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Interaction of Partially Purified Phytotoxins from *Phytophthora cactorum* on Apple Cell Plasma Membrane

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With 2 figures

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Abstract

The 'crude' filtrate (CF) of *Phytophthora cactorum* containing phytotoxin(s), having some properties similar to the toxins isolated from other *Phytophthora* species, was processed by three steps (acetone precipitation, dialysis and gel filtration chromatography). The CF fractions corresponding to the progressive steps of purification were tested for phytotoxicity on tomato seedlings and for activity on cell trans-membrane electrical potential (Em) of susceptible and resistant apple rootstocks (*Malus domestica*).

The fractions (F4), obtained from chromatography on Sephadex G50 fine and eluted in the zone corresponding to a molecular weight of 15 ± 2 kD, induced a specific alteration on susceptible apple cell membranes. These metabolites, even though incompletely purified, are able to induce a high and specific activity on susceptible apple cell Em only, not on resistant ones. As a consequence, they may be of potential use in screening for insensitive cells.

Zusammenfassung

Einfluß von Partiell Geläuterten Phytotoxinen von *Phytophthora cactorum* auf Plasmamembranen von Apfelzellen

In Rohfiltraten (CF) von *Phytophthora cactorum* wurden Phytotoxine mit ähnlichen Eigenschaften von Toxinen anderer *Phytophthora*-Arten nachgewiesen. Die Filtrate wurden in einem Drei-Phasen-Prozeß fraktioniert (Azentonniederschlag, Dialyse und Gelfiltration) und bezüglich Phytotoxizität an Tomatensämlingen und Beeinflussung des elektrischen Potentials (Em) auf Zellmembranen von anfälligen und resistenten Apfelwurzelstöcken (*Malus domesticus*) geprüft.

Die Gelfitrationsfraktion (F4) mit einem Molekulargewicht von 15 ± 2 kD führte zu einer

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spezifischen Veränderung bei Zellmembranen der anfälligen Apfelvarietät. Die in der Fraktion enthaltenen *P. cactorum*-Metaboliten induzieren nur bei Zellmembranen von anfälligen, nicht aber von resistenten, Apfelvarietäten eine heftige Veränderung des elektrischen Potentials. Sie könnten deshalb auch zur Selektion von resistenten Apfelvarietäten verwendet werden.

Pathogen produced metabolites and their site or mechanism of action at the host-cell level are important to develop new efficient *in vitro* screening methods for plant resistance.

Phytophthora spp. are known for the production of phytotoxic compounds (BALLIO *et al.* 1972, PAXTON 1972, RONNEBECK 1956, CSINOS and HENDRIX 1977, PLICH and RUDNICKI 1979, BEHNKE 1979). Mycolaminaran, a ß–1–3 glucan extracted from mycelia of *Phytophthora cinnamoni*, was studied for its capacity to induce wilting in different plants (KEEN *et al.* 1974). High quantities of this compound were shown to be present in the cytoplasm of various *Phytophthora* spp. (WANG and BARTINICKI-GARCIA 1974).

Elicitors of necrosis on excized leaves of tobacco have been isolated from the culture filtrates of incompatible species *P. cryptogea, P. cinnamoni* and *P. capsici* (BILLARD *et al.* 1988), and from *P. parasitica* var. *nicotianae* (RICCI *et al.* 1992). These factors, identified as proteins of about 10 kD, have been named cryptogein, cinnamomin, capsicein and parasiticein, respectively. The biological activities of pure cryptogein and capsicein have been examined on tobacco cells. They cause a rapid increase in pH and conductivity of the extracellular medium, and induce ethylene and phytoalexin production. These features are typically characteristic of the hypersensitive reaction (RICCI *et al.* 1992, BLEIN *et al.* 1991). In addition, it has been shown that these elicitors protect the plant against invasion caused by *Phytophthora nicotianae*, a tobacco-compatible pathogen, causal agent of tobacco black shank and unable to produce such elicitors (RICCI *et al.* 1992, RICCI *et al.* 1989, BONNET *et al.* 1986).

Several workers have studied the possibility that some or all the symptoms produced by compatible *Phytophthora* spp. are caused by toxins. CSINOS and HENDRIX (1977) demonstrated that toxins produced by *P. cryptogea* cause symptom development on excized tobacco leaves. Phytotoxic compounds from *P. citrophthora* were able to induce symptom development on lemon seedling (host) but not on tomato (non-host) (BREIMAN and BARASH 1981).

