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# IN VITRO SELECTION OF Phytophthora citrophthora ISOLATES RESISTANT TO PHOSPHOROUS ACID AND FOSETHYL-AL

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In vitro selection for resistance to fosetyl-Al (Aluminium tris-O-ethyl phosphonate, TEPA) and its main metabolite, phosphorous acid (PA), have been reported for different species of Phytophthora. After mutagenic treatment with N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), PA-resistant strains of P. palmivora (Dolan and Coffey, 1985) and P. capsici (Bower and Coffey, 1985) were recovered on a culture medium amended with PA.

The purpose of this paper is to describe the selection of P. citrophthora isolates resistant to PA or TEPA, and to characterize their properties in vitro or on detached Citrus leaves.

## MATERIAL AND METHODS

### In vitro sensitivity of Phytophthora citrophthora isolates towards PA and TEPA

Two single-zoospore isolates (P-35.1 and P-35.2) were derived from strain n° 289-35 of Phytophthora citrophthora R.E.&E.H. Smith, obtained from the "Centraalbureau voor Schimmelcultures", Baarn, The Netherlands.

For in vitro tests, PA or TEPA were added to corn meal agar (CMA) medium, and the final pH was adjusted to 6.2 with KOH before autoclaving. Agar disks (0.4 cm in diameter) taken from 5-day old colonies of P. citrophthora, were used as a source of inoculum. They were placed in the middle of agar plates, which were then incubated at 24°C. Linear radial growth was determined for each concentration of PA or TEPA (4 replicates) by measuring colony diameters at two perpendicular positions after 7 days of incubation. EC50 or EC90 values were calculated from linear regression analysis of the inhibition percentage of mycelial growth to the log concentration of the chemical.

### Characteristics of P. citrophthora isolates on detached citrus leaves

Leaves taken from rough lemon plants maintained in the greenhouse, were surface-sterilized for 15 min with 2 % NaClO, followed by two washes with sterilized distilled water. A disk (0.4 cm diameter) was excised from each surface-sterilized leaf, which was then floated, adaxial surface up, on a solution of PA or TEPA (buffered at pH 6.2 with 0.03 M MES (N-morpholonio ethane sulfonic

acid). After 2 h, the leaves were inoculated by placing a 0.4 cm diameter agar disk taken from 5-day old colonies of P. citrophthora at the site of the hole. After 5 days of incubation at 25°C under white fluorescent light with 16 h photoperiod, the diameter of each lesion was measured and the EC50 or EC90 values were calculated as described above.

**Mutagenesis and in vitro selection of P. citrophthora isolates resistant to PA or TEPA**

Zoospores of P. citrophthora were produced by the method of Harris (1986), and were treated with MNNG according to the method of Davidse (1981). The selection procedure was derived from the method used by Bower and Coffey (1985). Encysted zoospores ( $10^7$ in 10 ml) were exposed for 15 min to MNNG (30 ug/ml), and were then plated on 15 ml CMA selection medium containing TEPA or PA at EC90 (in terms of mycelial growth inhibition of the parental strain). The developing colonies were overlaid with a thin layer of selection medium and the fast growing colonies were then transferred once again on selection medium; isolates growing at a rate similar to that of the parental strain on unamended CMA were studied further.

Stability of the selected resistance was tested after 10 subcultures on CMA (devoid of TEPA or PA), either in terms of colony size on selection medium (amended with TEPA or PA at EC 90), or in terms of lesion size on leaves floated on TEPA or PA at EC50.

## RESULTS

While no PA-resistant or TEPA-resistant mutants were recovered in vitro by mass selection procedures in the absence of mutagenic treatment, a total of 24 PA-resistant isolates and 17 TEPA-resistant isolates of P. citrophthora were recovered, in separate experiments, when populations of zoospores were first mutagenised with MNNG (table 1). These 41 PA or TEPA-resistant isolates grew on EC90 culture medium (35 µg/ml of PA or 450 µg/ml TEPA) at the same speed as the parental strain on control medium devoid of PA or TEPA.

Three PA-resistant isolates and 2 TEPA-resistant isolates, when inoculated on Citrus leaves floated on EC 50 TEPA (13 µg/ml) or PA (5 µg/ml), developped lesions with the same size as those produced by the parental strain on leaves floated on control solution (MES at pH 6.2), while the 35 other isolates did not form lesions on TEPA or PA-treated leaves.

