

アルファルファ圃場の土壌より分離されたPhytophthora cryptogea Pethyb. & Laff. について

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Phytophthora cryptogea Pethyb. & Laff. Found in Alfalfa-field Soil

Naoyuki MATSUMOTO* and Toru SATO*

松本直幸*・佐藤 徹*：アルファルファ圃場の土壌より分離された

Phytophthora cryptogea Pethyb. & Laff. について

Abstract

Phytophthora sp. was isolated from an alfalfa-field soil in Shintoku, Hokkaido in 1978. The fungus exhibited a petaloid pattern with an abundant aerial mycelium on potato-dextrose agar. Young hyphae had no septa, but became septate with age. Hyphae on malt-extract agar tended to be gnarled and tuberculate (coralloid). Clusters of hyphal swellings of 7-10 μ m diam. were abundant on alfalfa seedlings. Sporangia (mean 53.7 $\times$ 32.4 μ m) were nonpapillate or inconspicuously papillate and noncaducous, and proliferation and sympodial elongation occurred. The majority of sporangia were ovoid or ellipsoidal, but some were elongated, flattened on one side, constricted, or kidney-shaped. The fungus grew on corn-meal agar from 5 to 36 C with an optimum growth temperature of 28 C. All of the isolates were of the A² mating type. The fungus was pathogenic to alfalfa and other plants. These characters suggested that the fungus was *P. cryptogea* Pethyb. & Laff. or *P. drechsleri* Tucker. Since many reports suggested that these two species were conspecific, comparisons were made by means of disc electrophoresis to obtain protein patterns of both identified species and the present isolates. Electrophoretic results confirmed the conspecificity of both species, and the isolates were identified as *P. cryptogea* due to priority.

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Introduction

During the survey for *Phytophthora megaspema* Drechsler, a causal fungus of Phytophthora root rot of alfalfa^{4,13)}, an unknown *Phytophthora* was found in an alfalfa field in 1978. In this field, alfalfa plants had disappeared, and weeds were prevalent in low-lying areas. The fungus formed no sex organs in single cultures, and sporangia were nonpapillate or inconspicuously papillate. These characters showed that it was closely related to *P. cryptogea* Pethyb. & Laff. or *P. drechsleri* Tucker. However, recent studies^{1,5,6,17)} suggest that these two species are similar or conspecific. We compared the present isolates with *P. cryptogea*** and *P. drechsleri*** by the electrophoretic technique in order to ascertain the identification of the isolates and to make a taxonomical review of both species.

Materials and Methods

Phytophthora sp. was isolated from the soil of low-lying areas in an alfalfa field

* Hokkaido National Agricultural Experiment Station, Hitsujigaoka 1, Toyohira-ku, Sapporo 061-01. 北海道農業試験場

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in Shintoku, Hokkaido by the alfalfa-seedling baiting technique¹²⁾ in May and September of 1978. Single-zoospore isolates (S-B, B-D, S-F, and S-16) were used in this study. Sporangia produced by placing mycelial V-8 juice agar (V-8 A) discs in nonsterile soil extract were measured for each isolate. Petri dishes containing corn-meal agar (CMA) were inoculated with mycelial CMA discs of 5 mm diam. cut from the margin of a 4-day-old colony. Colony diameters were determined after 3 days' incubation from 5 to 37 C. All 39 isolates were paired on V-8 A with either A¹ or A² mating type of *P. cinnamomi* Rands isolated from rhododendron and *P. cambivora* (Petri) Buisman from apple-orchard soils. Dual cultures were incubated at room temperature to inspect the presence of sex organs.

