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Oospore germination of homothallic *Phytophthora* species and the identity of *Phytophthora heveae* isolates from Taiwan

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Abstract. Germination of oospores of *Phytophthora cactorum, P. citricola, P. katsurae* and *P. heveae* was investigated. Germination of oospores of *P. citricola* was poor and no more than 2% germination was observed in this study. However, in the rest of three species oospores germinated up to 90% under favorable condition. Two types of oospore germination was observed; oospores of *P. heveae* germinated exclusively by producing germ tubes which directly developed into hyphae, whereas the oospores of *P. katsurae* and *P. cactorum*, besides being germinated by formation of germ tubes in some occasions, in most of the cases, germinated by producing a germ tube which in turn developed a sporangium on its tip. Aging of oospores and irradiation were the most important factors for enhancing oospore germination. Thirty-day-old oospores of the test species germinated better than those of 10 days old. Oospores of *P. katsurae* and *P. heveae* germinated up to 95% under illumination compared to that of 36% and 1% under darkness, respectively. Blue and green regions of light were the most effective in enhancing oospore germination. Moreover, red light also showed some effective in stimulating oospore germination. Isolates of *P. heveae* obtained from natural forest soils and citrus orchard soils were investigated. Taiwan isolates of *P. heveae* were similar to reference isolates of *P. heveae* in shape of sporangia and sexual organs as well as in soluble protein band pattern by electrophoresis. The existence of *P. heveae* was documented first time in Taiwan and the origin of this fungus in Taiwan was also speculated.

Key words: Homothallic *Phytophthora*; Oospore germination; *P. heveae*.

Introduction

Six homothallic species of *Phytophthora* so far have been recorded in Taiwan are *P. boehmeriae, P. cactorum, P. citricola, P. heveae, P. kasturae* and *P. vignae*. *P. boehmeriae* which caused leaf blight of *Boehmeria nivea* var. *concolar* Mak was erected by Sawada in 1929 and has not been noticed in Taiwan since then. *P. citricola* which caused leaf blight, crown rot and gamosis of citrus was also erected by Sawada in 1929. *P. citricola* was argued by Tucker as a synomy of *P. cactorum* (Tucker, 1931). However, Waterhouse (1954) reported that the shape of sporangia of *P. citricola* is distinct enough to be as a species of its own. *P. citricola* was recently found to be associated with strawberry fruit rot in Taiwan (Chang, 1988). *P. cactorum* mainly existed as a causal fungus of strawberry fruit rot ever since strawberry was introduced into Taiwan. *P. heveae*, the causal fungus

of fruit rot and black strip on rubber and rubber was reported as its sole host plant (Tucker, 1933). *P. heveae* was first time isolated in 1976 from natural forest soils in Lien Hwa Tsu Branch Station of the Taiwan Forest Experimental Institute at Yu Tsu, Nantou county. Later it was also isolated from soils of citrus orchards in Taichung areas. *P. heveae* was proved to be no pathogenicity to sweet orange (Ann, 1984). *P. katsurae* isolated from natural forest soils in Lien Hwa Tsu Forest Branch Station was idential to *P. castaneae*, the causal fungus of chestnut trunk rot in Japan, which was erected by Katsura as a new species and was later renamed as *P. katsurae* (Katsura, 1976; Ko and Chang, 1979). This paper was to present the features of oospore germination of some homothallic species of *Phytophthora* and morphological characters of Taiwan isolates of *P. heveae* in comparison with other homothallic species of *Phytophthora* found in Taiwan. Electrophoretic analysis of soluble protein band pattern was also conducted to compare Taiwan isolates of *P. heveae* with reference isolates of *P. heveae* as well as *P. citricola* and *P. cactorum*.

Materials and Methods

Oospore Germination

Test fungi. The cultures of *Phytophthora cactorum* and *P. citricola* used in this experiment were isolated from rotten strawberry fruit collected at Dah-hu, Miawli county and Shin-dien, Taipei county respectively. The cultures of *P. katsurae* and *P. heveae* were isolated by baiting with pieces of young citrus leaf disc from soils collected in natural forest plantation at Lien Hwa Tsu, Nantou county. Isolate Ann-1 of *P. heveae* was provided by Miss Ann. Isolates 1000 and 871 of *P. heveae* were offered by Dr. Ko of the University of Hawaii. All cultures were grown in V-8 juice agar.

