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Differential responses of citrus calli and protoplasts to culture filtrate and toxin of *Phoma tracheiphila* *

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Summary. Nucellar calli from two citrus cultivars with known tolerance to mal secco disease were chosen as experimental material, to test the pathogen's response to culture filtrate (CF) and partially purified toxin (PPT). The response of the two calli to the CF was in reverse order to the known response of the two cultivars to natural and artificial inoculations with *Phoma tracheiphila*. HPLC analysis of *P. tracheiphila* CF indicated the presence of a relatively high level of indole acetic acid (IAA). The response of the two calli and protoplasts derived from these calli to increasing amounts of IAA in the culture media was in the same order as that of calli and protoplasts to CF. In contrast, the responses to PPT of calli and protoplasts from these two types confirmed the relative tolerance of 'Femminello' lemon and 'Tarocco' orange trees to mal secco disease.

Key words: *Citrus* – In vitro selection – Lemon – *Phoma tracheiphila* – Phytotoxin

Introduction

Mal secco, caused by *Phoma tracheiphila* (Petri) Kanc. et Ghik., is a serious tracheomycotic fungal disease of *Citrus* known only in the Mediterranean region and the Middle East. Lemon [*Citrus limon* (L.) Burm. f.], citron (*C. medica* L.), bergamot (*C. bergamia* Risso), and sour orange (*C. aurantium* L.) are the most susceptible species, while sweet orange [*C. sinensis* (L.) Osb.] and mandarin (*C. reticulata* Blanco) are affected only when the susceptible sour orange rootstock becomes infected (Catara and Cutuli 1972; Salerno and Cutuli 1977). So far no genetic source of resistance to mal secco has been found among commercial lemon varieties (Perrotta and Tribulato 1977) and the only tolerant varieties, e.g., 'Monachello' and 'Interdonato' lemon, are of low horticultural value. Since lemon is highly apomictic and is a vegetatively propagated plant, classical selection methods for resistance, even after mutagenesis, would seem to offer a low probability of success.

During the last decade, recovery of disease-resistant plants by in vitro selection at the cellular level using a toxic compound, partially purified toxin or culture filtrate produced by a plant pathogen has been reported: *Nicotiana tabacum* to *Pseudomonas syringae* and *Alternaria solani*; *Zea mays* to *Helminthosporium maydis* race T; *Saccharum* sp. to *H. sacchari*; *Solanum tuberosum* to *Phytophthora infestans* and to *Fusarium oxysporum* (see Daub 1986, for review) as well as *Oryza sativa* to *H. oryzae* (Ling et al. 1985); *Brassica juncea*, *B. nigra*, and *B. carinata* to *Phoma lingam* (Sjodin and Glimelius 1988, 1989); and *Prunus persica* to *Xanthomonas campestris* pv *pruni* (Hammerschlag 1988, 1990). In all the above-mentioned cases, it was demonstrated that the metabolites produced by the pathogen are involved in the disease development and that the metabolites are active at the cellular level. Evidence shows that the toxin produced by *P. tracheiphila*, 'malseccin,' is involved in the pathogenicity (Nachmias et al. 1977a, b, 1979; Pennisi et al. 1988; Sesto et al. 1990). Ovule-derived nucellar calli of citrus are the most appropriate source for in vitro selection. It is possible to obtain highly friable, nucellar callus lines that maintain an embryogenic capacity for many years. The nucellar calli are also the only appropriate source of protoplasts which are able to divide and regenerate into normal plants (Vardi and Galun 1988, 1989). In addition citrus nucellar calli do not require auxin and cytokinin

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for sustained cell division (Vardi et al. 1982). This habituation eliminates any antagonism between the growth-inhibiting effects of the mal secco toxin and the growth-promoting effects of plant growth hormones added to the culture medium. The objective of the present research was to develop a selection methodology in order to obtain lemon lines that are tolerant to the mal secco disease. For this purpose, we compared the effect caused by culture filtrate (CF) and by partially purified toxin (PPT) on nucellar calli derived from lemon cv 'Femminello' and orange cv 'Tarocco' and on the corresponding protoplasts.