The EC50 values obtained with those 5 PA or TEPA-resistant pathogenic isolates of P. citrophthora, demonstrated cross resistance, as isolates selected on PA medium (PA-isolates) were TEPA-resistant, while the isolates selected on TEPA medium (F-isolates) were PA-resistant (table 2). Furthermore, the selected isolates arranged in a similar fashion in terms of resistance to either PA or TEPA. The resistance factors in vitro ranged from 10 to 19 for PA and from 8 to 14 for TEPA. Whatever the screening agent (PA or TEPA), selected pathogenic isolates were resistant to both PA and TEPA in the Citrus leaf test (table 3). Resistance

TABLE 1

SELECTION OF MUTAGENISED PHYTOPHTHORA CITROPHTHORA ISOLATES RESISTANT TO PHOSPOROUS ACID AND FOSETYL-AL ON CULTURE MEDIUM OR ON CITRUS DETACHED LEAVES

| Mutagen treatment | Screening agent | Number of treated zoospores /ml | Number of resistant isolates selected on medium with screening agent at EC90 a) | Number of resistant isolates on detached Citrus leaves floated on screening agent at EC50 b) |
|---|---|---|---|---|
| Nil | $H_3PO_3$ | $3.7 \times 10^7$ | nil | nil |
| | fosetyl-Al | $7.2 \times 10^6$ | nil | nil |
| c) MNNG | $H_3PO_3$ | $3.2 \times 10^7$ | 14 | 2 |
| | $H_3PO_3$ | $9.6 \times 10^6$ | 10 | 1 |
| | fosetyl-Al | $4.1 \times 10^7$ | 9 | 2 |
| | fosetyl-Al | $3.5 \times 10^7$ | 8 | nil |

a) Resistant isolates grew on medium with PA or TEPA at the same rate as the parental strain on control medium

b) Lesion diameter induced by resistant isolates increased on PA or TEPA treated leaves at the same rate as the lesions induced by the parental strain on leaves floated on control solution

c) MNNG: N-methyl-N'-nitro-N-nitrosoguanidine at 30 µg/ml

TABLE 2

EC50 VALUES OF MYCELIAL GROWTH OF PHYTOPHTHORA CITROPHTHORA ISOLATES RESISTANT TO EITHER $H_3PO_3$ OR FOSETYL-AL

| | $H_3PO_3$ | | fosetyl-Al | |
|---|---|---|---|---|
| a) Isolates | b) EC50 (µg/ml) | c) R factor | b) EC50 (µg/ml) | c) R factor |
| P-35.1 P-35.2 | 6.5 | 1.0 | 56.5 | 1.0 |
| PA-25 | 70.6 | 10.8 | 442.9 | 7.9 |
| PA-27 | 82.1 | 12.6 | 515.0 | 9.1 |
| PA-13 | 125.3 | 19.2 | 785.5 | 14.1 |
| F-19 | 74.5 | 11.4 | 461.9 | 8.2 |
| F-15 | 85.4 | 13.1 | 529.4 | 9.4 |

a) P-35.1 and P-35.2: parental strain. PA and F: resistant isolates selected on medium containing $H_3PO_3$ or fosetyl-Al, respectively.

b) EC50 values calculated from regression lines of the percentage of inhibition of radial mycelial growth on culture medium against the log of $H_3PO_3$ or fosetyl-Al concentration.

c) Resistance factor (R) calculated as the ratio between the EC50 of the resistant isolate and the mean EC50 of the two single spore parental isolates (P-35.1 and P.35.2).

TABLE 3

EC50 VALUES OF LESION SIZE ON CITRUS LEAVES OF PHYTOPHTHORA CITROPHTHORA ISOLATES RESISTANT TO EITHER $H_3PO_3$ OR FOSETYL-AL

| a) Isolates | $H_3PO_3$ | | fosetyl-Al | |
|---|---|---|---|---|
| | b) EC50 (µg/ml) | c) R factor | b) EC50 (µg/ml) | c) R factor |
| P-35.1 P-35.2 | 5.6 | 1.0 | 11.8 | 1.0 |
| PA-25 | 32.5 | 5.8 | 46.6 | 4.0 |
| PA-27 | 37.8 | 6.7 | 54.4 | 4.6 |
| PA-13 | 57.8 | 10.3 | 81.8 | 7.0 |
| F-19 | 34.3 | 6.1 | 49.3 | 4.2 |
| F-15 | 39.4 | 7.0 | 56.6 | 4.8 |

a)
P-35.1 and P.35.2: parental strains; PA and F: resistant isolates selected on CMV containing $H_3PO_3$ or fosetyl-Al, respectively.

b)
EC50 values calculated from regression lines of the percentage of inhibition of lesion size against the log of $H_3PO_3$ or fosetyl-Al concentration.

c)
Resistance factor (R) calculated as the ratio between the EC50 of the resistant isolate and the mean EC50 of the two single-spore parental isolates (P-35.1 and P.35.2).

