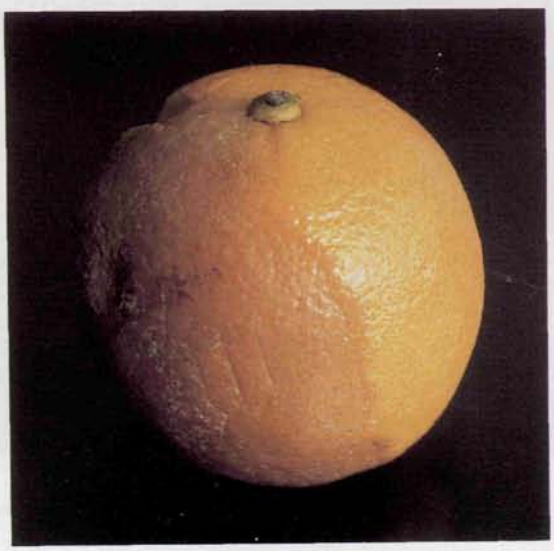

Fig. 1. Symptoms of brown rot of sweet orange fruit caused by *P. syringae.*

Serological tests

Extracts of the diseased parts of each sample reacted with *Phytophthora* antibody by ELISA and DIBA (Table 1). Healthy parts used as control gave no positive reaction. Aseptate hyphae were observed in and around the host tissues with green fluorescence by IFA (Fig. 2).

Table 1. Detection of *Phytophthora* in diseased tissues of sweet orange fruit by serological methods and identification of the pathogens

Sample No.	Symptom	Serological method [a]			Pathogen
		ELISA	DIBA	IFA	
1	Brown rot	0.68 (0.08) [b]	+ (−) [c]	+ [d]	*Phytophthora syringae*
2	Brown rot	0.56 (0.03)	+ (−)	+	*P. citrophthora*
3	Brown rot	1.06 (0.04)	+ (−)	+	*P. citrophthora*
4	Brown rot	0.57 (0.02)	+ (−)	+	*P. syringae*

a) Rabitte antiserum prepared for *P. capsici* were used in enzyme-linked immunosorbent assay (ELISA), dot immunobinding assay (DIBA) and immunofluorescent assay (IFA).
b) ( ) : Reaction of healthy tissue.
c) + : Extreme blue spot appeared, −: no blue spot.
d) + : Hyphae were observed with fluorescence.

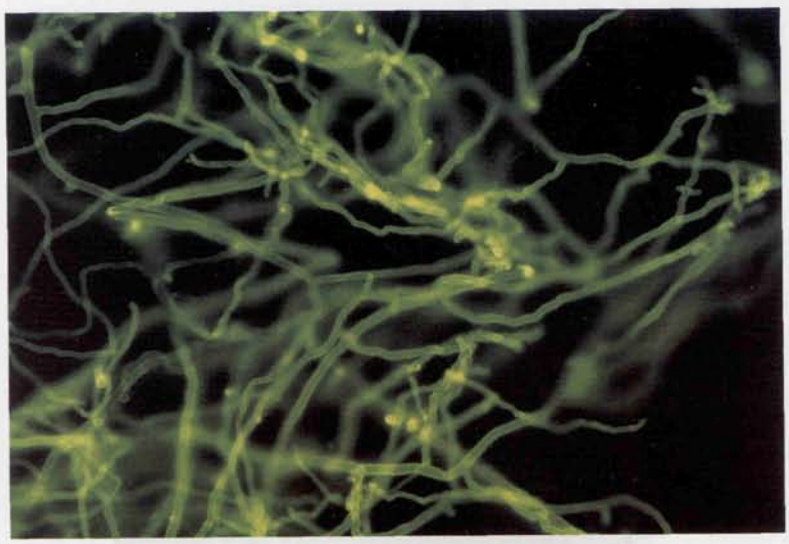

Fig. 2. Hyphae around the sweet orange fruit tissue with green fluorescence.

