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Nucellar callus of 'Femminello' lemon, selected for tolerance to *Phoma tracheiphila* toxin, shows enhanced release of chitinase and glucanase into the culture medium

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Abstract. *Phoma tracheiphila* is the causative agent of the disease mal secco. Citrus cultivars differ substantially in respect to their sensitivity to the pathogen *P. tracheiphila* and its toxin. Some cultivars (e.g., 'Femminello' lemon) are inherently sensitive while others (e.g., 'Tarocco' orange) are tolerant. Cell lines derived from nucellar tissue of 'Femminello', 'Tarocco' and a cell line selected for tolerance to the fungal toxin ('Femminello-S') were used to study host–pathogen interaction. Our results showed that calli or conditioned media of 'Tarocco' and 'Femminello-S' inhibited the size of co-cultivated fungal colonies when compared to 'Femminello'. In addition, conditioned medium of 'Tarocco' as well as 'Femminello-S', but not 'Femminello', promoted bursting of hyphal tips. A ten-fold increase in chitinase and glucanase enzymatic activity, as evaluated by radiometric assay and laminarin hydrolysis respectively, was detected in 'Femminello-S' extracellular extracts as compared to 'Femminello'. An increase in chitinase was also shown by immunoblot analysis. Our findings suggest a positive correlation between the presence of chitinase and glucanase in the conditioned media of the cultured cells and the tolerance of those cells to *P. tracheiphila* toxin.

Key words: Pathogenesis-related proteins (PR) – Glucanase – Chitinase – Plant cell culture – Disease tolerance

Introduction

Phoma tracheiphila (Petri) Kanc. et Ghik is a pathogen that causes a serious tracheomycotic disease of citrus trees known as mal secco. So-far no genetic source of tolerance to mal secco has been found among commercial lemon varieties (Perrotta and Tribulato 1977). However, some other citrus species are tolerant e.g., orange, mandarin, grapefruit. The use of classical genetic breeding to transfer this trait is difficult, due to the high polyembryonic characteristic of lemon and the other species, which leads to a high percentage of parental types in the F_1 population. In addition, the zygotes from such crosses will not easily produce hybrids of high commercial values. An alternative approach is in-vitro selection for resistance at the cellular level. Recovery of disease-resistant plants by in-vitro selection for resistance against toxic chemicals, and toxins or culture filtrates produced by plant pathogens, has been reported for various cell cultures (for review see Daub 1986; Galun and Breiman 1992). Nachmias et al. (1977) have isolated a partially-purified toxin (PPT) from *P. tracheiphila.*

Using tissue culture techniques, i.e., exposure of the sensitive 'Femminello' lemon cells to PPT, a cell line was established that was tolerant to the *P. tracheiphila* toxin and labelled 'Femminello-S' (Gentile et al. 1992b). The aim of the present work was to attain a better understanding of the mechanism involved in in-vitro selection by testing the two 'Femminello' cell lines (non-selected versus selected) as compared to cell lines derived from cultivars with known in-vivo tolerance to the mal secco disease. Unexpectedly, our preliminary experiments on co-cultivated fungal and plant cells showed that cells derived from the tolerant lines actually inhibited fungal growth. We therefore used three approaches to study the plant-pathogen interaction. In the first approach different combinations of co-cultivated lines were examined similarly to the dual culture experiments performed by Storti et al. (1988) in tomato cell lines resistant to *Fusarium oxysporum* f. sp. *lycopersici*. Secondly, we examined fungal growth in the presence of conditioned media ob-

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tained from cultured citrus cells. Thirdly, we examined extracellular proteins from the conditioned media of the cell-lines by immunoblot analysis using antisera specific for acidic endochitinase and for (1-3)-β-glucanase. Our findings suggest a positive correlation between the extrusion of chitinase and glucanase from the cultured cells into their respective media and the response of those cells to the *P. tracheiphila* toxin.

