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Is the *Phytophthora citrophthora* culture filtrate a reliable tool for the in vitro selection of resistant *Citrus* variants?*

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Summary. Nucellar calli from four *Citrus* cultivars with known resistance to the *Phytophthora citrophthora* pathogen were chosen as experimental material to test the pathogen's response to culture filtrate (CF). Sensitivity of the four calli to CF of the fungus was in reverse order to what is known on the susceptibility of the cultivars in vivo. Sensitivity of protoplasts derived from the same four calli to 2,4-dichlorophenoxyacetic acid (2,4-D) was in the same order as that of calli to CF. Protoplasts derived from calli selected for tolerance to CF showed a higher plating efficiency with increasing concentration of CF in the medium. TLC and GLC determinations showed the presence of indole acetic acid in the culture filtrate. Results indicate that CF of *P. citrophthora* cannot be used as a selection tool in vitro.

Key words: *Phytophthora citrophthora* – *Citrus* – Culture-filtrate – Indole acetic acid – Protoplast

Introduction

During the last few years attempts have been made to obtain resistant plants from selection at the cellular level (Behnke 1979, 1980 a, b; Sacristan 1982).

There are two prerequisites for successful selection in vitro: an effective selective agent and a reliable cell-to-plant regeneration system. In order to obtain disease-resistant plants by plating cells on toxin-containing medium, a causal relationship of the toxin to pathogenecity must be ascertained. Only a few of the toxins produced by fungal and bacterial pathogens have been investigated to the extent that their role in pathogenicity has been established and clearly defined by critical experimentation (Gregory et al. 1980; Yoder 1980; Durbin 1981; Walton and Earle 1984). Those fungal toxins which clearly indicated to be causal agents of diseases are host-specific toxins (Kono et al. 1981; Walton and Earle 1984). Indeed, when a large number of cells were exposed to host-specific toxins and an efficient cell-to-plant regeneration system was available, disease-resistant plants were obtained (Durbin 1981). Selection of maize cell cultures resistant to culture filtrate of *Helminthosporium maydis* race T and subsequent regeneration of plants seems to be a significant accomplishment in in vitro selection (Gengenbach et al. 1977; Bretell et al. 1980). Recently, successful in vitro selection of rice to *H. oryzae* (Ling et al. 1985) and *Avena sativa* to *H. victoriae* (Rines and Luke 1985) has been reported. In some cases when the toxin was not purified, culture filtrates or partially purified toxin were used for selection in vitro (Behnke 1980 b; Matern et al. 1978; Shepard et al. 1980; Sacristan 1982).

Various *Phytophthora* spp. have been shown to secrete phytotoxin metabolites in culture (Behnke 1979, 1980 b; Plich and Rudnicki 1979; Deaton et al. 1982) *P. citrophthora* (Sm. et Sm. Leonian) is the causal agent of trunk gummosis, collar and root rot, dumping-off and brown rot of *Citrus* spp. A culture filtrate of this fungus has been reported to contain both high and low molecular weight non-specific phytotoxic components (Breiman and Barash 1981; Breiman and Galun 1981). The development of the full sequence from isolated protoplasts to morphologically normal plants with various *Citrus* cultivars and species (Vardi et al. 1982) has enabled us to test the reliability of using *P. citrophthora* culture filtrate as an in vitro selection tool.

Materials and methods

Selection procedure

Nucellar-derived callus lines of four *Citrus* cultivars (Vardi et al. 1982) were used: 'Sour orange' (*C. aurantium* L.), 'Mur-

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cott' mandarin (*C. reticulata* Blanco), 'Shamouti' orange (*C. sinensis* (L.) Osbeck) and 'Villafranca' lemon (*C. limon* (L). Burm). The calli were maintained on solidified (1% agar) Murashige and Tucker's (1969) basal medium (BM) without hormones.

About 60 callus pieces (approximately 10 mg each) were each placed in 9 cm Petri dish containing either BM or BM supplemented with filter-sterilized culture filtrate (CF) of *Phytophthora citrophthora*. The CF was prepared as reported by Breiman and Barash (1981). The CF was concentrated by flask evaporation at 40 °C to 30% of the original volume. The pH of the concentrated filtrate was 5.9–6.0. Volume ratios of concentrated CF to BM were 1 : 2 and 1 : 1 (or 0.3 and 0.5).

Dishes were sealed with parafilm and incubated at 26 ± 1 °C, with 16 h/day of dim light. The best growing calli were selected for three passages of 4 weeks each, and then propagated for further selection and protoplast isolation.