Morphology of two species identified

Phytophthora citrophthora (Smith & Smith) Leonian (Fig. 3 A, B and C)

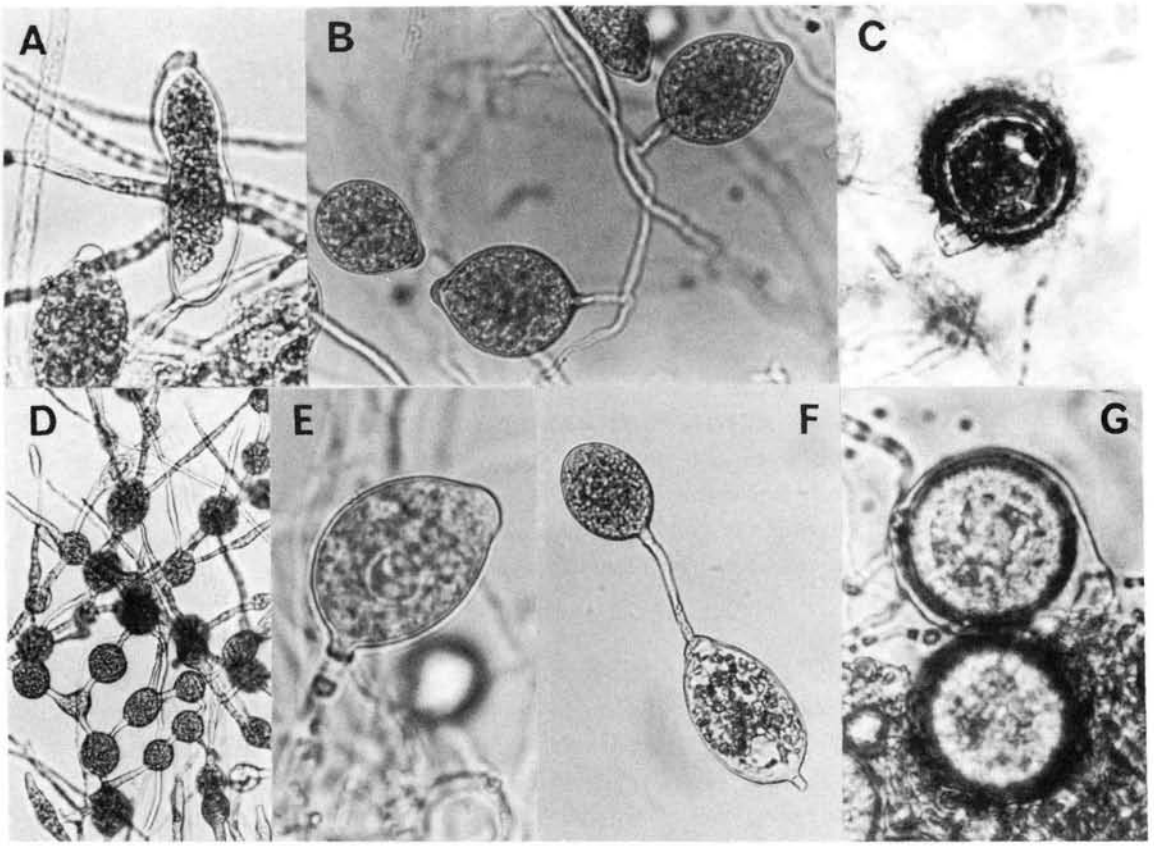

Fig. 3. Morphology of *P. citrophthora* (A−C) and *P. syringae.* (D−G).
A, B : Papillate sporangia (A : ×550, B: ×450).
C : Oogonium with amphigynous antheridium and oospore (×450).
D : Hyphal swellings (×750).
E : Semi-papillate sporangium (×750).
F : Sporangium germinating in a water with the formation of a secondary sporangium (×380).
G : Oogonia and nearly plerotic oospores (×800).

Two isolates (UO-9112 and UO-9116) of this species were recovered from two samples which showed positive reactions in the serological tests.

The fungi exhibited slightly radiate pattern on V-8 agar. Cardinal growth temperatures on V-8 agar were as follows: minimum below 5°C, optimum 25°C and maximum 30°C. No growth occurred at 35°C. In water, sporangiophores branched irregulary and sporangia (av. 61.3×39.5 μm, range 35.0-85.0×27.5-47.5 μm L/B ratio=1.55) were papillate, spherical, ellipsoid, ovoid, obpyriform or distorted shapes and had one or more apices. Internal proliferation of sporangia was not observed. Sex organs were produced

on HSA only when each isolate was paired with A2 mating type isolate of *P. capsici*. Oogonia (av. 39.3 μm, range 30.0-45.0 μm) in dual cultures were spherical with smooth walls and becoming yellowish brown with age. Antheridia (range 12.5-15.0×10.0-15.0 μm) were amphigynous. Oospores (av. 35.2 μm, range 30.0-40.0 μm) were plerotic. Only UO-9116 rarely formed yellowish chlamydospores (range 25.0-32.5 μm) on V-8 agar. Descriptions of characters were based on UO-9116.

