***Phytophthora cactorum* can synthesize substances needed for sexual reproduction but requires a stress factor to trigger the process**

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***Phytophthora cactorum* grown on basal agarose medium or in liquid basal medium produced oospores after being transferred to water agarose. The numbers of oospores produced under such conditions depended on the age of the culture prior to exposure to nutrient deprivation. When the concentration of basal medium used for cultivation of *P. cactorum* was increased, the numbers of oospores produced after being transferred to water agarose was also increased. *P. cactorum* grown on basal agarose medium also produced oospores when its mycelial growth was restricted after reaching the edge of Petri plates. In 5 cm plates oospore formation occurred in the third week, whereas in 9 cm and 14 cm plates oospores appeared in the fourth week. Most oospores were formed near the edge of the plates. The non-saponifiables extracted from mycelia of *P. cactorum* grown in liquid basal medium were stimulatory to oospore formation by *P. cactorum* and *Phytophthora parasitica*, whereas the saponifiables were stimulatory to *P. cactorum* only. Extracts from culture filtrate and basal medium were not stimulatory to oospore formation by either fungus. When the non-saponifiables were fractionated by Florisil column chromatography, only fraction 1 was not active. Fractions 2, 3 and 4 were stimulatory to oospore formation by both *P. cactorum* and *P. parasitica*. These results support the hypothesis that *P. cactorum*, and possibly other pythiaceae fungi as well, can synthesize substances needed for sexual reproduction but requires a stress factor to trigger the process.**

Keywords: *Phytophthora cactorum*, basal medium, stimulatory substances, sexual reproduction, stress factors

INTRODUCTION

Sterols were originally considered to be required for sexual reproduction in pythiaceae fungi and *Phytophthora cactorum* (Lebert & Cohn) Schroeter was the most commonly used organism in such studies (Elliott, 1983). This finding was accompanied by the discovery of the inability of this group of fungi to synthesize sterols (Hendrix, 1970; Elliott, 1983). However, it was subsequently found that sterols are stimulatory but not essential for sexual reproduction in *P. cactorum* because stimulatory effects can also be caused by phospholipids (Ko, 1985; Ko & Ho, 1983). Furthermore, sterols were not able to stimulate sexual reproduction of *Phytoph-*

thor *parasitica* Daster and *Phytophthora capsici* Leonian, which were stimulated by lecithins (Ko, 1985; Ko & Ho, 1983). Sexual reproduction of *Pythium aphanidermatum* (Edson) Fitzpatrick was also stimulated by some glycerides such as dipalmitin (Ko, 1986). Recently, it was found that after removal of contaminants by aminopropyl column chromatography the stimulatory activity of phospholipids was greatly increased (Jee *et al.*, 1997). These contaminants were isolated and shown to be inhibitory to oospore formation by *P. cactorum*. After removal of the inhibitory substances, most of the highly purified commercial fatty acids and related compounds became stimulatory to sexual reproduction of *P. cactorum* (Jee & Ko, 1997).

Although incapable of synthesizing sterols, pythiaceae fungi are known to be able to synthesize various types of terpenoids, fatty acids, phospholipids and other lipid compounds (Losel, 1988). *Phytophthora cinnamomi* has

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Abbreviation: FCC, Florisil column chromatography.

been shown to be capable of synthesizing geraniol and squalene from acetate (Wood & Gottlieb, 1978a, b). Synthesis of phospholipids, including lecithin and cephalin, by *P. parasitica*, and synthesis of fatty acids by *P. cactorum* have been reported by Hendrix & Rouser (1976) and Nes (1988), respectively. *Pythium ultimum* has also been reported to synthesize lecithin, cephalin and fatty acids (Bowman & Mumma, 1967). Since these compounds are all stimulatory to sexual reproduction of *P. cactorum* (Jee & Ko, 1997), it was considered possible that *P. cactorum* and possibly other pythiaceae fungi are capable of synthesizing substances needed for their own sexual reproduction and that a certain stress factor is needed to trigger this sexual process in these fungi growing on basal medium.

In this study, various stress factors were tested for their ability to induce oospore formation by *P. cactorum* on basal medium and extracts from mycelia of the fungus grown in liquid basal medium were tested for their ability to support oospore formation to determine if the hypothesis was correct.

METHODS

Organisms. Isolates of *P. cactorum* and *P. parasitica* and the basal medium used in this study were described in a previous paper (Jee *et al.*, 1997). Cultures continuously maintained on basal agarose (SeaKem HGT-P Agarose, FMC, BioProducts) medium were used as the source of inoculum. Under such conditions, a single isolate of *P. cactorum* or paired A1 and A2 isolates of *P. parasitica* did not form oospores on the basal medium unless a stimulatory substance such as lecithin was added, indicating that stimulatory substances had not been carried over from the original inoculum (Jee *et al.*, 1997).