The following plants were grown in pots containing steam-sterilized soil for inoculation experiments : alfalfa (*Medicago sativa* L.), red clover (*Trifolium pratense* L.), sweetclover (*Melilotus alba* Desr.), cowpeas (*Vigna sinensis* Savi. et Hassk.), beans (*Phaseolus vulgaris* L.), mungbeans (*P. aureus* Roxb.), adzuki beans (*P. angularis* Wight), soybeans (*Glycine max* Merr.), tomato (*Lycopersicon esculentum* Mill.), eggplant (*Solanum melongena* L.), green pepper (*Capsicum annuum* L.), cucumber (*Cucumis sativus* L.), cabbage (*Brassica oleracea* L.), sugar beets (*Beta vulgaris* L.), China-aster (*Callistephus chinensis* Nees), zinnia (*Zinnia elegans* L.), and safflower (*Carthamus tinctorius* L.). Young plants of about one-month-old were inoculated with mycelial CMA pieces at the base of the stem in the soil. Mycelium-free CMA pieces were used as controls. Inoculated plants were heavily watered. Pathogenicity to potato tubers (*Solanum tuberosum* L.) was also examined by wound inoculation. Reisolation was made on CMA acidified with lactic acid.

Since taxonomical characters of the fungus indicated the close relationship to *P. cryptogea* and *P. drechsleri*, disc electrophoresis was conducted as an aid for identification. The following isolates were used for comparison. *P. cryptogea* : P 10 and P 30 ; *P. drechsleri* : P 31 (IMI 129907) and P 42. Mycelia were grown in cleared V-8 juice broth at 25 C. After 7 days' incubation, they were rinsed in deionized water three times and stored at -30 C. Frozen mycelia were ground with sea sand in 0.1 M phosphate buffer pH 7.0. The mycelial suspension was centrifuged at 3,000 rpm for 10 min. to eliminate hyphal fragments and sea sand. Soluble protein fraction was obtained by the ultracentrifugation at 105,000 $\times g$ for 60 min. Protein concentration was determined using Folin phenol reagent¹¹⁾ and adjusted to 1,700 $\mu\text{g/ml}$. Disc electrophoresis followed the method of Davis²⁾. Polyacrylamide gel columns were prepared by filling glass tubes (9.5 cm long, 5 mm inner diam.) first with 1.3 ml of 7.5 % acrylamide solution (separation gel) followed by 0.3 ml of 2.5 % acrylamide solution (stacking gel). Subsequently, 0.35 ml of 2.5 % acrylamide solution containing 300 μg of fungal protein (sample gel) was laid on the stacking gel. Polymerization of each gel was made under fluorescent light at room temperature. Electrophoresis was conducted at 4 C with a power supply at 4 mA per tube. Tris-glycine buffer was used as an electrode buffer. The gels were removed from glass tubes when the tracking dye (bromophenol blue) had migrated 60 mm in the separation gel and were placed in fixer stain solution (amido black 10B, 1 g; in 100 ml of 7 % acetic acid) for 30-40 min. They were washed and destained in 7 % acetic acid using a current of 10 mA per column. Protein patterns were recorded using a densitometer, and Ef values were determined.

Results

Phytophthora sp. from the alfalfa-field soil showed a petaloid pattern with an

abundant aerial mycelium on potato-dextrose agar. Mycelia were aerial on V-8 A and submerged in CMA. On both media, the fungus exhibited no characteristic patterns. Young hyphae had no septa, but became septate with age. Hyphae on malt-extract agar tended to be gnarled and tuberculate (Fig. 1-A). Clusters of hyphal swellings of 7-10 μm diam. were abundant on alfalfa seedlings (Fig. 1-B and C). Sporangia (mean: $53.7 \times 32.4 \mu\text{m}$, range: $74.1-29.6 \times 42.0-19.8 \mu\text{m}$, mean ratio of length to breadth: 1.69; Table 1.) were nonpapillate or inconspicuously papillate and noncaducous, and proliferation and sympodial elongation occurred. The majority