Oospore production. All above mentioned homothallic species of *Phytophthora* produced abundant oospores when they were grown on 10 to 20% clean V-8 juice agar and incubated at 25°C in darkness. Oospores were observed and found mature morphologically 7 days after incubation.

Factors Affecting Oospore Germination

Aging and light effect on oospore germination. In our preliminary experiment 10-day-old oospores of *P. katsurae* and *P. heveae* were extracted by smashing culture agar to release oospores by using a Sorvall Omni-mixer, then thoroughly washed through a 300-mesh screen to eliminate hyhae and obtained desireable oospores. An appropriate concentration of oospore suspension was spreaded on selective medium (1 liter of 10% V-8 juice agar contains mycostatin 50 mg, PCNB 10 mg, Ampicillin 100 mg) or on water agar plate. A half of plates were placed in an incubator with 14 h photoperiod at 25°C and the remaining half was kept in darkness at the same temperature. Percentage of oospore germination was counted 5 days after incubation. Another set of experiment was undertaken by using 10-day and 30-day old oospores of *P. cactorum* and *P. heveae*. Preparation of oospore suspension and procedure for germination were the same as described previously. Percentage of oospore germination was counted 3, 5, 10, 15 and 20 days after incubation.

Light quality effect on oospore germination. In our preliminary observation we found that oospores of *P. katsurae* germinated well *in situ* in V-8 juice agar where they formed. To simplify experimental procedure test oospores were not extracted and released from agar medium. Thirty-day old oospores produced in 10% clean V-8 juice agar in 6-cm Petri plates were wrapped with black PVC insulation tape except a 2.5 cm^2 area on lid on which different Corning glass filter was laid on and through there light passed. The whole set-up was then placed under cool white fluorescent lamp inside an incubator with 14 h photoperiod at 25°C. Dark control was made by wrapped the plate completely with black PVC insulation tape. Corning glass filters used were #5543 (blue), #4010 (green) and #2408 (red). Percentage of oospore germina-

tion was taken 10 days after incubation by counting 100 oospores under a low power microscope in each treatment. Data were taken by duplication of whole experiments.

Electrophoresis

Test isolates of ***Phytophthora*** *species.* Isolates of *Phytophthora* tested are listed in Table 1.

Preparation of mycelial soluble proteins and procedures for electrophoresis. Test fungal isolates were grown in 150 ml 10% clean V-8 juice in a 250 ml flask. Each flask was inoculated with 10 pieces of 3 mm dia. 2 mm thick mycelial agar blocks cut from the margin of a vigorously growing colony by using a 3 mm dia cork borer. Each isolate was grown in 10 flasks. All cultures were incubated at room temperature for 7 days under darkness. Mycelial mats of each isolates was harvested by pouring through a layer of filter paper. Mycelial mats were rinsed three times with distilled water and dried with a vacuum pump. Dried mycelial mat was stored at −20°C for 48 h. The buffer soluble proteins were extracted by grinding frozen dried mycelial mats with a pestle in a mortar and added a little quarts sand at pH 7.0 (0.1 M potassium monobasic phosphate and 0.1 M sodium dibasic phosphate). The mixture was centrifuged at 15,000 rpm for 1 h. The resultant clear supernatant liquid from the fungal extract was decanted and immediately used for electrophoresis. Whole procedure was conducted at 4°C.

A vertical slab gel electrophoresis apparatus was used. Concentrations of mycelial buffer soluble proteins were determined by Coomassie brillant blue G250 method (Spector, 1978). A sample of 200 μl buffer soluble protein solution containing 17-70 μg protein +40% sucrose 40 μl +0.5% bromol phenol blue 15 μl was pippetted in each well in staking gel. Electrophoresis was carried out at 4°C, using a tris-glycine buffer at pH 8.2-8.5. A current of 25 mA for two gel slabs was applied until the tracking dye reached near the bottom edge of gel slab. The gel slabs were stained with Coomossie blue (1 g in 100 ml of acetic acid) for 30 to 40 min and destained with several changes of 7% acetic acid.