Stress factors. Culture discs (12 mm diameter, 5 mm thick) obtained from a 7-d-old culture established on basal agarose medium as described above were individually subjected to the following stress factors: (i) nutrient deprivation by placing the disc in an empty Petri plate, or Petri plate containing 25 ml sterile distilled water or water agarose (0.8% SeaKem HGT-P agarose) (Ho & Ko, 1980); (ii) high osmotic pressure by placing the disc on basal agarose medium amended with 1000 p.p.m. KCl; (iii) growth retardation by placing the disc on basal agarose medium amended with 10 p.p.m. metalaxyl (Subdue 2E, 25.11% active ingredient) or 150 p.p.m. streptomycin sulfate. After incubation for 7 d at 24 °C in darkness in a moist chamber, oospores were counted directly under a microscope. To obtain mycelial mats for similar tests, each 250 ml flask containing 25 ml liquid basal medium was inoculated with 10 pieces (approx. 2 × 2 × 5 mm) of *P. cactorum* culture established on basal agarose medium. After incubation for 7 d at 24 °C, the mycelial mats were picked up with a pair of forceps, washed in 25 ml sterile distilled water, transferred to tilted empty Petri plates to drain off free water and exposed to the same stress factors as described above. After incubation for 7 d at 24 °C, cultures containing oospores in Petri plates were ground with 30 ml distilled water in an Omni mixer at 4000 r.p.m. for 1 min. Oospore concentration in the suspension was determined with a microlitre pipette (Ko *et al.*, 1973). Only normal oospores were counted. Immature or aborted oospores were excluded. Three replicates were used for each treatment and all experiments were repeated with an independent batch of medium at least once.

Extraction of stimulatory substances from mycelia. Two plates of 14-d-old *P. cactorum* culture grown on basal agarose medium were triturated with 100 ml sterile distilled water in a sterilized Omni mixer at 4000 r.p.m. for 1 min. One millilitre of the culture suspension was added to a 250 ml flask containing 50 ml double strength liquid basal medium. After incubation for 14 d at 24 °C in darkness, mycelia in 100 ml flasks were harvested by filtration through Whatman no. 541 filter paper, washed with 200 ml distilled water and dried overnight at 60 °C. About 11.7 g dried mycelia was ground in a mortar and added to a 500 ml boiling flask containing 210 ml 95% (v/v) ethanol and 35 ml 50% (w/v) KOH. After gentle boiling under a reflux condenser for 2 h, the mixture was adjusted to 500 ml with deionized water. Non-saponifiables were extracted from the mixture by three aliquots of 35 ml petroleum ether (American Oil Chemists' Society, 1971). The saponifiable fraction was obtained by adjusting the aqueous portion to pH 2 with 6 M HCl and extracting the acidified solution with petroleum ether. Both non-saponifiable and saponifiable fractions were evaporated and the residues were dissolved in 1 ml ethyl acetate for bioassay.

The method previously described (Jee *et al.*, 1997) was used for the bioassays. Six basal medium discs (12 mm diameter, 5 mm thick) were evenly distributed in a Petri plate and 10 µl ethyl acetate solution containing 10 or 100 µg mycelial extract was spread over their surfaces. After evaporation of the ethyl acetate in a fume hood for 10 min, the discs were inoculated with a small piece of *P. cactorum* culture or pieces of A1 and A2 *P. parasitica* cultures about 2–3 mm away from each other. Inoculated discs were incubated for 7 d at 24 °C in darkness in a moist chamber for oospore formation. The number of oospores produced was determined as described above.

Florisil column chromatography (FCC). Chromatography of the non-saponifiables on Florisil (60–100 mesh, 500 g, Fisher Scientific) was performed following the method of Carroll (1961). Thirty grams Florisil was packed in a column (2 × 30 cm) with 60 ml hexane. Excess hexane was drained from the bottom before 70 mg non-saponifiables in 2 ml hexane was loaded on the top of the Florisil in the column. The column was washed with eluting solvents in the following order: 60 ml hexane, 200 ml 5% (v/v) ether in hexane, 200 ml 25% (v/v) ether in hexane and 360 ml 2% (v/v) methanol in ether.