Materials and methods

Establishment and maintenance of nucellar calli

Nucellar embryogenic calli from 'Femminello' lemon [*Citrus limon* (L.) Burm. f.] and 'Tarocco' orange [*Citrus sinensis* (L.) Osb.] were obtained as described by Vardi et al. (1982). Ovules were axenically excised from young fruits, 4–8 weeks after anthesis, and placed on solidified medium. The medium was that of Murashige and Tucker (1969) containing 5% sucrose, 1% agar (BM), and 500 mg l^{-1} malt extract. Prior to autoclaving, the pH was adjusted to 5.7 and supplemented with 10 g l^{-1} Difco agar. The nucellar calli that emerged from the cultured ovules were maintained on BM without growth substance and subcultured every 4 weeks.

Isolation of protoplasts

For protoplast isolation, calli were subcultured every 2–3 weeks. The procedure of protoplast isolation was described in detail by Vardi et al. (1982) and Vardi and Galun (1989).

Production of CF and PPT

The *P. tracheiphila* "80(+)" isolate was selected for the preparation of CF and PPT. This isolate was found to produce a high amount of protein in in vitro culture (Pennisi et al. 1988). The fungus cultures were maintained in 9-cm petri dishes containing carrot agar medium (400 g carrot cooked, mashed, and filtered through cheese cloth, then 40 g sucrose, 10 g agar, and distilled water were added to a final volume of 1 l). The cultures were then maintained at 4 °C.

For CF and PPT production, a 1-cm^3 disc of fungal culture was transferred to 1-l Roux bottles containing 100 ml of medium, as described by Nachmias et al. (1977a). The bottles were incubated under continuous light (1,200 lx) at 27 ± 1 °C. The CF was obtained after 27 days of static incubation by separation of mycelium from the liquid media.

The PPT was produced according to the method described by Nachmias et al. (1977a). The toxin was obtained from the second fraction collected from the chromatography on Sepharose 6B column (3 × 80 cm). Different preparations of PPT were standardized by determination of protein content, using bovine serum albumin (BSA) as standard (Lowry et al. 1951).

Analysis of indole-3-acetic acid, kinetin, and gibberellic acid from CF

The levels of indole-3-acetic acid (IAA), kinetin, and 3-gibberellic acid (GA_3) in CF were evaluated by HPLC analyses as described by Sandberg et al. (1987), Chen (1987), and Barendse (1987), respectively.

Exposure of calli to CF, PPT, and IAA

About 10 pieces of lemon and orange calli (approx. 5 mg each) were placed in 9-cm petri dishes containing BM as control: (i) BM supplemented with 25% CF and 50% CF of total BM volume; and (ii) BM containing different concentrations of PPT. The estimated molecular weight of PPT is 93,000 (Nachmias et al. 1977a), and based on this estimation 1, 3, and 5 μ*M* of PPT were employed. (iii) Various concentrations of IAA were tested: 0.016, 0.032, 0.064, and 0.128 ppm. CF, PPT, and IAA were filter sterilized and then incorporated into warm (45–50 °C) autoclaved BM.

Ten petri dishes were employed for each treatment. The dishes were sealed with Parafilm and incubated at 27 ± 1 °C, with 16 h/day fluorescent light (1,200 lx). Callus growth was assessed after 14, 21, and 28 days.

Protoplasts treated with CF and PPT

Protoplasts were isolated from 'Femminello' lemon and 'Tarocco' orange calli, and then washed and suspended in liquid protoplast isolation medium as described previously (Vardi et al. 1982; Vardi and Galun 1988, 1989), at a final density of 10^6 cells ml^{-1}. To evaluate the effect of CF, 0.5 ml aliquot of protoplasts (10^6 cells ml^{-1}) were suspended in liquid protoplast isolation medium (Vardi et al. 1982) that contained final concentrations of 0, 12.5, 25, and 37.5% CF. Protoplast samples were then scored 6, 12, and 24 h after incubation by fluorescein diacetate staining (Widholm 1972) to evaluate protoplast viability.

