Fosetyl-Al treatment of mycelium of *Phytophthora citrophthora* releases a higher scoparone elicitor activity from a fosetyl-Al sensitive strain than from an insensitive mutant.

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FOSETYL-AL TREATMENT OF MYCELIUM OF *PHYTOPHTHORA CITROPHTHORA* RELEASES A HIGHER SCOPARONE ELICITOR ACTIVITY FROM A FOSETYL-AL SENSITIVE STRAIN THAN FROM AN INSENSITIVE MUTANT.

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Fruits, Jan.-Feb. 1991, vol. 46, n° 1, p. 51-55.

ABSTRACT - The mycelium of a wild-type strain of *Phytophthora citrophthora* sensitive *in vitro* to fosetyl-Al (EC_{50} = 56 $\mu g.ml^{-1}$) and the mycelium of a fosetyl-Al insensitive mutant (EC_{50} = 780 $\mu g.ml^{-1}$) were incubated in fosetyl-Al at 50 $\mu g.ml^{-1}$. The eliciting activity of the filtrate of the incubation medium was evaluated by measuring its ability to elicit scoparone accumulation in leaf tissue of *Citrus jumbhiri* Lush. Our results indicate that, upon incubation in fosetyl-Al, mycelium of the sensitive strain of *P. citrophthora* released a highter scoparone eliciting activity than mycelium of the insensitive mutant. The strain-specific release of elicitors by fosetyl-Al may explain our previous results showing that preinoculation of *Citrus* leaves floated on fosetyl-Al with the sensitive strain of *P. citrophthora* protects them against subsequent infection by the insensitive mutant.

LE TRAITEMENT DU MYCELIUM DE *PHYTOPHTHORA CITROPHTHORA* AVEC DU PHOSETHYL-AL LIBERE UNE ACTIVITE ELICITRICE DE SCOPARONE PLUS ELEVEE CHEZ UN ISOLAT SENSIBLE AU PHOSETHYL-AL QUE CHEZ UN MUTANT TOLERANT.

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Fruits, Jan.-Feb. 1991, vol. 46, n° 1, p. 51-55.

RESUME - Le mycélium d'une souche sauvage de *Phytophthora citrophthora* sensible *in vitro* au phoséthyl-Al (EC_{50} = 56 $\mu g.ml^{-1}$) et celui d'un mutant tolérant au phoséthyl-Al (EC_{50} = 780 $\mu g.ml.^{-1}$) ont été incubés en présence de 50 $\mu g.ml^{-1}$ de phoséthyl-Al. L'activité élicitrice du filtrat du milieu d'incubation a été évaluée par la mesure de sa capacité à induire l'accumulation de scoparone dans des feuilles de *Citrus jumbhiri*. Nos résultats indiquent que, après incubation dans le phoséthyl-Al, le mycélium de la souche sensible relâche une plus grande quantité d'éliciteurs que le mycélium de la souche tolérante. La libération spécifique d'éliciteurs pourrait rendre compte de nos résultats antérieurs montrant que la préinoculation de feuilles de *Citrus* flottées sur le phoséthyl-Al avec la souche sensible de *P. citrophthora*, les protège contre l'infection subséquente par le mutant tolérant.

MOTS CLES - scoparone, fosetyl-Al, *Citrus, Phytophthora citrophthora.*

INTRODUCTION

Previous studies have shown that fosetyl-Al (aluminium tris-o-ethylphosphonate, trade name Aliette ®, Rhône-Poulenc, Lyon, France), has little *in vitro* effect on mycelium growth of most Oomycetes, although it controls some diseases they cause in plants (SCHWINN, 1979 ; FARIH *et al.*, 1981 ; SANDERS *et al.*, 1983 ; BOMPEIX and SAINDRENAN, 1984 ; GUEST, 1984 ; TIMMER and CASTLE, 1985). In treated plants, fosetyl-Al is degraded to phosphorous acid (H_3PO_3) which appears to be the active component involved in disease control (COHEN and COFFEY, 1986). The mode of action of fosetyl-Al (on the fungus, on the plant or on both) is still controversial.

