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Serological Diagnosis of the Diseases Caused by *Phytophthora* spp.

Etsuo KIMISHIMA and Yoshinori KOBAYASHI

Research Division, Yokohama Plant Protection Station

Abstract: Naturally infected plants showing root and/or stem rot, gypsophira, white trumpet lily and pumpkin, were collected from the domestic growing fields. Diseased parts of them reacted with *Phytophthora* antibodies by ELISA and DIBA. Aseptate hyphae with fluorescence were observed by immunofluorescence staining method using with *Phytophthora* antibodies. The pathogens isolated from gypsophila and white trumpet lily plants were identified as *P. nicotianae* var. *parasitica*. The pathogen from pumpkin was identified as *P. capsici* by the morphological and physiological characteristics. As a result, the serological methods are considered to be very useful for the rapid and sensitive diagnosis of the diseases caused by *Phytophthora* spp..

Key words: *Phytophthora*, diagnosis, ELISA, immunofluorescence staining method, DIBA

Introduction

The identification of *Phytophthora* species is based on the morphological and physiological characteristics, same as in the case of other fungi. However, it takes relatively long time and needs skillful techniques to identify *Phytophthora* species. In plant quarantine, quick diagnosis are requested. Therefore, the serological diagnostic methods have been considered to be useful as rapid and sensitive techniques.

Naturally infected plants, gypsophila (*Gypsophila elegans* M.B.), white trumpet lily (*Lilium longiflorum* THUNB.) and pumpkin (*Cucurbita moschata* DUCH.), were collected from the domestic growing fields and used for this study. The diseased and healthy parts of these plants were applied for the serological tests and the pathogens were identified. The reliability of serological diagnosis for the disease by *Phytophthora* sp. was evaluated on this experiment. Parts of this work have been reported elsewhere (KIMISHIMA, *et al.*, 1988a ; KIMISHIMA, *et al.*, 1989a).

Materials and Methods

Serological tests

It was tried to detect and diagnose the pathogen from the diseased plants directly by the serological test. Rabbit antisera prepared for *Phytophthora erythroseptica* PETHYBRIDGE and *P. capsici* LEONIAN (KIMISHIMA, *et al.*, 1989b) were used. Three techniques were used in this test : enzyme-linked immunosorbent assay (ELISA) (CLARK and ADAMS, 1977), the immunofluorescent staining method (IFSM) and dot immunobinding assay (DIBA) (HIBI and SAITO, 1985). The preparation of antigen samples and the procedures of the serological techniques were performed as previously described (NISHIO, *et al.*, 1983 ; KIMISHIMA, *et al.*, 1984 ; KIMISHIMA and KOBAYASHI, 1990a).

Isolation

Roots and/or stems were washed gently in running tap water. The discolored tissues (0.5-1 cm in length) were plated onto a modified selective medium (MASAGO, *et. al.*, 1977). Plates were incubated at 25°C for 2-4 days. The plugs cut from the advancing margin of a colony growing on the selective medium were transferred to plates containing water agar (WA). After a further incubation at 25°C for 2-3 days, pure isolates of *Phytophthora* sp. were obtained by the single hyphal tippings or single zoospore isolation.

Identification of *Phytophthora* species

Representative isolates were used for describing the morphology on V-8 agar (200 ml Campbell V-8 juice, 3.0 g calcium carbonate, 15 g agar per liter). Isolates were identified on the basis of mycelium characteristics, cardinal temperatures for vegetative growth, morphology and dimensions of sporangia and production of sex organs. For the examines of mycelium characteristics and cardinal temperatures, every isolates were cultured on V-8 agar for 3-5 days at various temperatures, ranging from 5 to 40°C. Sporangia were produced on V-8 agar or eggplant (*Solanum melongena* L.), at room temperature (20-25°C) for 5-7 days. To study the production of sex organs, the isolates were grown on V-8 agar to be paired individually with known A1 and A2 mating type cultures for 2 weeks at 25°C. The identification was made by keys (WATERHOUSE, 1963 ; KATSURA, 1971) and previous works (TASUGI and KUMAZAWA, 1938 ; WATERHOUSE and WATERSTON, 1964 ; STAMPS, 1985 ; KOBAYASHI, *et al.*, 1987).

