Inhibition of Reproduction of *Phytophthora* by the Calmodulin-interacting Compounds Trifluoperazine and Ophiobolin A

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Sporangium production in *Phytophthora palmivora* was inhibited by trifluoperazine (TFP) and ophiobolin A. TFP was inhibitory to a similar extent to cultures both in light and in darkness, although the stimulatory effect of calcium and the inhibitory effect of EGTA were much greater in the dark. Sporangium and oospore production in *P. cactorum* were inhibited by TFP. Thus development of both asexual and sexual reproductive structures involves a calcium/calmodulin interaction.

INTRODUCTION

Calcium is required for, or at least stimulates, asexual and sexual reproduction in Oomycetes, such as *Achlya* (Griffin, 1966), *Phytophthora* (Halsall & Forrester, 1977; Elliott, 1972) and *Pythium* (Yang & Mitchell, 1965; Lenny & Klemmer, 1966). An apparent exception is the *Pythium* sp. which causes an equine mycosis (Shipton, 1985). Physiological effects of calcium are generally mediated by its binding to calmodulin (Cheung, 1980; Means & Dedman, 1980). This paper reports the effect on reproduction of *Phytophthora* of the calmodulin-interacting compounds trifluoperazine (Levin & Weiss, 1976, 1977; Roufogalis, 1982) and ophiobolin A (Leung *et al.*, 1984, 1985).

METHODS

Cultures. The strains used were *Phytophthora palmivora* IMI 189732 (mating type A2) and *P. cactorum* IMI 270425.

Media. The growth medium generally used contained (l^{-1}): sucrose, 15.0 g; monosodium glutamate monohydrate, 1.50 g; KH_2PO_4 , 0.50 g; $MgSO_4 \cdot 7H_2O$, 0.25 g; trace element solution (Elliott, 1972), 1 ml; thiamin hydrochloride, 1 mg; sitosterol, 10 mg (added in 10 ml acetone).

The replacement solution contained (l^{-1}): KH_2PO_4 , 0.25 g; K_2HPO_4 , 0.25 g; $MgSO_4 \cdot 7H_2O$, 0.25 g; trace element solution, 1 ml. Thiamin hydrochloride was sometimes added. $CaCl_2$ was added to growth medium and replacement solution according to the design of the experiment.

Cultures were grown in 100 ml conical flasks containing 20 ml medium at 23 °C in the dark.

Two kinds of experiments testing inhibitory effects of compounds were performed. Either a solution of the compound was added to the growing culture (1.0 or 0.5 ml per flask) directly after several days' growth but before reproductive bodies appeared, or the medium was poured off and replacement solution (20 ml), incorporating the compound, was added.

In experiments where the cultures were exposed to light, they were placed in a growth room at 25 °C on a 12 h light/12 h dark cycle. The light intensity was approx. 80 W m⁻² ('Atlas' warm white fluorescent lights). The time at which the cultures were placed in the growth room varied from one experiment to another, with concomitant variation in the amount of light received before 12 h darkness.

Cultures were blended for several seconds in a Waring blender and fixed by addition of 2 ml ethanol/formalin (40% formaldehyde) (1:1, v/v). Portions (2.5 ml) of the suspension were dispensed into 5 cm Petri dishes, and the numbers of reproductive bodies counted, generally in 15 microscope fields (five in each of three dishes) 1.03 mm in diameter.

Abbreviation: TFP, trifluoperazine hydrochloride.

Chemicals. EGTA and trifluoperazine dihydrochloride (TFP) were from Sigma. EGTA was suspended in water and dissolved by addition of NaOH to pH 6.5. TFP was dissolved in sterile water.