Materials and methods

Origin and maintenance of nucellar calli

Embryogenic calli of nucellar origin were derived from orange, *Citrus sinensis* (L.) Osb. ('Tarocco'), and from lemon, *Citrus limon* (L.) Burm. f. cv 'Femminello Continella' ('Femminello'), as described in Vardi et al. (1982). The nucellar calli were subcultured every 4 weeks on Murashige and Tucker (1969) medium containing 4% sucrose, solidified with 1% Difco agar (MT). The in-vitro selection of the 'Femminello-S' line has been described elsewhere (Gentile et al. 1992b).

Dual culture

Dual culture experiments were carried out according to Storti et al. (1988) with some modifications. Two equal pieces of calli were placed oppositely and close to the periphery of a 5-cm Petri dish containing solidified MT medium. After 10, 20 and 30 days of culture in a growth chamber (27°±1 °C with a 16 h dim-light photoperiod) a small disk of *P. tracheiphila* mycelium [isolate "80(+)"; Pennisi et al. 1988] was placed in the center of each plate between the two callus pieces. Ten days later the fungal growth was monitored. Five plates were employed for each time and cell line replication.

Conditioned media

Suspension cultures were obtained by subculturing 200 mg of callus in 100 ml flasks containing 20 ml of liquid MT medium. The flasks were then horizontally shaken at 100 rpm. Thirty days from the beginning of culture, the liquid from individual flasks was centrifuged (1000 g, 5 min), the supernatants were collected and mixed with equal volumes of MT containing 1.8% agar. The mixed media from each of the flasks were then plated: 5 ml per 5-cm Petri dish. A small disk of *P. tracheiphila* mycelium was placed at the center of each dish. Five Petri dishes were employed for each cell-line. The growth of fungal colonies (cm^2) was evaluated after 10 days of incubation.

Extraction of proteins, polyacrylamide-gel electrophoresis and immunoblotting

Conditioned media were separated from suspension cultures as described by Gavish et al. (1992). Immunoblots were prepared essentially as described by Gavish et al. (1991) and modified by the "Enhanced chemiluminescence detection system" (Amersham) and autoradiographed.

Assay for chitinase and glucanase

Chitinase activity was measured by using the radiometric assay described by Boller et al. (1983). The activity of (1–3)-β-glucanase was measured according to Abeles et al. (1970).

Assay of antifungal activity

Microscope glass-slides were coated with a thin layer of carrot agar medium. A small disk (2 mm) of fungal mycelium was placed on the center of each slide. The slides were then placed above wet filter paper in 9-cm Petri dishes and the dishes were sealed with 'Parafilm'. The dishes were incubated (27°±1 °C, 16 h dim light) until the fungal colony reached a diameter of about 2 cm. Antifungal activity was tested by applying the protein-extract solution from the cell-suspension culture at the edge of the growing fungal colony and the hyphae were observed microscopically.

Results

Dual-culture and conditioned-medium experiments

Three callus lines were selected for dual-culture and conditioned-media experiments. Two of them were derived from citrus cultivars having known responses to the mal secco disease; the tolerant cultivar 'Tarocco' orange and the sensitive 'Femminello' lemon. The third line was 'Femminello-S' that resulted from in-vitro selection of 'Femminello' nucellar calli resistant to the partially purified toxin (Gentile et al. 1992b).

P. tracheiphila did not grow on unconditioned MT medium, while growth was normal in the presence of calli on MT medium or on carrot medium (Fig. 1 and Table 1). During dual-culture experiments we noted that fungal growth was stimulated by the presence of all citrus calli (Fig. 1B). However, fungal growth was inhibited by 'Tarocco' and 'Femminello-S' when inoculating plugs were transferred after at least 20 days of callus growth (Fig. 1C). If calli-containing plates were inoculated earlier, no inhibition was obtained and near-centrifugal growth of fungal hyphae was observed (Fig. 1B).