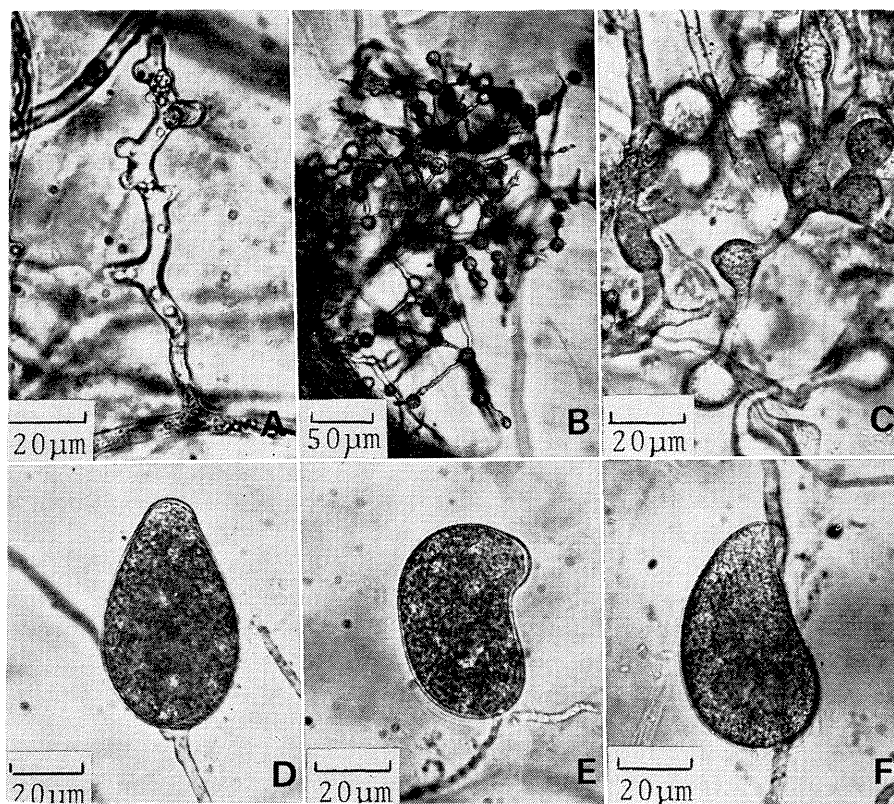


Fig. 1 Morphology of *Phytophthora* sp. from alfalfa-field soil : A. Gnarled and tuberculate hyphae ($\times 400$) ; B. Hyphal swellings in cluster ($\times 100$) ; C. Hyphal swellings ($\times 400$) ; D, E, F. Sporangial variation ($\times 400$).

Table 1. Sporangial sizes in *Phytophthora* isolates from alfalfa-field soil^{a)}

Isolate	Mean (μm)	Range (μm)	L/B ratio ^{b)}
S-B	51.1×30.2	$66.7-34.6 \times 42.0-22.2$	1.69
S-D	57.0×34.0	$74.1-37.1 \times 39.5-22.2$	1.69
S-F	54.3×30.1	$74.1-32.1 \times 42.0-22.2$	1.80
S-16	53.1×35.6	$74.1-29.6 \times 42.0-19.8$	1.49
Mean	53.7×32.4		1.69

a) Sporangia produced by placing mycelial V-8 juice agar discs in nonsterile soil extract were used for measurements, $N \geq 100$.

b) Mean ratio of length to breadth.

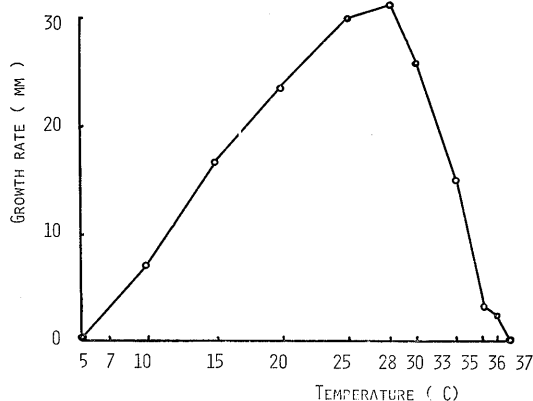


Fig. 2 Growth rate of *Phytophthora* sp. from alfalfa-field soil on corn-meal agar per 24 hr at different temperatures.

of sporangia were ovoid or ellipsoidal, but some of them were elongated, flattened on one side, constricted, or kidney-shaped (Fig. 1-D, E, and F). Atypical sporangia were more frequently found on alfalfa seedlings. Cardinal growth temperatures on CMA were as follows (Fig. 2): minimum about 5 C, optimum 28 C, and maximum 36-37 C. The fungus grew only slightly at 5 C and failed to grow at 37 C during 7 days. Thirty-nine isolates were all of the A² mating type; they mated with the A¹ *P. cinnamomi* isolate, but not with the A¹ nor A² isolate of *P. cambivora*. Oogonia in dual cultures were spherical with smooth walls.