Production of ophiobolin A (Leung *et al.*, 1984). *Helminthosporium maydis* (IMI 175569) was grown in the sucrose/ammonium tartrate medium of Luke & Wheeler (1955). The culture filtrate after 19 d growth was extracted (24 h) with ethyl acetate in a continuous extraction apparatus. The material extracted, after removal of solvent in a rotary evaporator, was taken up in methylene chloride, applied to a column (18 × 1.5 cm) of silica gel (Merck 60G) pre-conditioned with methylene chloride, and eluted with 8% (v/v) acetone in methylene chloride. Fractions containing material with the same properties on TLC as ophiobolin A were combined, and yielded 87 mg of pure crystals from 3 l of filtrate. This material had the same properties on TLC and the same mass spectrum (probe analysis on a Kratos MS 30 mass spectrometer; source temperature, 200 °C; 70 eV) as an authentic sample of ophiobolin A supplied by Dr C. L. Tipton. The ophiobolin A was dissolved in acetone; a dilution series was made in acetone and added to replacement solution, 2 ml to 150 ml.

RESULTS

Sporangium development in *P. palmivora* is greatly stimulated by light. Table 1 shows that in cultures kept in the dark, and where no calcium was added to the growth medium, the addition of CaCl₂ to the replacement solution had a highly significant stimulatory effect on sporangium development. Other experiments showed that when the growth medium included CaCl₂ none needed to be added to the replacement solution. *Phytophthora* sequesters calcium (Hemmes & Pinto da Silva, 1980) and presumably releases it, at least internally. The effect of calcium was concentration dependent (Fig. 1, curve C) and was inhibited by EGTA (Table 1).

In the light, on the other hand, no effect of calcium concentration could be demonstrated (Fig. 1, curve A), though in the experiment of Table 1 the inhibitory effect of EGTA in light was just

Table 1. *Sporangium formation in P. palmivora as affected by Ca²⁺ and EGTA in the replacement solution and by light*

Growth medium was without added calcium. Mycelium was grown for 6 d; the medium was then poured off and replacement solution added, containing 0.5 mM-Ca²⁺ or none, 2 mM-EGTA or none. Cultures in the dark were harvested 48 h later, those in the light after 24 h. Numbers of sporangia were counted in 15 fields for dark cultures, and in 12 fields for light cultures. There were nine replicate flasks for each treatment.

Addition(s) to replacement solutions		Mean no. of sporangia	
Ca ²⁺	EGTA	Dark	Light
+	—	95.6	163.0
+	+	55.6	120.1
—	—	63.2	152.2
—	+	50.0	128.4
Least significant difference (5%):		18.3	42.4

Table 2. *Effect of TFP and light on sporangium formation in P. cactorum*

The growth medium contained 10 g sucrose l⁻¹, 5.614 g sodium glutamate l⁻¹ and 1 mM-CaCl₂. After 9 d, medium was poured off and replacement solution, containing 1 mM-Ca²⁺ and thiamin (1 mg l⁻¹), added. Cultures were blended and fixed 4 d later.

Replacement solution	Mean no. of sporangia ± SE*	
	Dark	Light
Salts only	32.2 ± 3.19 (9)	113.2 ± 5.94 (9)
TFP (25 µM)	2.8 ± 1.34 (9)	27.8 ± 3.65 (8)

* The number of replicate flasks is shown in parentheses.

Fig. 1

Fig. 1. Effect of Ca^{2+} concentration on sporangium production in *P. palmivora*. Growth medium as in Methods without CaCl_2 and with 0.2 mM-EGTA. After 6 d, replacement solution, with Ca^{2+} as indicated, was substituted. Cultures in the light were fixed after 24 h, those in the dark after 48 h. Points are the means of seven or eight values. Curve A ($\circ$): no TFP, cultures in the light; mean square between calcium concentrations (5 d.f.) not significantly different from mean square within concentrations (42 d.f.) ($F = 0.83$). Curve B ($\bullet$): TFP 25 μM , cultures in the light; mean square between concentrations significantly greater ($P < 0.01$) than within (5 and 41 d.f.; $F = 7.27$). Curve C ($\square$): no TFP, cultures in the dark; mean square between concentrations significantly greater ($P < 0.01$) than within (5 and 35 d.f.; $F = 27.44$).