These results suggest that extracellular metabolites may be involved in the inhibition of fungal growth. We therefore examined the conditioned medium of each cell line. Cells were grown for 30 days as suspension cultures and the respective conditioned media were then used for fungal culture as described in Materials and methods. After 10 days of inoculation the fungal colonies reached sizes of 7.0, 4.8 and 5.2 cm^2 when plated in conditioned medium prepared from 'Femminello', 'Femminello-S' and 'Tarocco' respectively (Table 1). Statistical analysis showed that the fungal colony sizes on 'Femminello'-conditioned medium was significantly larger then on 'Tarocco'- and 'Femminello-S'-conditioned media. In comparison, during 10 days incubation on unconditioned MT medium there was no fungal growth (0.9 cm^2) while the size of fungal colonies on carrot medium was 9.8 cm^2.

Analysis of extracellular protein

After 20 days of growth, proteins were detected in the conditioned medium of all cultures. The 'Femminello' cell culture contained 15 μg/ml of protein while the medium of 'Femminello-S' or the medium of 'Tarocco' contained 47 μg/ml of protein. Total soluble extracellular

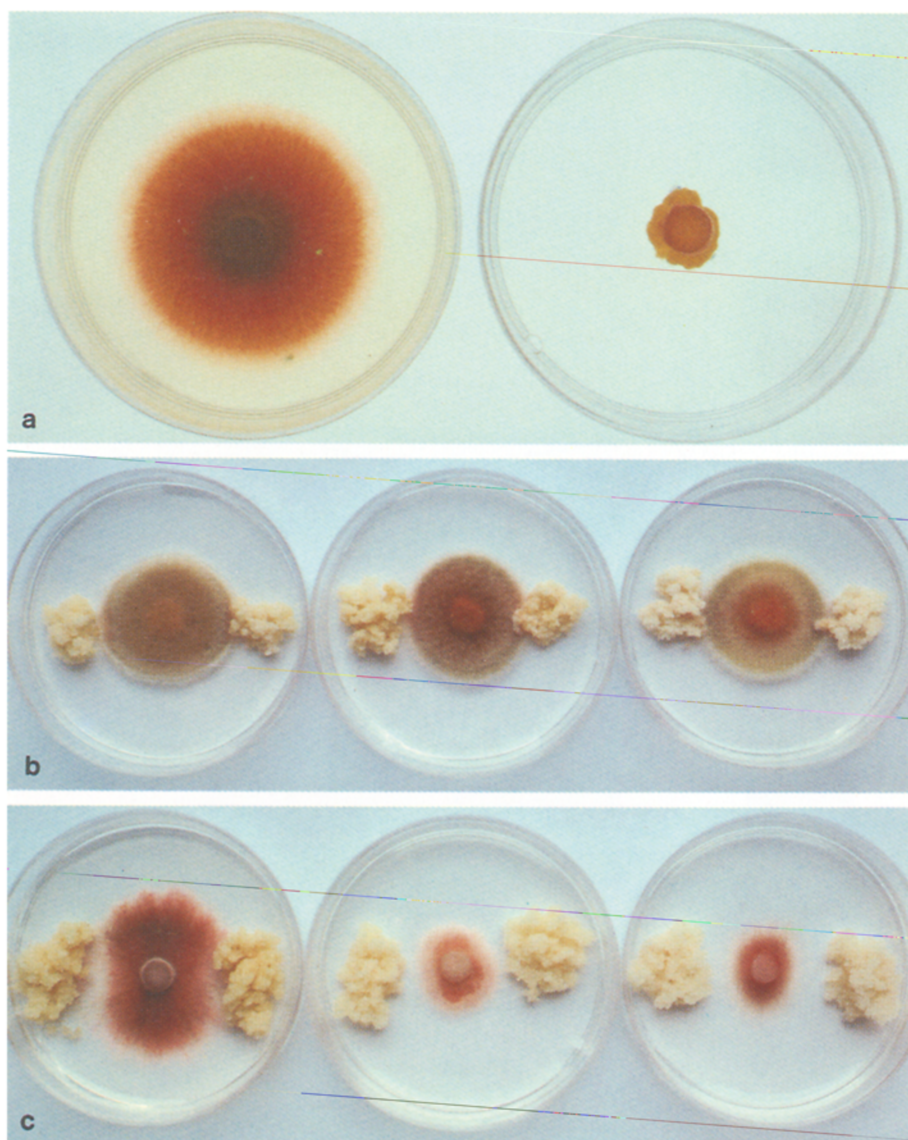

Fig. 1 A–C. Fungal growth in citrus calli/fungal mycelium combination. The dishes were photographed 10 days after fungus inoculation. **A** Growth on defined media. Left plate shows normal average size of fungal colony on control carrot medium and right plate shows near absence of fungal growth on unconditioned MT medium. **B** Fungal growth on MT medium by 10-day-old citrus calli. The calli were cultured for 10 days prior to fungal inoculation and co-cultured with fungus for an additional 10 days. Left plate 'Femminello', middle plate 'Femminello-S' right plate 'Tarocco'. **C** Fungal growth on MT medium in the presence of 30-day-old citrus calli. The calli were cultured for 30 days prior to fungal inoculation and co-cultured with fungus for an additional 10 days. Left plate 'Femminello', middle plate 'Femminello-S', right plate 'Tarocco'