RESULTS

Effect of stress factors on oospore formation on basal medium

Among the various stress treatments tested, only exposure to nutrient deprivation by transfer of culture to water agarose was effective in inducing oospore formation by *P. cactorum* grown in basal agarose medium or liquid basal medium. The numbers of oospores produced by the fungus grown in solid and liquid media were 82.7 ± 9.8 and 31.6 ± 4.2 ml⁻¹, respectively. After being transferred, *P. cactorum* grew sparingly from culture discs or mycelial mats and produced oospores in water agarose. Incubation of culture discs or mycelial mats in water or on basal agarose medium containing inhibitors or high concentrations of salts was not effective. Exposure of culture discs or mycelial mats to 4 or 36 °C for 24 h, or to air drying in a transfer hood for 6 h before being transferred to basal agarose medium also failed to induce oospore formation (data not

Table 1. Effect of culture age of *P. cactorum* grown on solid or liquid basal medium on oospore formation following transfer to water agarose

Culture age (d)	No. oospores (ml medium) ⁻¹ ± SD	
	Solid medium	Liquid medium
7	86.0 ± 10.9	33.7 ± 3.4
14	62.3 ± 7.2	99.2 ± 14.5
21	38.0 ± 7.2	73.5 ± 7.6
28	29.3 ± 4.2	50.5 ± 7.1
42	29.3 ± 4.3	35.1 ± 4.1

Table 2. Effect of the concentration of basal medium on oospore formation by *P. cactorum* 7 d after transfer to water agarose

Concentration of basal medium	No. oospores (ml medium) ⁻¹ ± SD	
	Solid medium	Liquid medium
Half	19.7 ± 1.7	11.0 ± 2.2
Standard	88.3 ± 13.7	42.4 ± 7.7
Double	144.6 ± 16.7	77.7 ± 10.1

shown). *P. cactorum* also did not form oospores when culture discs or mycelial mats on basal agarose were incubated at 4 or 36 °C (data not shown).

Factors affecting oospore formation on basal medium induced by nutrient deprivation

To study the effect of culture age on oospore formation, culture discs and mycelial mats were obtained, respectively, from basal agarose medium and liquid basal medium inoculated with *P. cactorum* and incubated at 24 °C for 7, 14, 21, 28 and 42 d. They were then transferred to water agarose for induction of oospore formation. For cultures grown on basal agarose medium, the optimum age for oospore production was 7 d (Table 1). Thereafter, the numbers of oospores produced decreased with increasing age of culture up to 28 d. There was no difference in the numbers of oospores produced by 28 and 42 d cultures. For cultures grown on liquid basal medium, the optimum age for oospore production was 14 d. Thereafter, the numbers of oospores produced decreased with increasing age of culture up to the oldest tested (Table 1). *P. cactorum* was also cultured on basal agarose medium or in liquid basal medium containing half, standard or double strength ingredients for 7 d before being transferred to water agarose for induction of oospore formation. The numbers of oospores produced increased when the concentration of nutrients increased in both cases (Table 2). In a separate test, mycelia growing in flasks containing different nutrient concentrations (one flask for each concentration) were dried at 60 °C overnight after 7 d incubation at 24 °C. The mycelial mat from standard

Table 3. Oospore formation in *P. cactorum* on basal agarose medium in different sized Petri plates

The amounts of medium in 5 cm, 9 cm and 14 cm plates were 10, 25 and 60 ml, respectively. No oospores were formed after 1 or 2 weeks incubation.

Incubation time (weeks)	No. oospores per plate ± SD		
	Plate diameter (cm) ...		
	5	9	14
3	46 ± 91	0	0
4	1133 ± 301	1420 ± 250	4386 ± 660
6	1150 ± 405	2760 ± 502	17802 ± 2394

Table 4. Effect of the amount of basal agarose medium on oospore formation by *P. cactorum* in 5 cm Petri plates

No oospores were formed after 1 or 2 weeks incubation.

Incubation time (weeks)	No. oospores per plate ± SD		
	Amount of medium (ml) ...		
	5	10	15
3	413 ± 106	1160 ± 245	1556 ± 210
4	560 ± 106	1406 ± 160	1637 ± 213
6	622 ± 123	1520 ± 433	3560 ± 1470

medium weighed about 1.45 mg. When the nutrient concentration in liquid basal medium decreased to half of the standard level, the dry weight of mycelia was decreased about 3.3 times. When the nutrient concentration was doubled, the dry weight of mycelia was increased about 1.7 times (results not shown).

Oospore formation on basal medium induced by growth limitation

Inoculum discs (5 mm diameter) of *P. cactorum* grown on basal medium were placed in the centre of 5, 9 and 14 cm Petri plates containing 10, 25 and 60 ml basal medium, respectively. Plates were observed under a microscope every week and oospores were counted for 6 weeks as described previously. Mycelia of *P. cactorum* reached the edge of 5 cm plates in 2 weeks, and that of 9 and 14 cm plates in 3 weeks.

During the growth period, no oospores were produced. However, oospores started to appear within 1 week after mycelia reached the edge of plates (Table 3). The larger the size of plates used, the larger the number of oospores produced.