The enhanced defence mechanisms observed in infected plants treated with fosetyl-Al include a rapid production of phytoalexins and lead to the establishment of an incompatible response. These observations support the hypothesis that host reactions are involved in protection by phosphonates (BOMPEIX *et al.*, 1980 ; GUEST, 1984 ; KHAN *et al.*, 1986 ; SAINDRENAN *et al.*, 1988 ; AFEK and SZTEJNBERG, 1989 ; SAINDRENAN *et al.*, 1990).

There is also evidence for a direct action of fosetyl-Al

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on the pathogens. Concentrations of H_3PO_3 in plant tissue treated with the phosphonate compounds were sometimes sufficient to account for disease control through direct toxicity (SMILLIE *et al.*, 1989 ; FENN and COFFEY, 1989). Similar conclusions were reached from experiments with fosetyl-Al insensitive mutants of *P. palmivora* and *P. parasitica* obtained by chemical mutagenesis (DOLAN and COFFEY, 1988 ; OUIMETTE and COFFEY, 1989). It was concluded that the inefficacy of fosetyl-Al and H_3PO_3 for disease control in plants inoculated with such H_3PO_3-insensitive mutants, provides strong evidence for a direct antifungal mode of action for fosetyl-Al.

We have shown previously that preinoculation of *Citrus* leaves floated on fosetyl-Al with a fosetyl-Al sensitive strain of *P. citrophthora* (P-35), not only inhibited infection by this sensitive strain, but also prevented subsequent infection by a pathogenic fosetyl-Al insensitive mutant of the fungus (PA-13) recovered on a culture medium amended with phosphorous acid (ALI, 1989 ; ALI *et al.*, 1989). These results suggested that inoculation of the fosetyl-Al sensitive strain on fosetyl-Al treated *Citrus* leaves induced a host reaction which inhibited the development of the subsequently inoculated fosetyl-Al insensitive mutant.

In the present study, we compared the parental fosetyl-Al sensitive strain (P-35) to the PA-13 insensitive mutant in terms of the release of elicitors of scoparone (a phytoalexin associated with resistance towards *P. citrophthora* in *Citrus* leaves) (AFEK and SZTEJNBERG, 1988).

MATERIALS AND METHODS

Fungal isolates and plant materials.

Two isolates of *P. citrophthora*, either sensitive (P-35) or insensitive (PA-13) to fosetyl-Al and H_3PO_3 (table 1) (ALI *et al.*, 1988 ; ALI, 1989 ; ALI *et al.*, 1989) were maintained on corn meal agar (CMA) at 25°C and were used to inoculate plants of rough lemon (*Citrus jumbhiri* grown as previously described (ALI *et al.*, 1988).

Scoparone elicitors released from mycelium of *P. citrophthora*.

After 5 days of growth on CMA in the dark at 25°C, aerial mycelium of *P. citrophthora* isolates, either sensitive or insensitive to fosetyl-Al, was removed from the surface of the colonies, using a scalpel, and was placed in a test-tube containing 10 ml of sterile Ribeiro's synthetic liquid medium amended with 0.03 M MES buffer, pH 6.2. The tubes were shaken vigorously in a Vortex mixer to obtain a mycelial slurry, and 1 ml of the slurry was pipetted into 49 ml of the modified Ribeiro's synthetic medium (RIBEIRO, 1975) in a 250 ml conical flask. Flasks were incubated without shaking in the dark at 25°C for 8 days. The liquid phase was removed and replaced by 0.03 M MES buffer, pH 6.2, containing 0,10,50 or 100 $\mu g.ml^{-1}$ fosetyl-Al (ten flasks per concentration) and the mycelium was then incubated further for 6 days. Cultures were bulked and the mycelial mats and fragments were collected on gauze ; they were oven-dried at 80°C for 24 h, and dry weights were determined.