Oospores almost filled the oogonia, but most of them were aborted. In many cases, oogonia failed to develop and remained in the antheridia.

The fungus readily killed the young plants of cowpeas, mungbeans, tomato, eggplant, green pepper, cucumber, sugar beets, China-aster, and safflower, while alfalfa, sweetclover, beans, adzuki beans, cabbage, and zinnia were infected, but not killed. The fungus was always reisolated from these plants. Red clover was not

Table 2. Pathogenicity of *Phytophthora* sp. from alfalfa-field soil to various plants^{a)}

Plants	Pathogenicity ^{b)}
Alfalfa (<i>Medicago sativa</i> L.)	+
Red clover (<i>Trifolium pratense</i> L.)	-
Sweetclover (<i>Melilotus alba</i> Desr.)	+
Cowpeas (<i>Vigna sinensis</i> Savi. et Hassk.)	++
Beans (<i>Phaseolus vulgaris</i> L.)	+
Mungbeans (<i>P. aureus</i> Roxb.)	++
Adzuki beans (<i>P. angularis</i> Wight)	+
Soybeans (<i>Glycine max</i> Merr.)	+
Tomato (<i>Lycopersicon esculentum</i> Mill.)	++
Eggplant (<i>Solanum melongena</i> L.)	++
Green pepper (<i>Capsicum annuum</i> L.)	++
Cucumber (<i>Cucumis sativus</i> L.)	++
Cabbage (<i>Brassica oleracea</i> L.)	+
Sugar beets (<i>Beta vulgaris</i> L.)	++
China-aster (<i>Callistephus chinensis</i> Nees)	++
Zinnia (<i>Zinnia elegans</i> L.)	+
Safflower (<i>Carthamus tinctorius</i> L.)	++
Potato tuber (<i>S. tuberosum</i> L.)	Pink rot

a) Plants of about one-month-old were inoculated with mycelial corn-meal agar pieces at the base of the stem in the soil.

b) ++ = Killed.

 + = Infected, but not killed.

 - = No infection.

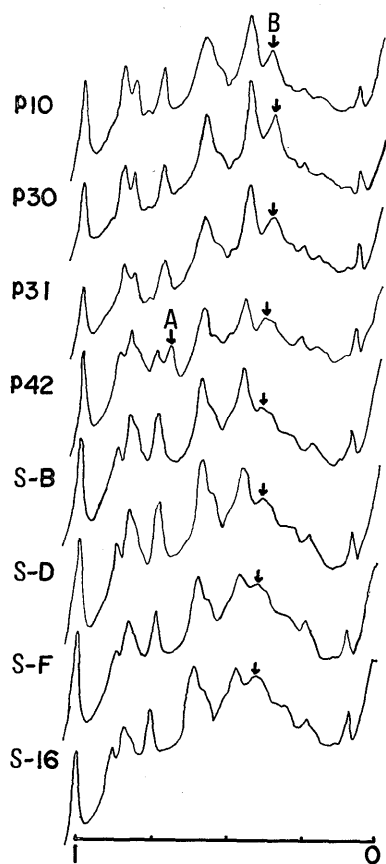


Fig. 3 Densitometer recordings of the electrophoretic protein patterns in *Phytophthora* isolates. P 10 and P 30 : *P. cryptogea*; P 31 and P 42 : *P. drechsleri*; S-B, S-D, S-F, and S-16 : *Phytophthora* isolates from alfalfa-field soil.

infected. Inoculated potato tubers showed "pink rot" symptoms, and the fungus was reisolated from the discolored portion (Table 2.)

Electrophoretic protein patterns were essentially the same in *Phytophthora* isolates used in this study (Fig. 3.). Isolate P 42 could be distinguished by band A ($E_f=71.2$), which did not exist in other isolates. Band B ($E_f=38.8$) was thick in P 10, P 30, and P 31, but not in P 42 nor our isolates.