Only a few studies on crown rot of apple have reported the biochemical mechanism of *Phytophthora cactorum* action, besides symptom description (PLICH and RUDNICKI 1979, BREIMAN and BARASH 1981, MEZZETTI *et al.* 1992). Strains of this fungus, isolated from infected apple plants and grown *in vitro*, produced toxic compounds that induced browning of veins and margins, desiccation of apple leaves and wilting of tomato cuttings (PLICH and RUDICKI 1979). It has been suggested that these phenomena can be caused by relatively low molecular weight molecules transferred acropetally, probably through the xylem and then accumulated in the leaves that rapidly desiccate (PLICH and RUDNICKI 1979).

From the chemical analyses of these compounds, a similarity with the toxins studied from other *Phytophthorae* spp. emerged: highly hydrophilic in nature, low solubility in organic solvents, thermostable at 70°C in a wide pH range (CRISTINZIO *et al.* 1992).

In this study we compared the properties of 'crude' and partially purified *P. cactorum* CF on cell trans-membrane electrical potential (Em) of susceptible and resistant apple rootstocks and on toxicity to tomato seedlings. The aim of our work was to: 1) provide additional evidence for the role of a toxic metabolite, produced by *P. cactorum*, in disease development and 2) produce a more purified toxic fraction that would be a more suitable selective agent for *in vitro* selection studies.

Materials and Methods

Culture filtrate (CF) preparation

Phytophthora cactorum (Leb. et Chon) Schroet. strain P39 (highly virulent, isolated from M.106), obtained from Dr H.S. ALDWINCKLE, NYSAES, Geneva, USA, was grown on a solid medium (Difco Bacto-agar 15 g/l, Campbell V8 juice 200 g/l and $CaCO_3$ 3 g/l) for 10 days in darkness at 25°C. For liquid inoculum preparation, fungal culture and filtration methods described by MEZZETTI *et al.* (1992) were used. Filtrates so prepared were freeze-dried and stored at -20°C.

CF partial purification

Acetone precipitation

The CF (300 ml) was mixed with acetone (1:2 v/v) at room temperature and allowed to precipitate overnight. The precipitates (P) and the supernatant (SN), each dried by evaporation under reduced pressure at 40°C, were redissolved up to the initial volume in H_2O. Aliquots of these fractions were bioassayed before further purification of the fraction inducing tomato wilting (SN).

SN dialysis

SN was dialyzed using a Serva spectra/por tube (Serva, 3MWCO-3500 D, 28.6 mm) bathed against 5 volumes of H_2O for 12 h and for 3 more baths of 2 h each. Both the permeable (SNPR) and the non-permeable (SNNPR) fractions were concentrated by evaporation under reduced pressure and redissolved up to their initial volume in H_2O. Both fractions were bioassayed and the fraction inducing tomato wilting (SNNPR) was used for the further purification step.

SNNPR gel filtration

An aliquot (10 ml) of SNNPR was applied to a Sephadex G-50 fine column (Pharmacia LKB Biotechnology, Uppsala, Sweden, 105 × 1.7 cm) that was eluted with double-distilled water at a flow rate of 22 ml/h in fractions of 2 ml. The fractions were monitored for UV absorption at 208 and 252 nm, because they were the wavelengths corresponding to the maximum absorption of the SNNPR submitted to gel filtration chromatography, and for phytotoxicity on tomato seedlings.

Plant bioassay

A tomato seedling bioassay was first used to test the phytotoxicity of the fractions obtained from the different purification steps. Epicotyls of well-developed seedlings, cv. 'Davis', were maintained overnight (at 22° ± 2°C) with the base of the stem immersed in a tube containing 1 ml of CF or fractions P, SN, SNPR and SNNPR. Phytotoxic activity was scored from 0 (no toxic) to 4 (highly toxic) on the basis of leaf wilting and necrosis compared to distilled water.

The 'crude' CF and the CF fractions corresponding to the above described purification steps were also tested for the ability to induce Em changes in cells from susceptible and resistant apple rootstocks. Shoot proliferation, cell isolation, staining and fluorescence measurements were carried out following the protocols developed by MEZZETTI *et al.* (1992). Briefly, mesophyll cells extracted from *in vitro* proliferating shoots of M.106 (susceptible to *P. cactorum*) and M.111 (resistant) were used. Cell suspensions of both rootstocks were stained with merocyanine 540 (MC-540, Eastman Kodak, Rochester, NY, USA), a specific fluorochrome able to detect changes in cell plasma membrane Em (DIMOND 1972, WAGGONER 1979, SIEBER 1987), and incubated for 24 h with the different fractions. Changes in cell fluorescence emission were monitored with a Zeiss Photomicroscope III equipped with epi-fluorescence and containing an HBO 100 mercury source for excitation, a BP 546/10 exciter filter and an LP590 barrier filter. Fluorescence emission was measured with an Hamamatzu photomultiplier tube (type R777) (HAMMERSCHLAG 1984).