Results

Oospore Germination

Radiation significantly enhanced oospore germination (Table 2). Preliminary result showed that 10-day old oospores of *P. katsurae* and *P. heveae* germinated only up to 60% and 58% 5 days after incubation under illumination, whereas only 4% and 3% of oospore germinated under darkness, respectively. It took 10 to 15 days to reach maximal germination. Oospores of all three species tested germinated well up to 90% under 14 h photoperiod (Tables 2, 3 and 4). Results also showed that

Table 1. *Isolates and sources of* ***Phytophthora*** *species used to examine electrophoretic soluble protein band pattern*

Species	Isolates	Sources
Phytophthora heveae	LWT-13A	Lien Hwa Tsu natural forest soils
	LWT-8A	
	LWT-3A	
	Ann-1	Citrus orchard soil at Taichung
	1000	ATCC
	871	ATCC
Phytophthora citricola		Rotten strawberry fruit at Shin-dien
Phytophthora cactorum		Rotten strawberry fruit at Dah-hu

Table 2. *Light effect on oospore germination of* ***Phytophthora katsurae*** *and* ***P. heveae***

Incubation time (day)	Percentage of oospore germination			
	Dark		Light	
	P. cactorum	*P. heveae*	*P. cactorum*	*P. heveae*
3	0	0	20	64
5	5	2	61	84
10	7	1	90	93
15	19	1	92	95
20	36	—	94	—

Table 3. *Maturity (or age) of oospores of* ***Phytophthora cactorum*** *and* ***P. heveae*** *in relation to their germination*

Incubation time (day)	Percentage of oospore germination			
	P. cactorum		*P. heveae*	
	10-day	30-day	10-day	30-day
5	34	72	10	59
10	37	89	24	60
15	52	94	34	58
20	64	95	45	62

Table 4. *Effect of light quality on oospore germination of* ***Phytophthora katsurae***

Light quality	Percentage of oospore germination
White	91
Blue	87
Green	85
Red	30
Dark	12

30-day old oospores germinated better than those of 10-day old ones of *P. heveae* and *P. cactorum* (Table 3). Two types of oospore germination were observed in present experiment; in *P. cactorum* and *P. katsurae* oospore germination initiated by emerging a germ tube, in most of the cases, then produced a new sporangium at its tip (Figs. 1a, b), whereas in *P. heveae* and in few cases of *P. cactorum* and *P. katsurae* oospores germinated by emerging more than one germ tubes from basal part of oospores, no sporangia developed on the tips of those germ tubes were observed (Figs. 1c, d). Germ tubes directly developed into hyphae. Blue and green light were the most effective in enhancing oospore germination of *P. katsurae*. Red light also showed some effectiveness (Table 4).

Electrophoresis

Phytophthora heveae. A homothallic species of genus *Phytophthora* was isolated from the natural forest soils collected at Lien Hwa Tsu Branch Station of Taiwan Forest Experimental Institute in spring of 1976. All of our isolates of *P. heveae* grew well on V-8 juice agar and corn meal agar at 25°C. They produced quite a number of sporangia on CMA under 14 h photoperiod and formed a great number of oospores in V-8 juice agar at 25°C under darkness. Sporangia more or less ovoid to

obpyriform papillate, 33-46×28-37 μ. Sexual organs always possess a relatively small antheridium and frequently at junction point of stalk and sharp low part of oogoium (Fig. 2b); oogonium 31-39×28-34 μ; oospores thick-walled, 23-27×24-28 μ; antheridia, 11-14×7-10 μ, amphigynous. Electrophoretic buffer soluble protein band pattern of isolates 1000 and 871 of *P. heveae* and isolates Ann-1, LWT-13A, LWT-8A and LWT-3A were almost identical and they were different from those of *P. cactorum* and *P. citricola*, two paragynous and homothollic species (Fig. 3). This evidence further supports the fact that isolates Ann-1, LWT-13A, LWT-8A and LWT-3A are *P. heveae*. Electrophoretic buffer soluble protein band pattern also showed that *P. citricola* and *P. cactorum* are very closely related.