proteins were analyzed by SDS-PAGE. A polypeptide of 30 kDa size was common to 'Femminello' and 'Femminello-S'; however in 'Femminello-S' two additional polypeptides with apparent molecular masses of 25 kDa and 37 kDa were evident. The respective profile from the 'Tarocco' cell culture was basically similar to that of 'Femminello-S' but the former also contained a number of bands with apparent masses of 45–50 kDa (Fig. 2).

Fractionated polypeptides (Fig. 2 A) were transferred to nitrocellulose sheets and reacted with antiserum specific for the acidic endochitinase of tobacco (PR-3; Fig. 2 B). A 25 kDa polypeptide was detected in TMV-treated tobacco plants which is the expected size of the acidic endochitinase (lane 1, Fig. 2). Two immunoreactive polypeptides of apparent molecular masses of 24 and 37 kDa were detected in the extracellular proteins of non-selected lemon ('Femminello') cells (lane 2, Fig. 2 B). In the selected lemon 'Femminello-S' cell line, the 37 kDa polypeptide was considerably enhanced (lane 3, Fig. 2 B) and two additional highly-immunoreactive polypeptides

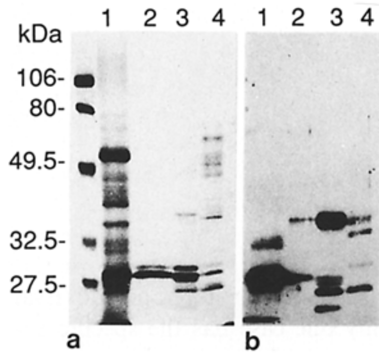

Fig. 2A, B. SDS-PAGE fractionation of extracellular protein. **A** Coomassie-stained fractionated protein extracts from total tobacco leaves and from extracellular medium of citrus calli. *Lanes 1*, tobacco; *2*, 'Femminello'; *3*, 'Femminello-S' and *4*, 'Tarocco'. **B** Immunoblot of fractionated proteins shown in **A** developed with chitinase antibodies

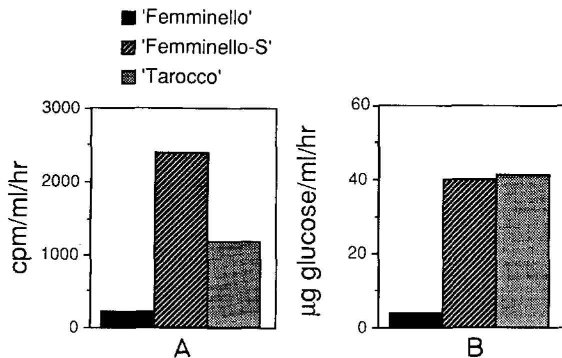

Fig. 3A, B. Endochitinase and glucanase activity in the medium of cultured citrus cells. Aliquots of extracellular medium from 20-day-old cells were analyzed for **A**, endochitinase and **B**, (1-3)-β-glucanase as described in Materials and methods