Phytophthora syringae (Kleb.) Kleb. (Fig. 3, D, E, F and G)

Two isolates (UO-1 and UO-9201) of this species were recovered from two samples which showed positive reactions in the serological tests.

The fungi exhibited characteristic petaloid pattern on V-8 agar. Cardinal growth temperatures on V-8 agar were as follows: minimum below 5°C optimum 20°C and maximum 25°C. In water, spherical to ellipsoid hyphal swellings were observed. Sporangiophores branched in a lax sympodium. Sporangia (av.55.2×38.3 μm,range 30.0-97.5×22. 5-52.5 μm L/B ratio = 1.4) were semi-papillate, non-deciduous, ovoid or obpiliform. Sex organs were aboundant in the host tissues. Oogonia (av. 30.1 μm, range24.0-45.0 μm) were spherical. Antheridia (av. 10.0×7.5 μm) were paragynous, spherical to oval. Oospores (av. 27.1 μm, range 22.5-40.0 μm) were plerotic or nearly plerotic. Descriptionsof characters were based on UO-9201.

Pathogenicity

P. citrophthora (UO-9112 and UO-9116) and *P. syringae* (UO-1 and UO-9201) caused brown rot on sweet orange fruit. The decay was first observed as a water-soaked discoloration of the rind, and then the affected area became light brown. All isolates of *P. citrophthora* and *P. syringae* caused dark green lesions on sweet orange, Iyo, Yuzu, Kumquat and Trifoliate orange leaves. *P. citrophthora* caused lesions on Natsudaidai and Hassaku leaves, but *P. syringae* did not cause. Control fruit and leaves showed no symptoms. The inoculated fungi were consistently reisolated from diseased plants.

DISCUSSION

Sweet orange fruit showing brown rot were found at plant quarantine inspection. It was possible to distingush the diseased fruit caused by *Phytophthora* by means of serological tests using ELISA, DIBA and IFA. These serological methods are thought to be used as immunodiagnostic tools for the citrus brown rot disease. Skaria and Miller (1989) also reported that ELISA was used to detect *Phytophthora* in citrus tissues.

Two *Phytophthora* species were isolated from diseased sweet orange fruit. Two isolates, UO-9112 and UO-9116, were identified as *P. citrophthora* (Smith & Smith) Leon. on the basis of their morphological characteristics and cardinal temperatures. There are no descriptions about sex organs of *P. citrophthora* in the key of Stamps et al. (1990) nor in the literature written by Waterhouse and Waterston (1964). However, production of sex organs of this species was reported by Laviola and Gallegly (1967), Savage et al. (1968), Isaka and Okamoto (1983) and Latorre et al. (1991). Liyanage and

Wheeler (1989) gave mating types for this species. In this study, UO-9112 and UO-9116 produced sex organs in a dual culture paired with A2 mating type isolate of *P. capsici.* From this result, these isolates are A1 mating type.

The morphological characteristics and cardinal temperatures of UO-1 and UO-9201 fit the descriptions given in the literature for *P. syringae* (Stamps, et al., 1990; Waterhouse and Waterstone, 1964a). *P. syringae* is known as a causal agent of brown rot of citrus fruit in USA (USDA, 1960; Klotz, 1978). This is the first report of *P. syringae* on citrus in Japan. Therefore, citrus brown rot, "Kassyoku-huhai byo" in Japanese, is proposed for this disease.

We wish to thank Dr. Helga Förster, University of California, Riverside, for providing the isolates of *P. parasitica.*

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和 文 摘 要

オレンジ果実から血清学手法により検出された *Phytophthora citrophthora* と *P. syringae* について

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輸入検疫において発見された褐色腐敗症状を呈するオレンジ果実の病原菌を同定するため、常法による病原菌の分離、同定と疫病菌抗血清を用いた血清試験を行った。試験の結果、米国産オレンジ4サンプルがELISA, DIBA および蛍光抗体法のいずれの血清試験においても供試抗血清と顕著に反応した。陽性反応を示した各サンプルからの分離菌は形態的および生理的特徴から *Phytophthora citrophthora* および *P. syringae* と同定された。各分離菌はオレンジ果実に原寄主と同様の病徴を再現し、再分離された。*P. syringae* による病気の名称として褐色腐敗病を提唱した。