The incubation fluids were then filtered through a sintered filter to remove mycelial fragments and an eliciting fraction was prepared from this cell-free incubation filtrate, as described by ANDERSON-PROUTY and ALBERSHEIM (1975) and CAHILL and WARD (1989). The incubation filtrate was reduced to approximately 1/4 of the original volume, using a rotary evaporator at 35°C. The concentrated filtrate was dialysed (10,000 mol.wt.cut off) for 2 days against 4 x 2 l changes of distilled water at 4°C. Three vol. ethanol 95% were then added to one vol. dialysate, and the resulting mixture was kept at 4°C for 3 days, in order to allow the precipitate to settle. The clear supernatant liquid was siphoned off and discarded. The bottom phase was centrifuged for 1 h at 10,000 g, and the pellet was resuspended in 1 ml of 0.03 M MES buffer, pH 6.2 (containing 10 $\mu g.ml^{-1}$ neomycin sulphate). This eliciting preparation (EP) was used in subsequent tests of elicitor activity.

Carbohydrate and protein content of the elicitor preparations.

The carbohydrate concentrations of EP were determined as D-glucose equivalent, using the phenol-sulfuric acid technique (DUBOIS *et al.*, 1956). The protein content of EP was determined by the method of BRADFORD (1977).

Assay of elicitor activity.

Leaves taken from rough lemon plants grown in the greenhouse were surface-sterilized for 5 min in 2% NaClO, followed by three washes with sterilized distilled water. They were then arranged in glass trays on moist filter papers. A longitudinal incision, 5 mm long, was made with a scalpel along the main vein of each leaf, lifting up vein tissue and some adjacent epidermis.

TABLE 1 - EC_{50} values of inhibition of mycelial growth of *Phytophthora citrophthora* P-35 or PA-13 on corn meal agar in the presence of either fosetyl-Al or H_3PO_3.

	EC_{50} ($\mu g.ml^{-1}$) (a)	
P. citrophthora	fosetyl-Al	H_3PO_3
P-35 (sensitive)	56.5	6.5
PA-13 (tolerant)	785.5	125.5

(a) - EC_{50} values of inhibition of radial mycelium growth were obtained from equations of a regression line plotting percent inhibition versus concentration.

Twenty µl of EP containing 10, 20 or 30 µg glucose equivalent were placed in the incision with a micropipette. Control leaves were treated similarly with MES buffer containing neomycin sulphate.

After 4 days in the dark at 25°C, a leaf disk, 2 cm in diameter, containing the wounded site and the elicitor droplet, was excised from each leaf and scoparone concentration was determined in 1 g.f.wt. of tissue collected from several treated leaves, by the method of AFEK and SZTEJNBERG (1988).

RESULTS

Statistical analysis indicates that the mean initial dry weight of mycelium for both fungal strains (0.311 mg for the P-35 isolate and 0.522 mg for the PA-13 strain) did not change significantly after 6 days of incubation in MES buffer containing different concentrations of fosetyl-Al (table 2).

Carbohydrate and protein content of EP were measured (table 3). Incubation of mycelium in fosetyl-Al increased carbohydrate and protein content in EP, the increase being larger for the P-35 sensitive strain than for the PA-13 insensitive mutant of *P. citrophthora*.

The eliciting activity of EP was estimated by measuring scoparone accumulation in 1 g aliquots of leaf tissue (table 4). Results show scoparone accumulation only in leaf tissue treated with EP of strain P-35 incubated in fosetyl-Al, but not in tissue treated with EP of P-35 incubated in MES, or with EP of PA-13 mutant incubated in either MES or fosetyl-Al.

DISCUSSION

Various studies have reported that treatment with fosetyl-Al and H_3PO_3 protect plants against infection by several Oomycetes. It was hypothesized that fosetyl-Al treatment may act indirectly by inducing host defence

TABLE 2 - Dry weight of mycelium of *Phytophthora citrophthora* after incubation for 6 days (a) in the presence of fosetyl-Al

fosetyl-Al ($\mu g.ml^{-1}$)	Dry weight of mycelium (mg)	
	Strain P-35 (c)	Isolate PA-13 (c)
0	0.4574 ± 0.17 (d) (b)	0.5596 ± 0.04 (e)
10	0.3676 ± 0.13 (d)	0.6638 ± 0.28 (e)
50	0.3150 ± 0.11 (d)	0.7513 ± 0.42 (e)
100	0.2666 ± 0.05 (d)	0.6727 ± 0.30 (e)