Discussion

Morphological characters of sporangia of *Phytophthora* sp. from the alfalfa-field soil and its heterothallic nature suggest *P. cryptogea* or *P. drechsleri*. Growth responses at different temperatures are somewhat similar to those of *P. drechsleri*¹⁹⁾. Cardinal growth temperatures for *P. cryptogea* were lower²⁰⁾. Tucker¹⁹⁾ considered that the temperature requirements of different strains of the same species do not vary widely, however, temperatures for growth are not always the criterion for distinguishing species: Ribeiro *et al.*¹⁵⁾ recently reported a high-temperature isolate of *P. megasperma*, whose optimum growth temperature was 8-11 C higher than ordinary *P. megasperma* isolates on alfalfa^{3,13)}. Klisiewicz¹⁰⁾ casted doubt on the value of temperatures for growth in separation of *P. cryptogea* and *P. drechsleri* although he distinguished the two species by sporangial morphology. Bumbieris¹⁾ could not find a significant difference in growth temperatures between *P. cryptogea* and *P. drechsleri*, suggesting that some variation exists in temperature requirements in different isolates of the same species.

On the basis of morphology, as well as responses to different growth temperatures and malachite green, he proposed the conspecificity of both species. Galindo and Zentmyer⁶⁾ tentatively identified *Phytophthora* isolates on pepper plants as *P. drechsleri* although they had certain characteristics of *P. cryptogea*. Flowers *et al.*⁵⁾ isolated *Phytophthora* sp. from beans and identified as *P. cryptogea* in spite of the growth at 35 C. Shepherd¹⁷⁾ observed the similar mating patterns of *P. cryptogea* and *P. drechsleri*, concluding that these taxa were conspecific. Thus, the conspecificity of both species was suggested in many reports.

Electrophoretic protein patterns are known to be species-specific in many species of *Phytophthora*^{7,8)}. Similar protein patterns were obtained in the present study from *P. cryptogea*, *P. drechsleri*, and *Phytophthora* sp. from the alfalfa-field soil. We conclude that these two species are conspecific, and the alfalfa-field isolates were identified as *P. cryptogea* due to priority.

This represents the first report on *P. cryptogea* in Japan although Katsura⁹⁾

previously reported *P. drechsleri* on watermelon. It differs from the ordinary *P. cryptogea* (= *P. drechsleri*) in that abundant sex organs were readily formed on various agar cultures. As *P. cryptogea* has a wide host range^{5,10,14,16,18}), this pathogen should be watched for in any fields with low-lying or poorly drained areas.

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和 文 摘 要

アルファルファ圃場の土壌より分離された
Phytophthora cryptogea Pethyb. & Laff. について

松本直幸・佐藤 徹

1978年、北海道新得町のアルファルファ圃場の土壌より未知の *Phytophthora* 属菌が分離された。本菌はジャガイモ煎汁寒天培地上では花卉状の菌叢を示した。Hyphal swellings はアルファルファ実生苗上で cluster 状に豊富に形成された。遊走子のうは乳頭突起が不明瞭で、多くは卵形～楕円形、担子梗より脱落せず、proliferation と sympodial elongation により新しい遊走子のうを生じた。V-8 ジュース寒天培地に無殺菌の土壌抽出液を処理して形成される遊走子のうの大きさは平均 $53.7 \times 32.4 \mu\text{m}$ であった。分離した39菌株はすべて A² mating type で、*P. cinnamomi* A¹ 菌株との対峙培養により球形平滑の蔵卵器、底着の蔵精器を生じたが、完全な卵胞子の形成は稀であった。トウモロコシ煎汁寒天培地上での生育適温は28C、最低生育温度5C、最高36～37Cであった。本菌は土壌接種でアルファルファをはじめ多くの植物に病原性を示し、ジャガイモ塊茎には有傷接種で pink rot 症状をおこした。上述の特徴から本菌は *P. cryptogea* か *P. drechsleri* に相当し、ディスク電気泳動により輸入した両菌との識別を試みた。両菌と本分離菌株の菌体蛋白パターンはほぼ一致し、両種が同種であるという説を裏づける結果が得られたので、本分離菌株を先名権により *P. cryptogea* Pethyb. & Laff. と同定した。