Pathogenicity tests

Representative isolates were selected and used for the following two pathogenicity tests. Test a ; Mycelial mats together with V-8 agar were placed onto wounded and unwounded leaves or fruit of each plant. The wounded plant surface was prepared by injuring with a razor or a bundle of 20 needles. Inoculated plants were maintained in moist condition at room temperature. The development of lesions was observed. Test b ; The infested soil was prepared by mixing 400 ml of autoclaved soil with 10 ml of agar cultures. The agar cultures were previously prepared by growing the test fungus on V-8 agar at 25°C for 3-5 days. The seedlings were planted in the infested and uninfested soils. These inoculation experiments were carried out in a glasshouse (15-30°C) and observed for 3 weeks.

Results

Symptoms on plants

Gypsophila : In August 1986, root rot was found on gypsophila in Sizuoka prefecture. Wilting of whole plant and discoloration of stem were also found on the affected plant. Papillate sporangia were observed on the diseased parts of them.

White trumpet lily (Fig. 1-D) : In June 1987, stem rot was found on white trumpet lilies at Okino-erabu island, Kagoshima prefecture. In infected plant the leaves and stem were discolored, water-soaked, dark brown and rotted. Aseptate hyphae were observed in the

affected tissues, but no sporangia.

Pumpkin (Fig. 1-G) : In June 1988, root rot was found on pumpkins at Hitachi, Ibaraki prefecture. The stem and root were water-soaked, discolored and rotted. Aseptate hyphae and sporangia were observed in the affected tissue.

Serological tests

As shown in Table 1, extracts of the diseased parts of gypsophila and white trumpet lily showed high ELISA value, 0.16 and 1.90, respectively. Healthy parts of them showed very low value, below 0.05. Aseptate hyphae with fluorescence were observed in the white trumpet lily tissues by IFSM. Hyphae isolated from gypsophila were stained yellow-green by IFSM. On the other hand, extracts of the diseased parts of pumpkin reacted with *Phytophthora* antibodies by DIBA. Healthy parts of it used as a control gave no positive reaction. The above results are clearly showing that all symptomatic plants are infected with *Phytophthora.*

Identification of *Phytophthora* species

Phytophthora nicotianae var. *parasitica* (DASTUR) WATERHOUSE isolated from gypsophila (Fig. 1-B, C) and white trumpet lily (Fig. 1-E, F)

Five isolates were recovered from the symptomatic gypsophila plants and 3 isolates were recovered from the symptomatic white trumpet lily plants. Each of 2 representative

Table 1. Results of the serological tests for the diseases caused by *Phytophthora* spp. and identification of the pathogens

Plant	Symptoms	Microscopic[1] examination	Serological tests[2]			Pathogen
			ELISA	IFSM	DIBA	
Gypsophila	root rot	○	0.16(0.05)[3]	+[4]	nt[5]	*P. nicotianae* var. *parasitica*
White trumpet lily	stem rot	×	1.90(0.04)	+	nt	*P. nicotianae* var. *parasitica*
Pumpkin	root rot	○	nt	nt	+(−)[6]	*P. capsici*
Gerbera[7]	root rot	×	0.68(0.08)	nt	nt	*P. cryptogea*
Zucchini[8]	fruit rot	×	0.35(0.03)	+	nt	*P. capsici*
White trumpet lily[9]	stem rot	×	1.20(0.09)	+	nt	*P. megasperma*
Litchi[10]	fruit rot	×	0.47(0.03)	+	nt	*Peronophythora litchii*
Carnation[11]	root rot	×	0.10(0.10)	nt	−(−)	*Pythium aphanidermatum*