(a) cultures, 8-day old, were filtered. Mycelium was resuspended in MES buffer, pH 6.2, containing increasing concentration of fosetyl-Al, and was incubated for 6 days. The initial mean weight of mycelium was 0.3110 mg for P-35 isolate and 0.5220 mg for PA-13 mutant, respectively.
(b) data are the mean results of 4 independent experiments with 10 replicates ± standard deviation.
(c) P-35 : parental fosetyl-Al sensitive strain ; PA-13 : insensitive mutant.
(d) and (e) not significantly different from the respective initial dry weights of mycelium

TABLE 3 - Carbohydrate and protein content of the eliciting preparation (EP) obtained from the incubation filtrate of either sensitive strain (P-35) or tolerant mutant (PA-13) of *P. citrophthora*.

fosetyl-Al ($\mu g.ml^{-1}$)	Glucose equivalent (mg) (a)		Protein (mg) (b)	
	P-35 (e)	PA-13 (e)	P-35 (e)	PA-13 (e)
	(c)			
0	0.1204 ± 0.04	0.1454 ± 0.80	(d)	(d)
10	0.1697 ± 0.12	0.1672 ± 0.10	0.0580 ± 0.018	(d)
50	3.0325 ± 0.18	0.8710 ± 0.21	0.1516 ± 0.030	0.0345 ± 0.020
100	3.1629 ± 0.23	0.9880 ± 0.24	0.1581 ± 0.040	0.0494 ± 0.029

(a) total carbohydrate content in 1 ml of EP (prepared from 500 ml of incubation filtrate) was determined in EP aliquot as glucose equivalent by the phenol sulfuric acid technique.
(b) total protein content in 1 ml of EP (prepared from 500 ml of incubation filtrate) was determined in EP aliquot by the method of BRADFORD (1977).
(c) data are the mean results of 4 independent experiments with 2 replicates ± standard deviation
(d) protein concentration below limits of detection.
(e) P-35 : parental sensitive strain ; PA-13 : insensitive mutant.

TABLE 4 - Scoparone accumulation in wounded *Citrus* leaves by eliciting preparation (EP) from the fosetyl-Al sensitive isolate (P-35), or from the insensitive (PA-13) mutant of *Phytophthora citrophthora* (a).

Origin of EP	Total glucose equivalence (μg) in EP	μg scoparone in 1 g (f.wt.) aliquot of leaf tissue (b)	
		P-35 (c)	PA-13 (c)
Mycelium without fosetyl-Al	10	8.0 ± 0.28	8.3 ± 0.71
	20	10.0 ± 0.37	7.0 ± 0.25
	30	10.6 ± 0.33	12.8 ± 0.87
Mycelium with fosetyl-Al	10	53.0 ± 18.13	16.6 ± 0.82
	20	70.2 ± 22.61	15.4 ± 1.65
	30	71.4 ± 18.52	17.3 ± 3.89

(a) - twenty microliters of EP were applied on each wound.
(b) - scoparone produced after 4 days in 1 g (f.wt.) aliquots of leaf tissue (pooled from several leaves), as estimated by the method of AFEK and SZTEJNBERG (1988). Application of MES buffer on the wound (control without elicitor) produced 0.14 μg scoparone in 1 g of leaf tissue.
Data are the mean result of 4 independent experiments with 2 replicates ± standard deviation.
(c) - P-35 : fosetyl-Al sensitive strain ; PA-13 : insensitive mutant. Mycelium was incubated in MES buffer with or without fosetyl-Al (50 μg. ml^{-1}).

mechanisms (BOMPEIX *et al.*, 1980 ; GUEST, 1984 ; KHAN *et al.*, 1986 ; VERNENGHI *et al.*, 1987 ; SMILLIE *et al.*, 1989 ; SAINDRENAN *et al.*, 1988). Such resistance may be linked to the induction of osmiophilic substances and phytoalexins.