TABLE 4

STABILITY OF RESISTANCE TO $H_3PO_3$ OR FOSETYL-AL AFTER 10 SUBCULTURES ON MEDIUM DEVOID OF $H_3PO_3$ OR FOSETYL-AL

| a) Isolates | Mean diameter (mm) on CMA | | | | Mean size of lesions (mm) on Citrus leaves | | | |
|---|---|---|---|---|---|---|---|---|
| | $H_3PO_3$ (EC90:30µg/ml) | | fosetyl-Al (EC90:420µg/ml) | | $H_3PO_3$ (EC50:6µg/ml) | | fosetyl-Al (EC50:15 µg/ml) | |
| | b) Sub 0 | b) Sub 10 | Sub 0 | Sub 10 | Sub 0 | Sub 10 | Sub 0 | Sub 10 |
| P-35.1 | 6 | 6 | 5 | 5 | 10 | 10 | 11 | 10 |
| P-35.2 | 5 | 6 | 6 | 5 | 13 | 12 | 13 | 12 |
| PA-25 | 48 | 29 | 46 | 25 | 25 | 15 | 28 | 18 |
| PA-27 | 45 | 45 | 48 | 46 | 29 | 27 | 27 | 28 |
| PA-13 | 58 | 59 | 58 | 58 | 32 | 34 | 31 | 31 |
| F-19 | 46 | 47 | 46 | 46 | 29 | 27 | 29 | 29 |
| F-15 | 48 | 46 | 47 | 47 | 27 | 28 | 27 | 29 |

a) P-35.1 and P-35.2: parental strains. PA and F resistant isolates selected on CMA containing $H_3PO_3$ or fosetyl-Al, respectively.

b) Sub 0: isolate tested immediately after isolation from selection medium with $H_3PO_3$ or fosetyl-Al.
Sub 10: isolate tested after 10 subcultures on control medium.

factors in the leaf test ranged from 5.8 to 10.3 for PA, and from 4 to 7 for TEPA. The pathogenic selected isolates arranged in a similar fashion for resistance to PA or TEPA, and this arrangement was parallel to that obtained in the mycelial growth test on CMA (table 2 and table 3). Four of the 5 pathogenic PA and TEPA-resistant isolates of P. citrophthora maintained their resistance after 10 single-zoospore subcultures (table 4).

## DISCUSSION

The mode of action of the systemic antifungal agent fosetyl-Al is a yet uncertain. Early studies detected only a low in vitro antifungal activity against fungi which were readily controlled in the field, and so headed at a possible action of TEPA by stimulating the defense mechanisms of the treated inoculated host (Bompeix et al., 1981). However, it is now established that PA (the main metabolite of TEPA) at low concentration inhibits mycelial growth of many Phytophthora species on a low phosphate medium (Fenn and Coffey, 1984). The ability to develop PA-resistant fungal strains in vitro could be of significance interpreting the mode of action of PA and fosetyl-Al in treated plants.

PA-resistant mutants of P. capsici (Bower and Coffey, 1985) and P. palmivora (Dolan and Coffey, 1985), selected in vitro after chemical mutagenesis with MNNG, were infectious to PA or TEPA-treated plants.

Our present study shows that PA or TEPA-resistant isolates of P. citrophthora were obtained only after mutagen treatment of zoospores. Moreover, whatever the screening agent (PA or TEPA) used in vitro during the selection procedure, a close parallel effect of PA and TEPA was observed on mycelial growth in vitro or on lesion size on Citrus leaves.

This good correlation between in vitro and in vivo resistance to PA or TEPA treatments, suggests that PA and TEPA have a similar mode of action, by acting directly on P. citrophthora, as previously reported for other Phytophthora species (Fenn and Coffey, 1984).