1) Typical sporangia of *Phytophthora* spp. were observed on the naturally infected plant and it is possible to consider the pathogen as *Phytophthora* sp. (○), or it is impossible to consider it as *Phytophthora* sp. because of no sporangia (×).
2) Rabbit antisera prepared for *P. erythroseptica* and *P. capsici* were used in enzyme-linked immunosorbent assay (ELISA), immunofluorescent staining method (IFSM) and dot immunobinding assay (DIBA).
3) ( ) : Reaction of healthy plant tissue.
4) + : Hyphae were observed with fluorescence.
5) Not tested.
6) + : Extreme blue spot appeared, − : no blue spot.
7)-11) Previously reported elsewhere. 7) : (KIMISHIMA and GOTO, 1991), 8) : (KOBAYASHI, *et al.*, 1987), 9) : (KIMISHIMA, *et al.*, 1988b), 10) : (KOBAYASHI, *et al.*, 1986), 11) : (KIMISHIMA, *et al.*, 1990b).

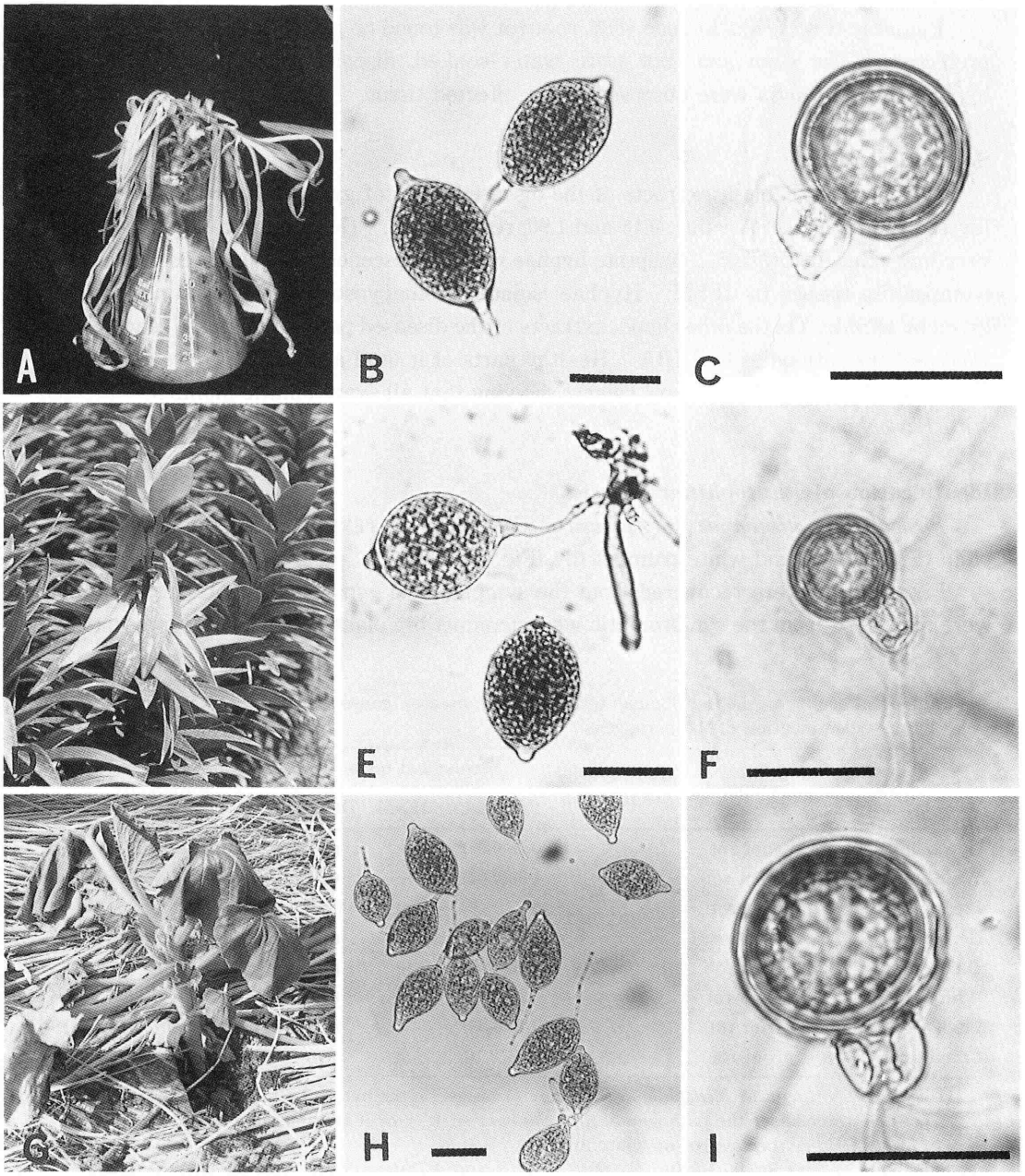

Fig. 1. Symptoms of plant affected with *Phytophthora* sp. and morphological features of the pathogens. A–C, *P. nicotianae* var. *parasitica*: A, Symptoms of root rot on gypsophila inoculated; B, Papillate sporangia, C, Oogonium and amphigynous antheridium. D–F, *P. nicotianae* var. *parasitica*: D, Symptoms of stem rot on white trumpet lily; E, Papillate sporangia; F, Oogonium and amphigynous antheridium. G–I, *P. capsici*: G, Symptoms of root rot on pumpkin; H, Papillate sporangia; I, Oogonium and amphigynous antheridium. Bars on the figures represent 30 μm.

Table 2. Morphological and cultural characters of *Phytophthora* species isolated from gypsophila, white trumpet lily and pumpkin