Table 1. Growth of *P. tracheiphila* on defined and conditioned media. Values indicated by the same letters are not statistically different by Duncan's test at $P = 0.05$

Source of medium	Growth (cm^2)	Statistical grouping
'Femminello' [a]	7.0	c
'Femminello-S' [a]	4.8	b
'Tarocco' [a]	5.2	b
MT medium	0.9	a
Carrot agar medium	9.8	d

[a] Filtered conditioned medium was prepared from 30-day-old citrus cell cultures grown in MT medium; the filtered medium was solidified with agar prior to fungal inoculation

of 20 and 24 kDa were observed. In the 'Tarocco' culture medium four polypeptides reacted with the antiserum (lane 4, Fig. 2B); two of them had apparent molecular masses similar to those of the selected lemon (24 and 37 kDa) and two had intermediate apparent masses of about 28 and 31 kDa (Fig. 2).

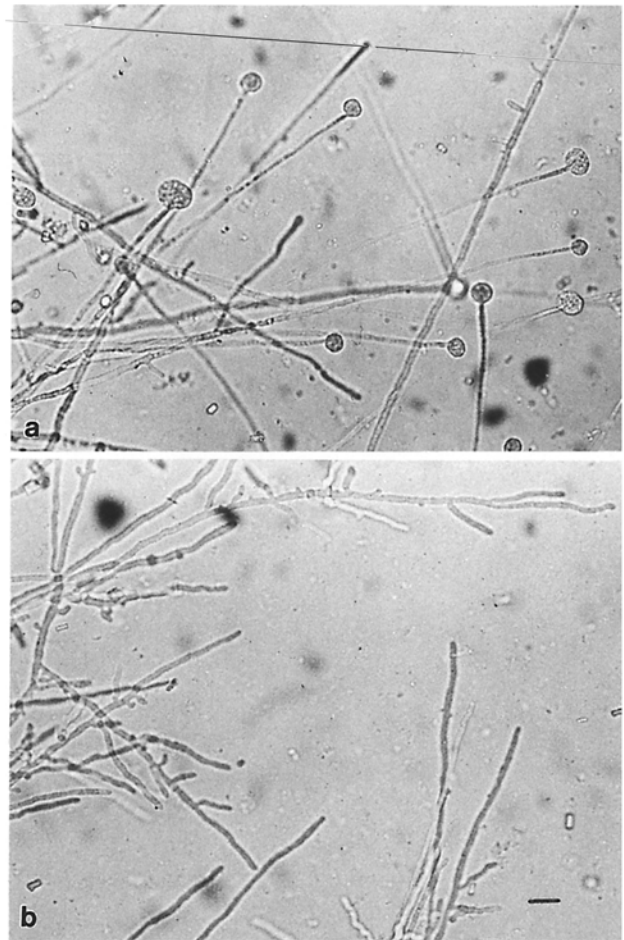

Fig. 4A, B. Light micrographs of hyphae 5 min after application of 10 µl of approximately 30 µg of proteins extracted from the conditioned medium of 'Femminello-S' (**A**) and 'Femminello' (**B**). Bar is equal to 10 microns

Chitinase and (1-3)-β-glucanase activity

Conditioned media from citrus cells cultured for 20 days was analyzed by a radiochemical assay for endochitinase. A ten-fold increase of specific chitinase activity in the culture medium from 'Femminello-S' as compared to 'Femminello' was detected. The value obtained from the culture medium of 'Tarocco' cells was half of that obtained from 'Femminello-S' (Fig. 3A). The activity of (1-3)-β-glucanase was evaluated by using laminarin as a substrate. The amount of reducing groups released from the laminarin substrate indicated a ten-fold increase of glucanase activity in the medium of 'Femminello-S' cells as compared to 'Femminello'. The glucanase activity in the medium of 'Tarocco' orange cultures was similar to that of 'Femminello-S' (Fig. 3B).