Discussion

Aging or maturity of oospores, and radiation have been emphasized as critical factors for oospore germination in species of *Phytophthora* (Ribeiro, 1983; Zentmyer and Ervin, 1970). Backwell (1943) noted that oospores of *P. cactorum* needed a minimum of 9 months for full maturation to have a good germination. Shaw (1967) demonstrated that 25-day old oospores of *P. cactorum* germinated up to 67% under illumination. Oospore germination of *P. cactorum*, *P. heveae* and *P. katsurae* in this study reached more than 90% when oospores extracted from 30-day-old cultures were tested under 14 h photoperiod of cool white fluorescent light irradiation at 25°C. Blue light was found to be the most effective in enhancing oospore germination of *P. katsurae*. Green light was also found to be highly effective in stimulating oospore germination; however, light passed through Corning glass filter #4010 included some amount of spectrum in blue light. Narrow spectum interference filter needed to be used to clarify the effectiveness of green light in enhancing oospore germination. By using monochrome light, Ribeiro

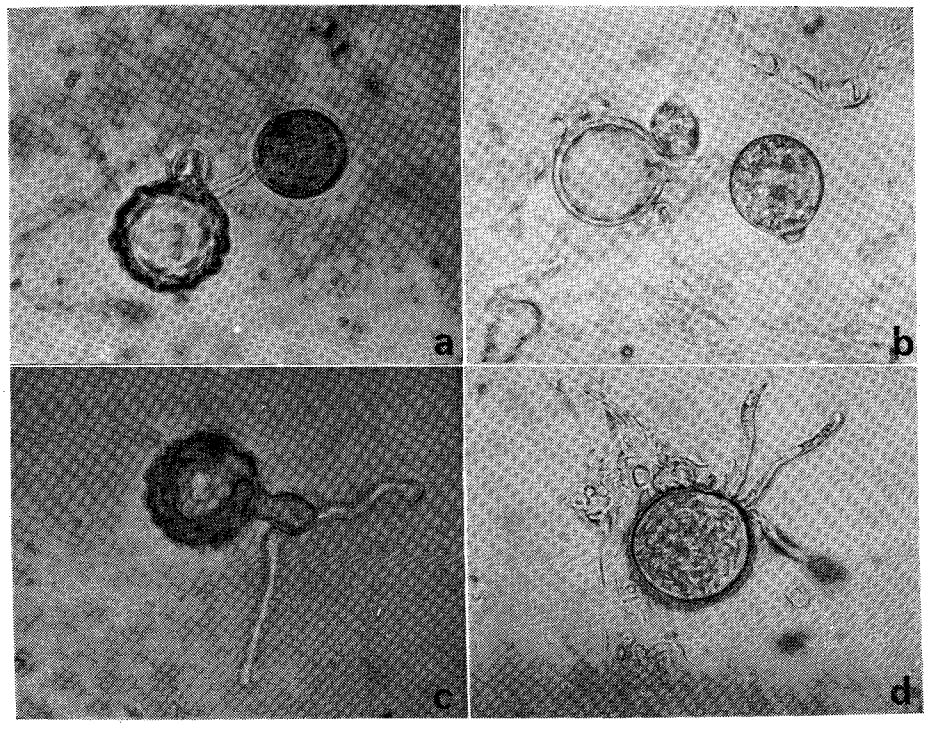

Fig. 1. Oospore germination of *Phytophora katsurae* (a, c) and *P. cactorum* (b, d) (500×).