	Isolate		
	Gypsophila	White trumpet lily	Pumpkin
Sporangium	27.5-70×20-37.5[1] (43.8×29.4)	28-64×20-44 (44.6×34.3)	30-80×17.5-37.5 (54.3×27.7)
L/B ratio[2]	1.49	1.3	1.96
Oogonium	27.5-35.0 (32.3)	15-25 (21.4)	27.5-37.5 (30.4)
Antheridium	10-20×10-17.5	10-15×10-17.5	10-17.5×12.5-17.5
Oospore	22.5-30 (27.4)	12.5-22.5 (21.4)	20-32.5 (25.7)
Chlamydospore	(25.6)	(29.8)	absent
Temp.			
optimum	25°C	25-28°C	25°C
grows well	>35°C	>35°C	>35°C

1) Range (Av.) μm.
2) Mean ratio of length to breadth.

isolates was identified as *P. nicotianae* var. *parasitica* (DASTUR) WATERHOUSE on the basis of deciduous, broadly ovoid, not noticeably narrowed at the apex, papillate sporangia; cardinal growth temperatures; and the production of yellowish-brown chlamydospores but not sex organs in single culture. All isolates studied formed oospores only when paired with an A1 mating type isolate of *P. nicotianae* var. *parasitica* (MATSUZAKI collection, P-1) or *P. capsici* (IFO 30696) (Table 2).

P. capsici LEONIAN isolated from pumpkin (Fig. 1-H, I)

Three isolates resembling a *Phytophthora* species were recovered from roots and stems of the symptomatic pumpkin plants. Representative isolate was identified as *P. capsici* LEONIAN on the basis of deciduous, ellipsoidal, papillate sporangia, cardinal temperature; but no sex organs in single culture. The isolate studied formed oospores only when paired with an A1 mating type of *P. capsici* (IFO 30696). Chlamydospores were absent (Table 2).