Lysis of the hyphal tips

Microscope slides coated with a thin layer of carrot agar and inoculated with *P. tracheiphila* (as described in Ma-

terials and methods) were used in the hyphal tip lysis tests. Small drops of protein extract (containing approximately 30 μg/ml protein) derived from the conditioned media of each of the three cell lines were applied at the edge of the growing mycelium. The extracellular proteins from 'Femminello-S' (Fig. 4) and 'Tarocco' (data not shown) caused abrupt swelling of the fungal tips. Some of the fungal tips started to burst within a few minutes after the application of the protein extract. Neither swelling nor bursting of hyphal tips were recorded after the application of the protein extract from 'Femminello' cultures.

Discussion

Nucellar-derived citrus cell lines have been shown to accumulate stage-specific extracellular proteins. One group of extracellular proteins was found to play an important role in somatic embryogenesis (Gavish et al. 1991). Additional enzymatic activities in the conditioned medium include peroxidases and polypeptides that cross-react to specific classes of tobacco pathogenesis-related proteins (Gavish et al. 1991). Their constitutive expression has been detected in a few additional cell culture systems (Esaka et al. 1990). In contrast, in many reported cases the secretion of PR proteins in cell culture was conditionally induced by pathogens, elicitors, hormones, or stress, as reported in tobacco (Antoniw et al. 1981; Ohashi and Matsuoka 1987), parsley (Kombrink and Hahlbrock 1986), and rice (Nishizawa and Hibi 1991). The exact function of these extracellular polypeptides is unknown though in one case it was shown to be involved in in-vitro carrot embryogenesis (De Jong et al. 1992).

Here we compare two isogenic cell lines from one cultivar and another cell line from a related cultivar that differ in their ability to grow in the presence of a fungal toxin. We showed that both the selected line, 'Femminello-S', and the line derived from a cultivar with intrinsic fungal tolerance, 'Tarocco' orange, repressed the growth of *P. tracheiphila*. In each case repression was correlated with an immunochemically-detected increase in the PR protein, chitinase, We also show a concomitant increase in the enzymatic activity of chitinase and of (1-3)-β-glucanase in the culture media of the tolerant cell lines. Furthermore, this increased activity was correlated with a direct repression of fungal growth as illustrated by lysis of the hyphal tips. Thus the observed repression of gross fungal growth is consistent with the increase in extracellular accumulation of cell wall hydrolytic enzymes. The results obtained are consistent with the observation that *P. tracheiphila* contains chitin as one of its major cell wall components (Bartnicki-Gracia 1968). Total soluble extracellular protein from two additional callus lines, the sensitive sour orange and tolerant 'Murcott' tangor, was analyzed by SDS-PAGE and by immunobloting with acidic endo-chitinase of tobacco (data not shown). Interestingly, we found that the profile from the sensitive sour orange was similar to the profile of 'Femminello' while the profile from the tolerant 'Murcott' tangor was similar to that of 'Tarocco' orange.

The function of PR proteins in plants is obscure. They were originally detected in pathogenesis responses in a wide range of plants; however, recent evidence for their stage-specific accumulation may indicate an alternative role for these genes in plant development (Eyal and Fluhr 1991). In only one case has the specific enhancement of chitinase activity been shown to promote plant-resistance to a fungal disease (Broglie et al. 1991); therefore, it remains to be seen whether plants regenerated from the resistant cell lines will show resistance at the whole plant level and whether this resistance will be correlated with elevated levels of chitinase. Evidence shows that the partially-purified toxin produced from the culture filtrate of *P. tracheiphila* is involved in its virulence (Nachmias et al. 1977; Pennisi et al. 1988; Sesto et al. 1990). It was also shown that the response of calli and calli-derived protoplasts (Gentile et al. 1992a), as well as leaf protoplasts, to the *P. tracheiphila* toxin (Sesto et al. 1990) is in full agreement with what is known about the response of citrus cultivars to the mal secco disease. Recently, we reported that protoplasts isolated from leaves of regenerated 'Femminello-S' plants displayed a wide range of segregation with a noticeable proportion of tolerant plants (Gentile et al. 1992b). However, it still remains enigmatic why selection for tolerance to a toxin, whose mode of action is presently unknown, would result in cell cultures that over-secrete hydrolases.

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