For the evaluation of the effect of PPT, 0.5 ml aliquot of protoplasts (10^6 cells ml^{-1}) was suspended in 0.5 ml aliquots of 0.7 *M* mannitol solution containing final concentrations of 0, 0.5, 1.0, 2.0, or 4.0 μ*M* PPT. After 1 h the protoplasts were washed with a protoplast washing solution (Vardi et al. 1982) and resuspended ($5 \cdot 10^5$ cells ml^{-1}) in the same solution. Protoplast samples were then scored after 3, 6, 18, 24, or 48 h by fluorescein diacetate as noted above.

Results

Effects of CF and IAA on callus growth

The effect of *P. tracheiphila* CF on the growth rate of lemon and orange calli was tested (Fig. 1). After 21 and 28 days, growth of lemon calli on medium containing 50% CF was almost double that of control calli, whereas the growth of orange calli was not affected. These unexpected results obtained with lemon calli led us to question whether or not the CF contained plant-growth hormones. HPLC analysis showed that GA_3, kinetin, and IAA are indeed present in the CF. We found very low values of GA_3 and kinetin (0.012 and 0.015 ppm, respectively) but a rather higher level of IAA – 0.064 ppm. Therefore, we decided to test the response of these two callus lines to different concentrations of IAA (Fig. 2). Lemon calli, after 28 days of culture in medium containing 0.032 ppm IAA, showed the highest growth rate. (This IAA level corresponds to the level of IAA present in medium with 50% CF.) However, increasing the IAA concentration to 0.128 ppm caused a reduction of callus growth to reach almost the control value. The growth rate of orange callus was also promoted by the addition

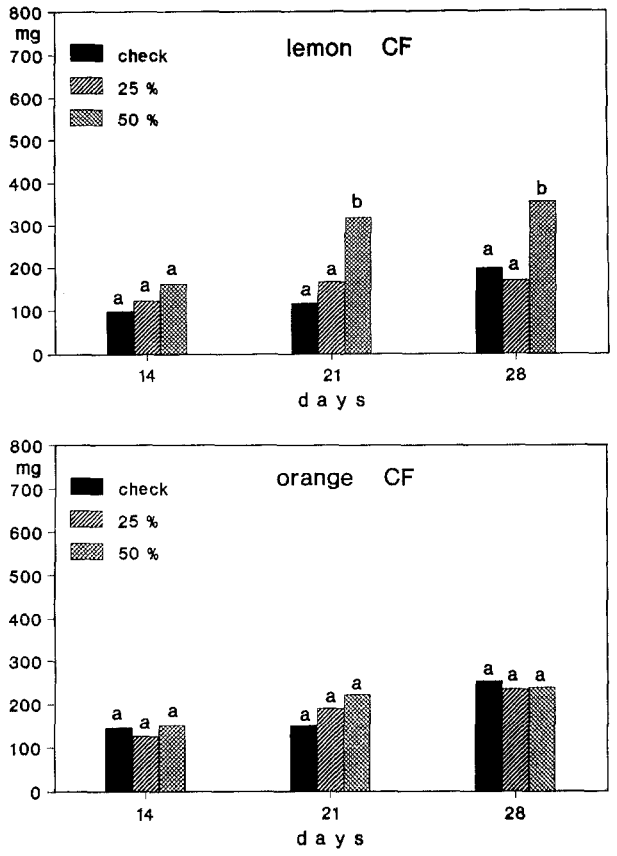

Fig. 1. The effect of two culture filtrate (CF) concentrations, in the culture medium, on callus growth as compared with the check. The results are presented as fresh weight of lemon (*top*) and orange (*bottom*) calli after 14, 21, and 28 days. *Bars* with a common letter do not differ significantly ($P = 0.05$)