However, the resistance factors calculated for PA or TEPA on the basis of inhibition of the radial growth on culture medium in vitro, were higher than those calculated in terms of inhibition of lesions size on Citrus leaves, and this difference might reflect a stimulation of the host defense mechanisms.

Indeed, with Ridomil, a fungicide known for its direct toxicity towards P. citrophthora, the resistance factors relating to in vitro inhibition of mycelial growth were similar to those obtained on the basis of lesion size, using the Citrus leaf test (Van Cauwelaert, 1986), thus indicating a direct effect on the fungus.

The strongest evidence presented to date that an antifungal compound can act by stimulating host defense mechanisms, is the reversion of the inhibition of disease development by inhibitors of plant resistance metabolism. Early studies reported that aminooxyacetic acid (AOA) and glyphosate, presumptive inhibitors of phenylpropanoïd synthesis, reversed the antifungal activity of TEPA in tomato leaflets infected with P. capsici (Bompeix et al., 1981). However, further studies with the tomato-P. capsici host-parasite system established that the toxicity of suboptimal concentration of PA was not reversed by glyphosate, whereas AOA could inhibit PA uptake into the fungus.

The effets of AOA and glyphosate remain to be assessed with the Citrus-P. citrophthora system, but our overall results so far support the hypothesis of Bompeix and Saindreman (1984), suggesting that the mode of action of TEPA combines both a direct toxicity effect towards the pathogen, and the triggering of host defense mechanisms in the host.

The in vitro selection of P. citrophthora isolates resistant to both $H_3PO_3$ and fosetyl-Al does not imply a risk of such selection in the field. Indeed, we failed to obtain TEPA-resistant isolates by mass selection of non-mutagenised zoospores. Moreover, most TEPA-resistant isolates of P. citrophthora were non pathogenic on detached TEPA-treated Citrus leaves. The competitive fitness and the pathogenicity of infectious TEPA-resistant isolates of P. citrophtora remain to be assessed on TEPA-treated Citrus plant.

## REFERENCES


Bompeix, G., Ravisé, A., Raymal, G., Fettouche, F. et Durand, M., 1980. Modalités de l'obtention des nécroses bloquantes sur feuilles détachées de tomate par l'action du tris-o-éthyl phosphonate d'aluminium (phoséthyl d'aluminium), hypothèses sur son mode d'action in vivo. Ann. Phytopathol. 12, 337-351.

Bompeix, G., Fettouche, F. et Saindreman, P., 1981. Mode d'action du phosethyl-Al. Phytiatrie-Phytopharmacie 30, 257-272.

Bompeix, G. and Saindreman, P., 1984. in vitro antifungal activity of fosetyl-Al and phosphorous acid on Phytophthora species. Fruits 39, 777-786.

Bower, L. and Coffey, M., 1985. Development of tolerance to phosphorous acid, fosetyl-Al and metalaxyl in Phytophthora capsici. Can. J. Plant Pathol. 7, 1-6.

Cohen, Y. and Coffey, M., 1986. Systemic fungicides and the control of Oomycetes. Ann. Rev. Phytopathol. 24, 311-338.

Davidse, L., 1981. Resistance to acylalanine fungicides in Phytophthora megasperma f. sp. medicaginis. Neth. J. Plant Pathol. 87, 11-24.

Dolan, T. and Coffey, M., 1985. In vitro and in vivo assessment of Phytophthora palmivora strains resistant to phosphorous acid. Phytopathology 75, 1330 (Abst.).

Fenn, M. and Coffey, M., 1984. Studies on the in vitro and in vivo antifungal activity of fosetyl-Al and phosphorous acid. Phytopathology 74, 606-611.

Harris, D., 1986. Methods for preparing, estimating and dilution suspensions of Phytophthora cactorum zoospores. Trans. Br. Mycol. Soc. 86, 482-486.

Joseph, M. and Coffey, M., 1984. Development of laboratory resistance to metalaxyl in Phytophthora citricola. Phytopathology 74, 1411-1414.

Van Cauwelaert, T. 1986. Essai d'obtention de souches résistantes de Phytophthora citrophthora (Smith & Smith) Leonian et de Phytophthora cactorum (Libert & Cohn) Schoter vis-à-vis de l'Aliette et du Ridomil. Travail de fin d'Etudes, Faculté des Sciences agronomiques de l'Etat, Gembloux, Belgique.