Pathogenicity

Test a: When the isolate (SK-1) from gypsophila was inoculated on gypsophila, stem and root rot and wilting of whole plant were developed (Fig. 1-A). When the isolate (EL8705) from white trumpet lily was inoculated on a leaf of white trumpet lily and on a fruit of tomato (*Lycopersicon esculentum* MILL), eggplant, sweet pepper (*Capsicum annuum* L.) and cucumber (*Cucumis sativus* L.), dark-brown lesions surrounded by water-soaked areas were formed. When the isolate (IS-1) from pumpkin was inoculated on a fruit of pumpkin, eggplant and cucumber, similar symptoms were formed and then these areas were covered with mycelia and sporangia.

Test b: SK-1 was pathogenic to alfalfa (*Medicago sativa* L. subsp. *sativa*) and cowpea (*Vigna sinensis* ENDL.). EL-8705 was pathogenic to alfalfa, cowpea, pea (*Pisum sativum* L.) and kidney been (*Phaseolus vulgaris* L.). IS-1 was highly pathogenic to pumpkin,

cucumber, sweet pepper and tomato. Root rot and damping-off were found on all affected plants by the unwounded inoculation.

No symptom was found on the noninoculated plants. The causal fungi were consistently reisolated from the diseased plants.

Discussion

It is known there are various methods to diagnose plant disease. The diagnosis of fungal diseases is generally carried out by microscopically studying the morphology of its mycelium, fruiting structures and spores. In case of *Phytophthora* diseases, if the typical sporangia would not be confirmed by microscopic examination, it is very hard to decide the pathogen to be *Phytophthora* sp.. It is said that the serological diagnosis is one of the rapid and sensitive means, especially in plant virus diseases. We have prepared the antisera for *Phytophthora erythroseptica* and *P. capsici* showing genus specific reactivities.

In this paper, it is examined about the reliability of serological test to diagnose the *Phytophthora* diseases. Results of serological tests (ELISA, IFSM, DIBA) show that the diseased plants, gypsophila, white trumpet lily and pumpkin showing root rot and stem rot, were infected with *Phytophthore* species. Then, those pathogens were identified as *P. nicotianae* var. *parasitica* for the former two plants and *P. capsici* for the last one by their morphological and physiological characteristics (Table 2). Similar results have been shown on gerbera (KIMISHIMA and GOTO, 1991), zucchini (KOBAYASHI, *et al.*, 1987), white trumpet lily (KIMISHIMA, *et al.*, 1988b) and litchi (KOBAYASHI, *et al.*, 1986) (Table 1). However, carnation showing root rot caused by *Pythium aphanidermatum* did not reacted with *Phytophthora* antibodies by ELISA and DIBA (KIMISHIMA. *et al.* 1990b).

Serological methods described here were found to be able to diagnose the diseases caused by *Phytophthora* spp. or *Peronophythora* sp.. And they are considered to be very useful as rapid and sensitive means for diagnosis of the *Phytophthora* diseases.

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和文摘要

疫病の血清診断

君島悦夫・小林慶範
横浜植物防疫所調査研究部

根腐れ及び茎腐症状を示すカスミソウ，テッポウユリ及びカボチャを国内の栽培圃場から採集した。これらの病植物を用い，疫病菌抗血清を用いた血清診断を試みると共に，病原菌の分離・同定を行い，血清診断の結果と比較した。診断方法はELISA，蛍光抗体法及びDIBAによった。試験の結果，病植物はELISA及びDIBAで疫病菌抗血清と顕著な反応を示した。また，蛍光抗体法により無隔の菌糸が蛍光色に染色されて観察された。形態的及び生理的特徴から，カスミソウ及びテッポウユリからの分離菌は双方とも *Phytophthora nicotianae* var. *parasitica* (DASTUR) WATERHOUSE と同定され，カボチャからの分離菌は *Phytophthora capsici* LEONIAN と同定された。本試験の結果，疫病の診断に抗血清を用いる方法は迅速で精度の高い方法と考えられた。