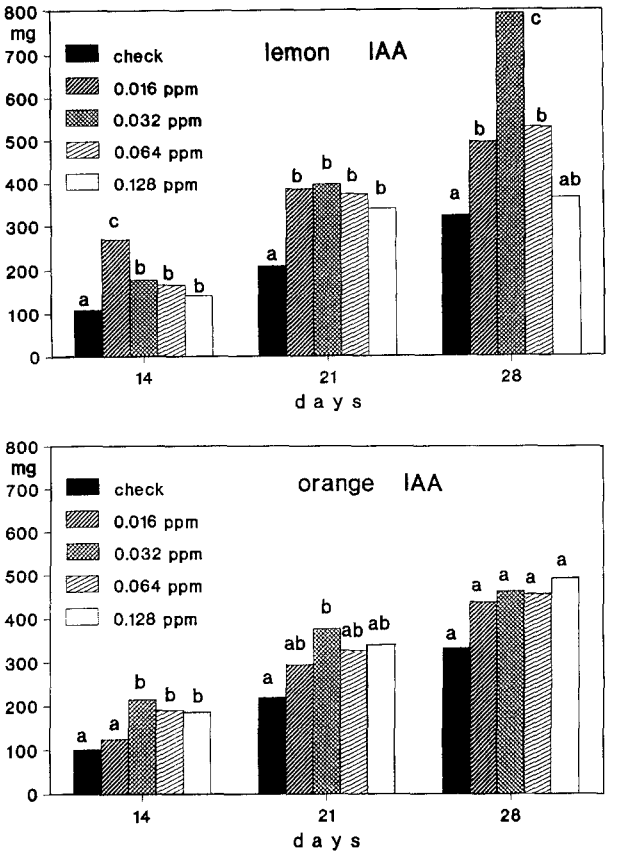

Fig. 2. The effect of different indole-3-acetic acid (IAA) concentrations, in the culture medium, on callus growth as compared with the check. The results are presented as fresh weight of lemon (*top*) and orange (*bottom*) calli after 14, 21, and 28 days. *Bars* with a common letter do not differ significantly ($P = 0.05$)

of IAA, but this promotion was significant only after 14 days and diminished after 28 days.

Retardation of callus growth by PPT

In order to study the differential effect of PPT on lemon and orange calli, these calli were cultured in the presence of 0, 1, 3, and 5 μ*M* PPT. Callus growth was evaluated after 28 days of culture. In both types of calli, reduction in growth occurred in cultures at increasing PPT concentrations (Table 1). The two calli types reacted differently to the high PPT concentrations (3.0 and 5.0 μ*M*); while lemon calli ceased to grow, the orange calli did grow but at a reduced rate. Only a few lemon colonies were able to grow at the lowest (1 μm) concentration tested.

Reduction of protoplast viability by CF

The effect of CF on protoplast survival was based on the evaluation of cell viability by fluorescein diacetate (FDA) staining. Lemon- and orange-calli-derived protoplasts were exposed to different CF concentrations (0, 12.5, 25, and 37.5%) as described in 'Materials and methods.' The

Table 1. Growth of lemon and orange calli in the presence of PPT. Calli (initial f.w. 50 mg/plate) were weighed after 28 days and the results are expressed as mg (f.w.) increase (per plate) during culture

Callus line	Toxin concentrations (μ*M*)			
	0	1	3	5
Lemon	211.1	172.8	−1.3	−11.0
Orange	144.6	72.6	35.7	30.3

results show that with the two highest CF concentrations used (25 and 37.5%), protoplast mortality occurred mainly during the first 12 h, and that lemon protoplasts were less sensitive than orange protoplasts to CF (Fig. 3).

Effect of PPT on protoplast survival

The viability of protoplasts isolated from lemon and orange calli following toxin treatment (0, 0.5, 1.0, 2.0, and 4 μ*M*) was assessed as described in 'Materials and