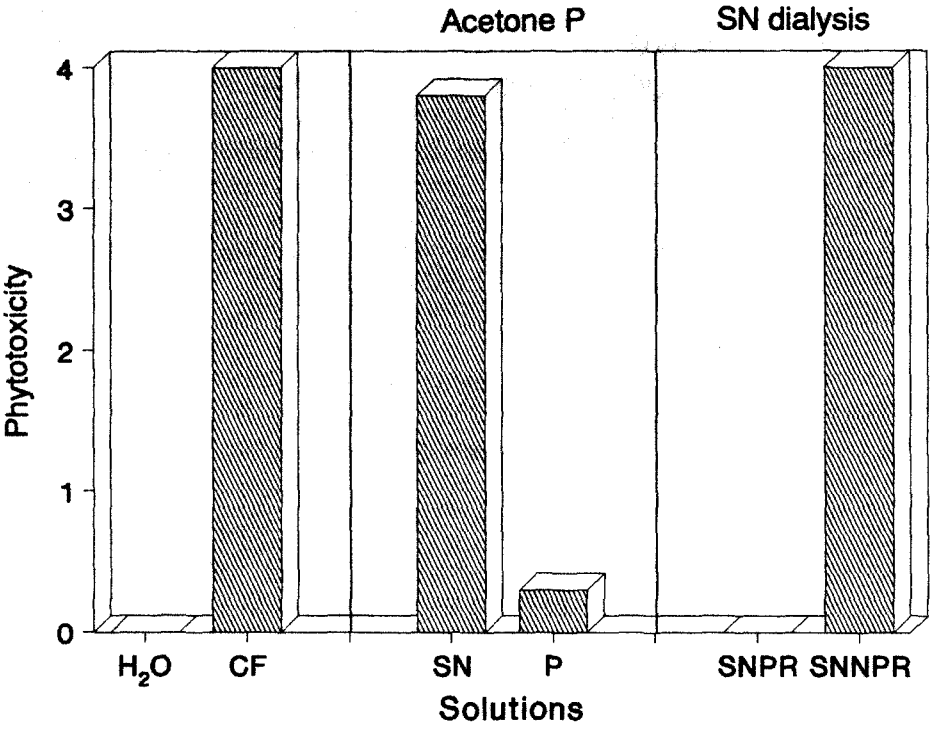

Fig. 1. Tomato seedling response to the 'crude' filtrate (CF) of *Phytophthora cactorum* and its partially purified fractions: SN = acetone supernatant, P = acetone precipitate; SNPR = permeable solutions of the supernatant, and SNNPR = non-permeable solution of the supernatant. Phytotoxic rating: 0 = no toxic, 4 = highly toxic.

All data are expressed as percentage of fluorescence emission of the treated cells compared with control cells maintained in half-strength MURASHIGE and SKOOG (1962) salts supplemented with 0.45 M sucrose.

Results

CF partial purification and tomato seedling bioassay

Tomato seedlings were highly sensitive to the 'crude' filtrate (CF) and to the supernatant (SN) fraction following acetone precipitation, with the latter inducing a complete wilting of the seedlings at the end of the incubation period (Fig. 1).

The phytotoxic compound(s) was non-permeable through the dialysis tube used (3500 D). In fact, high toxicity on seedlings was shown by the non-permeable SNNPR fraction only, while no toxicity was shown by the permeable SNPR fraction.

About 80 (2 ml each) fractions originated from the subsequent gel filtration of the SNNPR were collected in seven homogenous groups (F1–F7) for UV absorption and toxic activity studies (Fig. 2). The F4 group, including the elution volumes from 62 to 92 ml, corresponding to the molecular weight of about 15 ± 2 kD, estimated approximately by combining the cut-off of the dialysis tube (3500 D) and the elution volume of the phytotoxic fractions (92 ml), showed the highest UV absorption at 208 nm and produced the highest toxic effect on tomato seedlings. A lower toxicity was shown by the F2 group including the elution volumes from 14 to 38 ml with a smaller peak always at 208 nm.