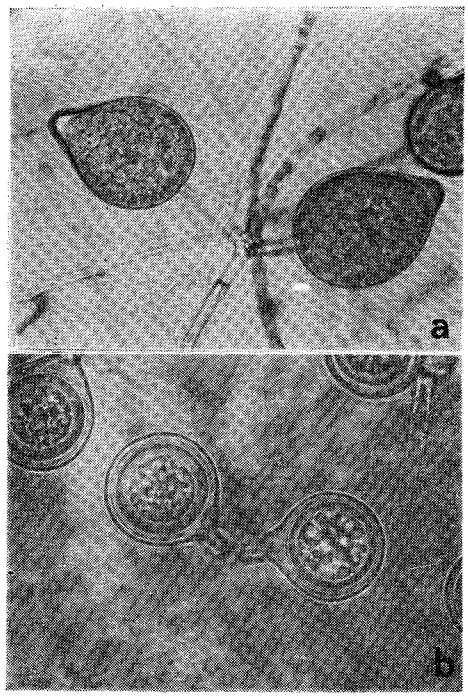

Fig. 2. Sporangia (a) and sexual organs (b) of *Phytophthora heveae*. (500×)

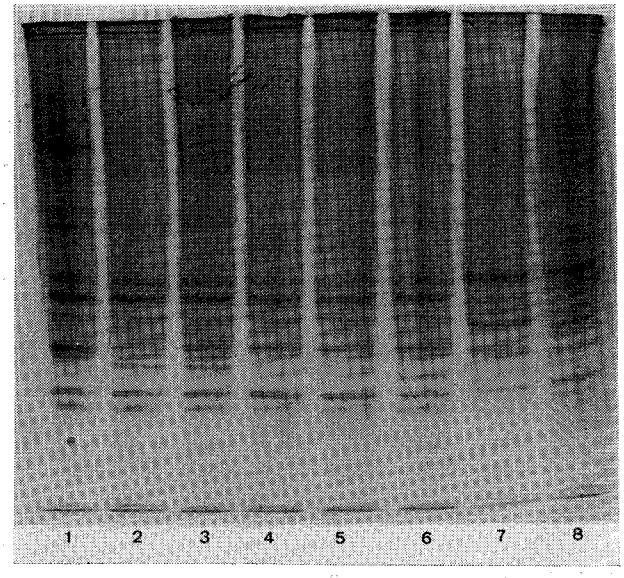

Fig. 3. Electrophoretic soluble protein band pattern of *Phytophthora heveae* (4, 5) *P. citricola* (7), *P. cactorum* (8) and isolates LWT-3A (1), LWT-8A (2), LWT-13A (3) and Ann-1 (6).

et la. (1975, 1976) demonstrated that blue light and low level of red light were the effective regions in stimulating oospore germination. Present study also revealed some effectiveness of red light in enhancing oospore germination of *P. katsurae*.

Electrophoretic buffer soluble protein band pattern showed that isolates Ann-1, LWT-13A, LWT-8A and LWT-3A were identical with isolates 1000 and 871 of *P. heveae*. Shape of sporangia and sexual organs also very similar to those described as *P. heveae*. Rubber tree was reported to be as the sole host plant of *P. heveae*. Until Ann's isolation of this fungus from citrus orchard soils collected in Taichung and Nantou counties, and our isolates obtained from natural forest soils at Lien Hwa Tsu no record of this fungus has been documented in Taiwan. It was proved that this fungus was not pathogenic to sweet orange (Ann, 1984). Rubber tree has been introduced into this island decades ago but has never been cultivated in large scale plantation. We do not have any clue ot trace the path of distribution of this fungus to citrus orchards in Taichung and Nantou counties, and the soils in Lien Hwa Tsu natural forest plantation. However, the establishment of *P. heveae* in this island is undoubted. No record has been documented on its infection to any other crops and plants in Taiwan so far.

Literature Cited

Ann, P. J. 1984. Species, mating types and pathogenicity of *Phytophthora* distributed in citrus orchards in Taiwan. Trans. Br. mycol. Soc. **82**: 631-634.

Blackwell, E. 1943. The life story of *Phytophthora cactorum* (Leb. & Cohn) Schroet. Trans. Br. mycol. Soc. **26**: 71-89.

Chang, H. S. 1988. *Phytophtkora* species associated with strawberry fruit rot. Bot. Bull. Academia Sinica **29**: 61-67.

Katsura, K. 1976. Two new species of *Phytophthora* causing damping-off of cucumber and trunk rot of chestnut. Trans. mycol. Soc. Japan **17**: 238-242.