Fig. 2

Fig. 2. Effect of TFP on sporangium production in *P. palmivora*. Growth medium contained 1 mM- CaCl_2 . Cultures were grown for 7 d in the dark; the medium was then replaced. Replacement solutions had 0.5 mM- Ca^{2+} . Half the cultures were placed in the light ($\circ$), half were returned to the dark ($\bullet$). Cultures were blended and fixed 2 d later. There were six flasks for each treatment, eight for the controls. The control values were 261.6 ± 9.26 sporangia for cultures in the light, 119.5 ± 9.38 for the dark cultures. Values for light and dark treatments are expressed as percentages of their respective control means. The vertical lines through each point represent one standard error.

significant. Nevertheless, both in the light and in the dark TFP was strongly inhibitory to sporangium development at 100 μM (Fig. 2), and the degree of inhibition was dependent on calcium concentration (Fig. 1, curve B).

In the light, ophiobolin A inhibited sporangium development in *P. palmivora* (more than 90% inhibition at 10 μM ; Fig. 3).

TFP inhibited oospore and sporangium development in *P. cactorum* (Fig. 4, Table 2).

DISCUSSION

The inhibition of reproduction by two calmodulin-interacting compounds, TFP and ophiobolin A, suggests that calmodulin is involved in the promotion of reproduction in *Phytophthora*. [Roufogalis (1982) has cautioned against invoking calmodulin on the basis of TFP inhibition alone.] Calmodulin is believed to be of universal occurrence in eukaryotes. Stimulation of sporangium formation by light is well known (Harnish, 1965; Huguenin & Jacques, 1973; Alizadeh & Tsao, 1985). TFP inhibited sporangium development in *P. palmivora* to a similar extent in the light and in the dark. Calcium is thus implicated under both conditions. However, the effect of calcium concentration and the degree of inhibition by EGTA were less in the light than in the dark. No particular precautions were taken to eliminate traces of calcium by procedures such as acid washing of glassware, and an agar disc inoculum (about 4 mm diameter

Fig. 3

Fig. 3. Effect of ophiobolin A on sporangium production in *P. palmivora*. Growth medium contained 1 mM-CaCl₂. Cultures were grown for 6 d in the dark; the medium was then replaced and cultures transferred to the light. The replacement solutions had 0.25 mM-Ca²⁺. Cultures were blended and fixed after 2 d. Values are means for seven flasks at each concentration.

Fig. 4

Fig. 4. Effect of TFP on oogonium/oospore (●) and sporangium (○) production in *P. cactorum* (two different experiments). In the experiment where oospores were counted, the fungus was grown on the medium described in Methods with 1 mM-CaCl₂. TFP was added to the cultures after 12 d (no oogonia were present at this time), and oogonia/oospores were counted in cultures blended and fixed 2 d later. There were seven flasks at each concentration. In the sporangium experiment, the growth medium contained 10 g sucrose and 5.614 g sodium glutamate l⁻¹ and 1 mM-CaCl₂. After 8 d the medium was poured off and replacement solution (with 0.5 mM-Ca²⁺ and 1 mg thiamin l⁻¹) added. Cultures were kept in the dark. Sporangia were counted in cultures blended 5 d later. There were seven flasks at each concentration, eight for the control.

and 2 mm thick) was used; trace amounts of calcium were likely to have been present. Although the sporangia formed in the dark could have resulted from light stimulation during manipulation of the cultures, it seems possible that in the dark a different induction mechanism with a greater calcium dependence could be in operation. But whatever the induction mechanism, a calcium/calmodulin interaction affects an essential step in the development process.

Light is, on the other hand, inhibitory to oospore formation (Harnish, 1965; Huguenin & Jacques, 1973; Yu *et al.*, 1981) and oogonium development is undoubtedly induced by a different mechanism from sporangium formation. Nevertheless, my results with *P. cactorum* indicate that a process dependent on a calcium/calmodulin interaction plays an essential part in oospore development. Kerwin & Washino (1986) have shown that calmodulin inhibitors inhibit oospore development in *Lagenidium giganteum*; the effect is greater on oospore maturation than on the earlier stages of development of the gametangia.

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