Activity of CF, SN, P, SNPR and SNNPR on apple cell membranes

Using the MC-540 optical probe method, both CF and SN were observed to induce an initial fluorescence increase followed by a continuous decrease in M.106 cells (Table I). Changes in fluorescence were not observed in M.106 cells exposed to the P fraction.

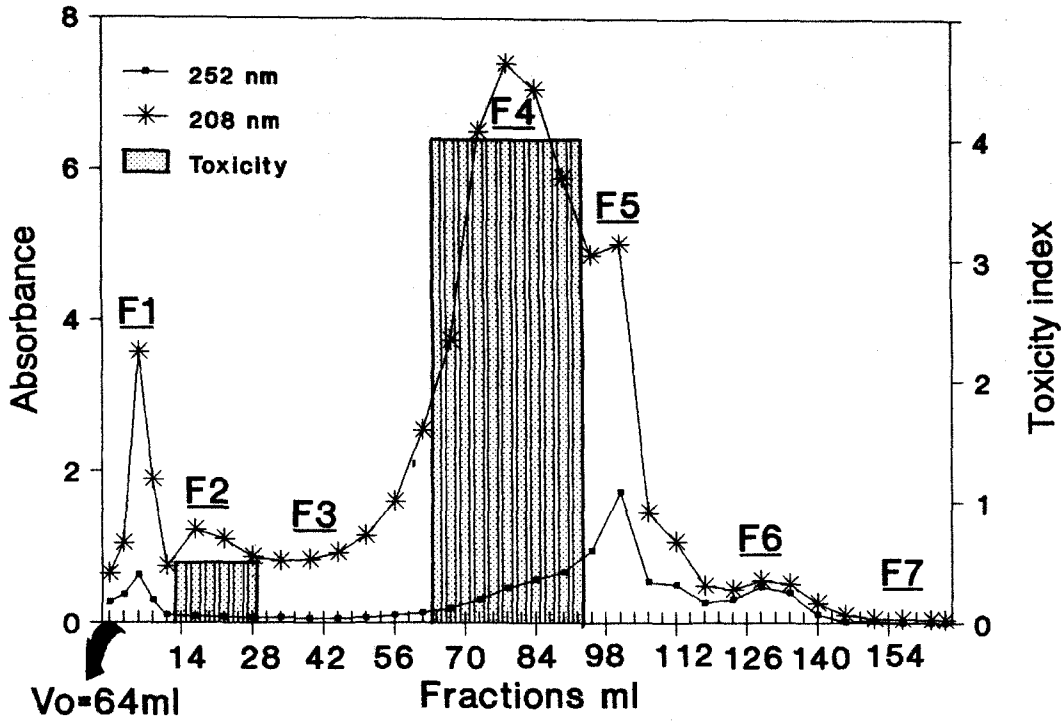

Fig. 2. Gel filtration chromatography of the SNNPR-solution of *Phytophthora cactorum* filtrates. The bars show the different phytotoxicity levels of the group of fractions detected with the tomato seedlings bioassay. Phytotoxic rating: 0 = no toxic, 4 = highly toxic.

Table 1

Fluorescence emission of M.106 (susceptible) cells after incubation with 'crude' and partially purified CF of a *Phytophthora cactorum* virulent strain (P39). Twenty-five cells per treatment were measured with four replications (% of control ± SE)

Treatment	5min	3h	24h
CF—P39	119.6±10.1	83.0±6.5	63.7±8.3
CF—P39 SN	117.0±6.5	89.0±7.5	70.3±9.8
CF—P39 P	104.3±5.0	98.6±3.8	92.6±5.8
CF—P39 SNPR	121.3±12.7	106.3±3.5	98.6±3.2
CF—P39 SNNPR	95.3±11.0	77.6±4.0	69.0±4.3

SN = acetone supernatant; P = acetone precipitate; SNPR = permeable fraction of the supernatant; SNNPR = non-permeable fraction of the supernatant.

The SNPR fraction induced an initial increase in fluorescence, but during incubation fluorescence intensity stabilized at the control level (Table I). In contrast, the SNNPR metabolite(s) induced an immediate and continuous decrease in fluorescence (increase in negative plasma membrane Em) (Table I).

Changes in fluorescence were not observed in M.111 (resistant apple rootstock) cells exposed either to CF or to the CF solutions obtained following acetone precipitation and dialysis (Table 2).