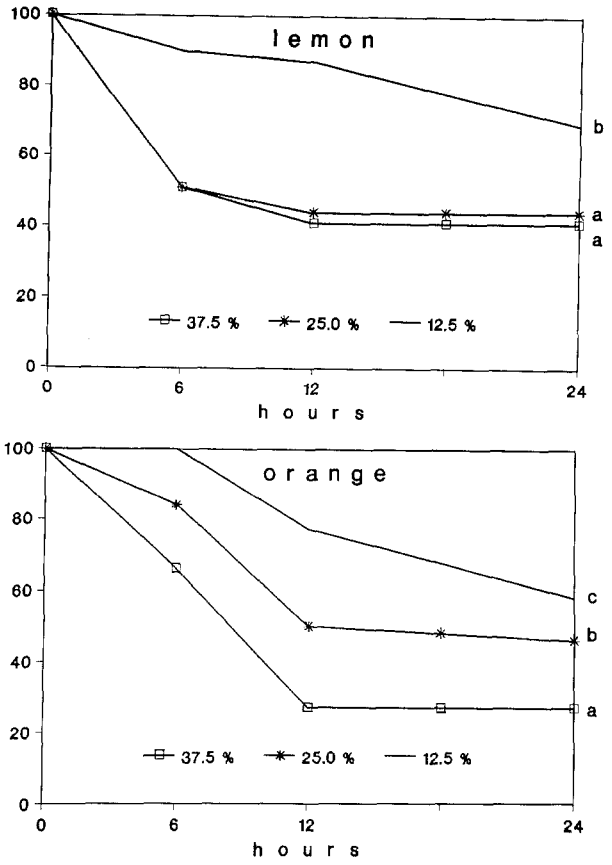

Fig. 3. The viability of protoplasts, derived from lemon and orange calli, after incubation for 6, 12, and 24 h in medium containing different concentrations of culture filtrate (CF). The CF was added to the protoplast cultures immediately after isolation. Data are expressed as percent of control (=100%). Lines labeled with the same letter do not differ significantly ($P = 0.05$)

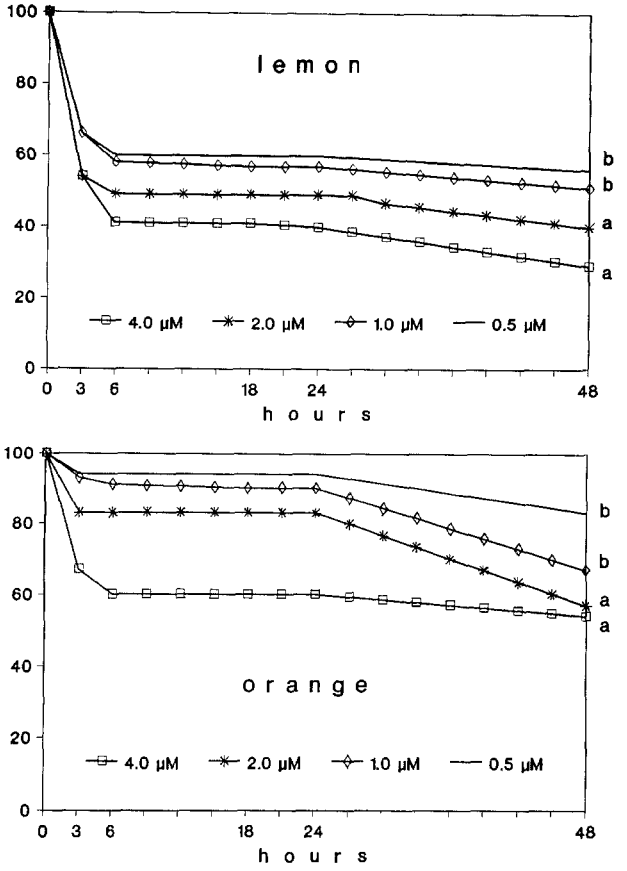

Fig. 4. The viability of protoplasts, derived from lemon and orange calli, after 1 h of incubation in medium containing different concentrations of partially purified toxin (PPT). The PPT was added to the protoplast cultures immediately after isolation. Protoplast viability was scored at different times after PPT treatment. Data are expressed as percent of control (=100%). Lines labeled with the same letter do not differ significantly ($P = 0.05$)

methods.' Samples were scored after 3, 6, 18, 24, and 48 h. The different response of the protoplasts derived from the two genotypes can be detected, as soon as 3 h after toxin treatment, even with the lowest (0.5 µ*M*) PPT concentration used. Lemon protoplasts were found to be more sensitive (at all PPT concentrations used) than orange protoplasts (Fig. 4).

Discussion

Phytotoxin and CF, which cause rather typical and well-defined symptoms on the appropriate susceptible hosts, have potential for the study of the relationships among the reactions of protoplasts, calli, and the same genotypes as do complete plants. Calli and protoplasts are usually much more sensitive to the pathotoxins than other types of plant material and are thus very suitable for use in bioassays (Earl et al. 1978; Yoder 1981).