To determine the relationship between the amount of basal medium used and the number of oospores produced, 5 cm plates each containing 5, 10 or 15 ml basal medium were inoculated with *P. cactorum* as described

Table 5. Activity of non-saponifiables of mycelial extract of *P. cactorum* on oospore formation by *P. cactorum* and *P. parasitica* following FCC

Fraction	Eluting solvent	Lipid class eluted	Recovery (%)	No. oospores (10 µg sample) ⁻¹ ± SD	
				<i>P. cactorum</i>	<i>P. parasitica</i>
1	Hexane (60 ml)	Hydrocarbons	1.2	0	0
2	5% ether in hexane (200 ml)	Sterol esters	3.8	9875 ± 1540	5750 ± 957
3	25% ether in hexane (200 ml)	Sterol, triglycerides	12.5	46875 ± 5105	11250 ± 645
4	2% methanol in ether (360 ml)	Mono-, diglycerides	82.5	4875 ± 1875	76 ± 35

above. The fungus started to produce oospores after the mycelia reached the edge of the plates regardless of the amount of basal medium used (Table 4). The more the basal medium present in a plate, the more the oospores produced.

To determine the distribution of oospores on water agarose, 5 cm plates each containing 10 ml basal medium were inoculated with *P. cactorum* as described above. Most oospores were produced near the edge of the plates. The numbers of oospores produced near the centre, between the centre and the edge and near the edge were 3.7 ± 0.6 , 24.0 ± 5.0 and 166.2 ± 30.5 ml⁻¹, respectively.

Stimulation of oospore formation by substances extracted from mycelia

The non-saponifiables extracted from mycelia of *P. cactorum* grown in liquid basal medium were stimulatory to oospore formation by *P. cactorum* and *P. parasitica* [18250 ± 1779 and 10510 ± 1472 oospores (100 µg sample)⁻¹, respectively, after incubation for 7 d at 24 °C in darkness], whilst the saponifiables were stimulatory to *P. cactorum* only [5277 ± 745 oospores (100 µg sample)⁻¹]. Extracts from culture filtrate and liquid basal medium were not stimulatory to oospore formation by either fungus. When the non-saponifiables from mycelia were fractionated by FCC, fraction 4 contained the largest amount of the components followed by FCC fractions 3, 2 and 1 (Table 5). Only FCC fraction 1 was not active. The other fractions were stimulatory to oospore formation by both *P. cactorum* and *P. parasitica*. FCC fraction 3 was most active followed by FCC fractions 2 and 4.

DISCUSSION

In this study, it was found that the extract from mycelia of *P. cactorum* grown in liquid basal medium was stimulatory to oospore formation by both *P. cactorum* and *P. parasitica*. *P. cactorum* also produced oospores on basal medium when it was transferred to the nutrient-free solid medium of water agarose (Ho & Ko, 1980) or when its growth was limited after reaching the edge of culture plates. These results support the hypothesis that

P. cactorum, and possibly other pythiaceae fungi as well, can synthesize substances needed for sexual reproduction but requires a stress factor to trigger the process.

When the concentration of basal medium used for culture of *P. cactorum* was increased, the numbers of oospores produced after being transferred to water agarose was also increased. This suggests that the amount of stimulatory substances synthesized is positively correlated with the amount of basal nutrients available. When basal-medium-grown cultures of *P. cactorum* were transferred to sterile distilled water, the fungus did not continue to grow and no oospores were produced. This suggests that new growth is needed for oospore formation and that nutrient stress created by diffusion gradients in water (Ko & Lockwood, 1967) is too strong to be suitable for initiation of sexual reproduction.

Induction of sexual reproduction in *P. cactorum* by growth limitation appears to be a relatively slow process because in 9 cm plates the fungus formed oospores in the fourth but not third week of incubation. Most researchers recorded their observations of induction of sexual reproduction in *P. cactorum* within 1 or 2 weeks (Elliott *et al.*, 1964; Harnish, 1968; Leal *et al.*, 1964). This may explain why oospore formation by pythiaceae fungi in basal medium has not been reported previously.

The non-saponifiables in mycelial extracts of *P. cactorum* were stimulatory to oospore formation by *P. cactorum* and *P. parasitica*, whilst the saponifiables were stimulatory only to *P. cactorum*. This result suggests that at least two different kinds of stimulatory substances were synthesized by *P. cactorum*. Moreover, different FCC fractions of the non-saponifiables showed differences in ability to support oospore formation by *P. cactorum* and *P. parasitica*, indicating that there is more than one non-saponifiable stimulatory substance existing in the mycelial extract. The identity of these compounds remains to be investigated. The activity of stimulatory substances extracted from mycelia of *P. cactorum* was comparable to that of fatty acids and related compounds tested by Jee & Ko (1997).

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