With M.106 cells, chromatographic fractions F2 (low toxicity) and F3 (no toxicity) did not induce any change in fluorescence compared with untreated cells (Table 3). The F4

Table 2
Fluorescence emission of M.111 (resistant) cells after incubation with 'crude' and partially purified CF of a *Phytophthora cactorum* virulent strain (P39). Twenty-five cells per treatment were measured with four replications (% of control ± SE)

Treatment	5 min	3 h	24 h
FC—P39	100.6±5.0	95.6±1.5	96.0±7.6
FC—P39 SN	99.6±4.9	100.0±10.3	97.6±5.8
FC—P39 SNNPR	97.3±10.0	102.6±4.5	96.6±7.2

SN = acetone supernatant; SNNPR = non-permeable fraction of the supernatant.

Table 3
Fluorescence emission of M.106 (susceptible) cells after incubation with culture filtrate (CF) fractions obtained by gel filtration of the non-permeable solution of the acetone supernatant. Twenty-five cells per treatment were measured with four replications (% of control ± SE)

Treatment	5 min	3 h	24 h
FC—P39	119.6±10.0	83.0±6.5	63.6±8.3
FC—P39 SNNPR (F2)	98.3±2.3	101.0±2.6	101.6±2.8
FC—P39 SNNPR (F3)	99.6±9.1	102.0±6.9	101.0±5.6
FC—P39 SNNPR (F4)	88.0±7.9	83.0±6.1	70.3±2.3

SNNPR = non-permeable solution of the acetone supernatant; F2 = slightly toxic; F3 = not toxic; F4 = highly toxic.

solution (highly toxic) induced an immediate and continual decrease in fluorescence (5 min = 88 % ± 7.9; 3 h = 83 % ± 6.1; 24 h = 70.3 % ± 4.3), similar to that induced by SNNPR.

Discussion

Phytophthora cactorum 'crude' filtrate (CF) was able to induce tomato seedling wilting and change of susceptible apple plasma membrane Em. These alterations have been induced by different compounds released in the CF by the fungus.

The 'crude' CF and SN fraction produced an immediate decrease in negative plasma membrane Em (= increase of fluorescence) followed by a continuous increase in negative plasma membrane Em (= decrease of fluorescence) of M.106 susceptible cells. These membrane alterations were induced by different CF metabolites. In fact, cells incubated with SNPR showed only an initial increase in fluorescence, whereas cells incubated with SNNPR showed a continuous decrease. Comparing these results with those obtained from the phytotoxicity assay on tomato seedlings, it can be suggested that the SNPR fraction contains secondary metabolites not involved in fungal phytotoxicity. In contrast, the continuous decrease in fluorescence caused by the highly phytotoxic SNNPR fraction suggests the presence of such metabolite(s) with a specific capacity to induce an increase in negative plasma membrane Em, probably with a primary role in disease development. This hypothesis is supported further by the fact that cells of the resistant rootstock M.111 were not affected by the CF fractions toxic to M.106 cells.

Subsequent gel chromatography fractionation allowed further purification of the CF. Fractions F1–F7 were characterized by a different toxicity on tomato seedlings and on apple

cell membranes. Solution F4 was able to induce wilting and fluorescence changes similar to that observed with the SNNPR fraction. The weakly phytotoxic F2 and the strongly phytotoxic fraction F4 showed hydrophilic and polymeric properties; the first showed an apparent molecular weight between 30 and 15 ± 2 kD, the second at 15 ± 2 kD. Moreover these active fractions were thermo- and pH stable. At this purification grade, it is not possible to attribute to them a definite chemical nature.

Cell plasma membrane alteration produced by these toxic fractions was similar to the alteration induced on the same cells by valinomycin (MEZZETTI *et al.* 1992). This ionophore molecule is known to affect the plasmalemma K^+ permeability inducing an increase in negative plasma membrane Em followed by a stimulation of ATPase activity (LEV and BURZHINSKY 1967, CHANGE 1977).

Further characterization of the chemical nature of these toxic compounds is necessary for a more complete determination of the mechanism that induces changes of membrane potential, and whether the plasma membrane alone is affected or other organelle are also affected as observed with some phytotoxins (HOLDEN and SZE 1989).

Crude culture filtrates have been used successfully in some *in vitro* selection studies (SACRISTAN 1982, HARTMAN *et al.* 1984, SHOHET and STRANGE 1989), but crude culture filtrate of another *Phytophthora* species (*P.citrophthora*) proved to be an ineffective selective agent because of the presence of indole-3-acetic acid in the filtrate which stimulated cell growth (VARDI *et al.* 1986).

Although the phytotoxic metabolites of the *P. cactorum* CF are incompletely purified, the partially purified toxic fraction can be considered a very efficient selective agent for *in vitro* early selection and studies (DAUB 1986).

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