AFEK and SZTEJNBERG (1989) reported that the phytoalexin scoparone accumulated in *Citrus* plants inoculated with *P. citrophthora* in the presence of fosetyl-Al, thus providing a chemical marker for the induction of resistance. These authors suggested that fosetyl-Al may act in two ways : an increase of host defense mechanism at low concentrations, or a fungistatic effect of higher concentration.

We have studied two pathogenic *P. citrophthora* isolates (the fosetyl-Al sensitive P-35 strain and the fosetyl-Al insensitive mutant PA-13), and have shown that preinoculation of *Citrus* leaves floated on fosetyl-Al with the P-35 sensitive strain prevented subsequent infection by the PA-13 insensitive mutant (ALI, 1989 ; ALI *et al.*, 1989).

Our present study compared the effects of incubation in fosetyl-Al on the release of scoparone elicitors by mycelium of the sensitive P-35 strain, or the insensitive PA-13 mutant of *P. citrophthora*.

When incubated without fosetyl-Al, the mycelium of either fungi released little scoparone eliciting activity, and both were pathogenic towards *Citrus* leaves floated on water agar buffered with MES.

Upon incubation in fosetyl-Al (50 μg.ml^{-1}), mycelium of the sensitive P-35 strain, (unlike mycelium of PA-13 insensitive isolate) released eliciting activity leading to a significant accumulation of scoparone in *Citrus* leaf tissues. Accordingly, *Citrus* leaves floated on fosetyl-Al were resistant to strain P-35, but sensitive to isolate PA-13 (ALI *et al.*, 1988).

Our data are consistent with the hypothesis that scoparone accumulation in fosetyl-Al treated *Citrus* leaves is linked to their resistance to P-35 strain, while sensitivity to isolate PA-13 is associated to low scoparone induction. This does not preclude, however, that other mechanisms might also be involved in conferring resistance or pathogenicity, as scoparone was merely considered in our work as a marker of defence reaction.

Our overall results provide an explanation for the cross protection phenomenon we observed upon successive inoculation of the P-35 sensitive strain and the PA-13 insensitive mutant of *P. citrophthora* in *Citrus* leaves floated on fosetyl-Al. The hypothesis that such cross protection stems from the specific release of phytoalexin elicitors by the sensitive strain in the presence of fosetyl-Al is currently investigated further.

ACKNOWLEDGMENTS

M.K. ALI is indebted to the «Commissariat Général aux Relations Culturelles Internationales» of the «Communauté Française de Belgique» for research grants. We thank A.M. POLLART for able technical assistance, and Rhône-Poulenc C° for samples of technical grade Aliette ®.

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Reçu : août 1990
Accepté : février 1991

EL TRATAMIENTO DEL MICELIO DE *PHYTOPHTHORA CITROPHTHORA* CON FOSETIL-AL AMPLIFICA UNA ACTIVIDAD ESTIMULADORA DEL SCOPARONE MAS ELEVADA EN UN AISLADO SENSIBLE AL FOSETIL-AL QUE EN UN AISLADO TOLERANTE.

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Fruits, Jan.-Feb. 1991, vol. 46, n° 1, p. 51-55.

RESUMEN - El micelio de una cepa salvaje de *Phytophthora citrophthora* sensible *in vitro* al fosetil-Al (EC 50 = 56 µg. ml) y el de un mutante tolerante al fosetil-Al (EC 50 = 780 µg.ml.) fueron incubados en presencia de 50 µg.ml de fosetil-Al. La actividad estimulante del filtrado del medio de incubación fué evaluada con la medición de su capacidad para inducir la acumulación del SCOPARONE en las hojas del *Citrus jambhiri* LUSH. Nuestros resultados indican que después de la incubación en el fosetil-Al, el micelio de la cepa sensible desprende una mayor cantidad de estimuladores que el micelio de la cepa tolerante. La amplificación específica de estimuladores podría dar cuenta de nuestros resultados anteriores mostrando que la pre-inoculación de hojas de *Citrus* puestas sobre el fosetil-Al con la cepa sensible de *P. citrophthora*, los protege contra la infección subsecuente por el mutante tolerante.