Ko, W. H. and H. S. Chang. 1979. *Phytophthora katsurae*, a new name for *P. castaneae*. Mycologia **71**: 840-844.

Ribeiro, O. K. 1983. Physiology of asexual sporulation and spore germination in *Phytophthora*. In D. C. Erwin, S. Bartnicki-Garcia, and P. H. Tsao, (eds.), *Phytophthora*: Its Biology, Taxonomy, Ecology, and Pathology.

American Phytopathological Society, St. Paul, MN, pp. 55-70.
Ribeiro, O. K., G. A. Zentmyer, and D. C. Erwin. 1975. Comparative effects of monochromatic radiation on the germination of oospores of three *Phytophthora*. Phytopathology **65**: 904-907.
Ribeiro, O. K., G. A. Zentmyer, and D. C. Erwin. 1976. The influence of qualitative and quantitative radiation on reproduction and oospore germination of four *Phytophthora* species. Mycologia **68**: 1162-1173.
Sawada, K. 1929. Descriptive catalogue of the Formosa fungi, part 3, Special Bull. No. 27. Agri. Expt. Stn. Gov't Formosa.
Shaw, D. S. 1967. A method of obtaining single oospore cultures of *Phytophthora capsici* using water snails. Phytopathology **57**: 454.
Spector, T. 1978. Refinement of the Coomassie blue method of protein quantitation. A simple and linear spectrophotometric assay for 0.5 to 50 μg of protein. Anal. Biochem. **86**: 142-146.
Tucker, C. M. 1931. Taxonomy of genus *Phytophthora* de Bary. Univ. Mo. Agri. Expt. Stn. Bull. **159**: 208 pp.
Tucker, C. M. 1033. The distribution of the genus *Phytophthora*. Res. Bull. 184. Univ. Mo. Agri. Expt. Stn.
Waterhouse, G. M. 1954. *Phytophthora citricola* Sawada (syn. *P. cactorum* var. *applanata* Chester). Trans. Br. mycol. Soc. **40**: 349-357.
Zentmyer, G. A. and D. C. Erwin. 1970. Development and reproduction of *Phytophthora*. Phytopathology **60**: 1120-1127.

同株性疫病菌卵孢子之發芽及橡膠樹疫病菌存在於臺灣之確認

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六種臺灣有的同株性疫病菌中測試四種的卵孢子發芽情況，除 *Phytophthora citricola* 之卵孢子發芽率極低，不到百分之二外， *P. cactorum*, *P. heveae* 和 *P. katsurae* 之卵孢子發芽率均高，達百分九十以上。*P. heveae* 的卵孢子均以發芽管方式發芽， *P. cactorum* 和 *P. katsurae* 之卵孢子除少數以發芽管方式發芽，均首先形成一發芽管，發芽管之先端再形成一孢囊 。 影響疫病菌卵孢子發芽之主要因子有二：即卵孢子之成熟度（孢齡）和光照 。 三十日孢齡之卵孢子發芽率遠高於孢齡十日者。*Phytophthora katsurae* 和 *P. heveae* 之三十日孢齡之卵孢子在光照下發芽率達百分之九十五，而在無光照下者分別依序僅達百分之三十六及百分之一。藍光與綠光是促進卵孢子發芽之最有效波長。紅光顯然也有些許促進效果。*P. heveae* 橡膠樹疫病菌首次從蓮花池天然林土壤中分離得到。後又證實存在於臺灣中部數處桔園土壤中，其形態和參考已知之 *P. heveae* 菌株相類似 ，與原始菌株之形態描述也相同 。 電泳分析所得之可溶性蛋白帶分佈和已知之參考菌株 P1000 和 P871 者幾近完全相同 。 是故可確認 *P. heveae* 已存在於臺灣土壤中。橡樹在臺灣並未廣泛種植，早年引進做實驗林，後也有種植爲庭園樹，*P. heveae* 之存在，僅能以引進橡樹時同時引進解釋。蓮花池天然林土壤和桔園土壤分離的 *P. heveae* 是首次確認本菌已存在於臺灣土壤的記載。