A number of investigators have obtained successful selections using culture filtrates to select resistant material (Sacristan 1982; Hartman et al. 1984; Hammerschlag 1988). Culture filtrate of *P. tracheiphila* was found to contain 'malseccin', an extracellular nonspecific phytotoxin that is involved in the pathogenicity (Nachmias et al. 1977a, b, 1979; Pennisi et al. 1988; Sesto et al. 1990). 'Malseccin' is a partially purified nonspecific phytotoxin, containing glycopeptides of high (93,000) and low (350–700) molecular weight, which causes typical symptoms.

The results obtained by electron microscope (Perrotta et al. 1978; Nachmias et al. 1980; Barash et al. 1981; Sesto et al. 1990) reveal that this nonspecific PPT causes damage to the cell membranes, inducing chlorosis and necrosis in lemon leaves, inducing electrolyte leakage from lemon leaf or carrot discs, and causing uncoupling of electron transport in chloroplasts (Nachmias et al. 1977b).

The *P. tracheiphila* isolate used by us was found to extrude a high level of protein into the CF (Pennisi et al. 1988). Since purified toxin from this fungal pathogen is not yet available, we analyzed the effect of CF and PPT on nucellar calli and on the corresponding protoplasts. We chose calli and protoplasts from a susceptible and a tolerant citrus type (i.e., lemon and orange, respectively), in order to develop a reliable system for selection of genotypes tolerant to the mal secco disease. Such a system may provide a useful tool that is not only beneficial to citriculture but also for studying, at the cellular level, the mechanism of tolerance in isogenic cell-lines.

The response of the two calli to the CF was in reverse order to the known response of the two cultivars to natural and artificial inoculations with *Phoma* (Catara and Cutuli 1972). Callus derived from the susceptible 'Femminello' lemon grew better with increasing amount of CF, as compared with both control and callus derived from the more tolerant 'Tarocco' orange. The latter was not affected by the increasing amount of CF. These results are in accordance with the report of Vardi et al. (1986) on CF of *Phytophthora citrophthora*. It should be noted that all citrus nucellar calli and protoplast systems are habituated and do not require the addition of plant growth substances for cell division. Moreover, in many cases the addition of auxin inhibited cell division (Vardi and Raveh 1976; Kochba et al. 1980). Culture filtrates of fungi are rich in secondary metabolites as well as in growth-inhibiting and -stimulating substances (for review, see Yoder 1980, 1983). Growth regulators such as cytokinins (Johnston and Trione 1974), auxin (Gruen 1959; Epstein and Miles 1967), and gibberellic acid (Brian et al. 1954) have been found also in the culture filtrates. In the present study, HPLC analysis of *P. tracheiphila* CF indicated the presence of very low concentrations of kinetin and GA_3 and a relatively high level of IAA. Comparison of the results presented in Figs. 2 and 3 shows a similarity in response with increasing amount of CF and IAA in the culture media. In both experiments the highest growth rate of lemon was obtained on media containing 0.032 ppm IAA.

In contrast to the differential reaction of lemon and orange (calli and protoplasts) to CF, the responses of calli and protoplasts from these two citrus types to PPT confirmed the relative tolerance of 'Femminello' lemon and 'Tarocco' orange trees to mal secco. None of the orange calli exposed to PPT ceased to grow, although reduction of callus growth was noticed with increasing amounts of PPT. Complete inhibition of lemon colonies was recorded with the highest PPT concentration used, and only a few of the colonies grew in the lowest PPT concentration tested.

Likewise, lemon and orange protoplasts reacted to the addition of CF in the incubation medium, in contrast to the response of the two respective trees to mal secco infection: lemon protoplasts were less sensitive to CF than were orange protoplasts. In contrast, protoplast response to PPT was in agreement with the known response to 'Femminello' lemon and 'Tarocco' orange (e.g., Sesto et al. 1990). Studies of the effects caused by the *P. lingam* toxin 'sirodesmin' PL (Sjodin et al. 1988) revealed that this toxin is not specific to plants that are sensitive to this pathogen: protoplasts from all the investigated material were likewise affected. In contrast, the viability of citrus protoplasts (including leaf protoplasts) after treatment with 'malseccin' is in full agreement with what is known about the tolerance of the cultivar to the mal secco disease. It seems, therefore, that in citrus a selective toxic effect exists and PPT can be utilized not only for screening but also for in vitro selection. Work along this line has been already started by us.

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