Protoplast isolation and culture

Protoplast isolation and culture were as described by Vardi et al. (1982). Protoplasts were plated in 6 cm Petri dishes containing BM medium with or without CF. Colony formation was scored as described by Vardi and Raveh (1976), 4–5 weeks after plating.

Indole-3-acetic acid (IAA) analysis. Radioactive indole-3-acetic acid (50 µl IAA-2-C^{14}, specific activity of 54 mCi/mmol, which contained 0.72 µg of IAA) was added to the filtrate of the fungus. The filtrate was brought to pH 3.0 with 10 N HCl; it was then extracted three times with ethyl acetate; the pooled ethyl acetate was extracted with 0.1 N sodium bicarbonate which was then acidified with 10 N HCl to pH 3.0, and again extracted with ethyl acetate as described above. The ethyl acetate was dried with sodium sulfate (Na_2SO_4) and evaporated to dryness. Further procedures of purification with HPLC, with pentafluorotoluene, TLC and gas liquid chromatography, and a final calculation by the isotope dilution method, were performed as described previously (Epstein and Cohen 1981). The GLC was done on a Varian 3300 equipped with an electron capture detector and connected to a Spectra Physics integrator model 4270. The column was a fused silica 12 M × 0.32 mm ID, SE-30 stationary phase capillary column. Oven temperature was 180°, injector temperature 250°, and detector temperature 280 °C. A standard of fluorinated IAA was prepared from the radioactive IAA and its concentration was calculated from its radioactivity and specific activity. This standard was used for computing the amount of IAA in the samples obtained from the HPLC peaks.

Results

In vitro response of nucellar calli for resistance to toxin

Calli from four *Citrus* varieties with known tolerance to the *P. citrophthora* pathogen (Klotz 1978) were selected for the experiment: 'Sour orange' – resistant, 'Murcott' mandarin – moderately susceptible, 'Shamouti' orange – susceptible, and 'Villafranca' lemon – highly susceptible.

Calli pieces were plated on media containing CF as described in "Material and methods". The growth of calli from all four cultivars was drastically reduced in the presence of CF and a complete inhibition of growth occurred in 'Sour orange' and 'Murcott' mandarin calli. Several attempts to grow these two calli in the presence of CF were unsuccessful. The fresh weight of nonselected 'Villafranca' callus growing in the presence of the two CF concentrations used was reduced by 86% compared with the control whereas in the case of 'Shamouti', 0.33 CF reduced growth by 61% and 0.5 CF by 90% (Figs. 1 and 2).

Recurrent selection through three passages on media containing 0.33 CF was pursued with the 'Shamouti' orange and 'Villafranca' lemon. The best growing calli were multiplied for further experiments at the protoplast level.

The effect of CF on selected and nonselected callus-derived protoplasts

Protoplasts were isolated from selected and nonselected calli of 'Shamouti' orange, 'Villafranca' lemon and nonselected callus of 'Murcott' mandarin. The protoplasts were plated in media containing 0.03, 0.06 and 0.12 parts of the original concentrated CF. Plating efficiency obtained from nonselected protoplasts of 'Shamouti' orange and 'Villafranca' lemon showed a slight stimulation effect at 0.03 CF and reduction at 0.12 CF compared with the control. Nonselected 'Murcott' protoplasts were found to be highly sensitive to CF (Fig. 3).

The plating efficiency of protoplasts derived from the selected calli increased as a function of CF concentration and reached 170% and 1,070%, compared with the control at 0.12 CF for 'Shamouti' orange and 'Villafranca' lemon, respectively (Fig. 3). It seems that

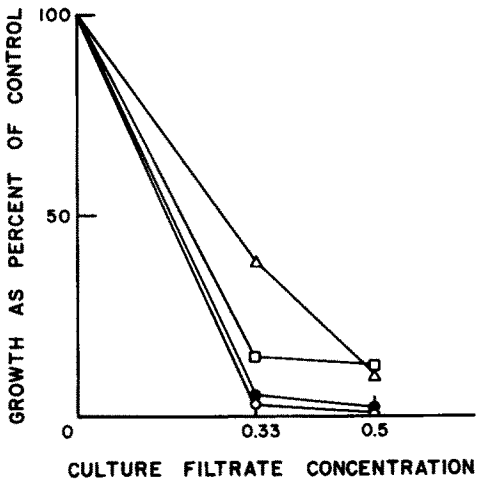

Fig. 1. Growth of nonselected nucellar calli of 'Villafranca' lemon (□), 'Shamouti' orange (△), 'Murcott' mandarin (○) and 'Sour orange' (●) as a function of CF concentration. Data are fresh weight values after 1 month of growth, expressed as percent of control (= 100%)

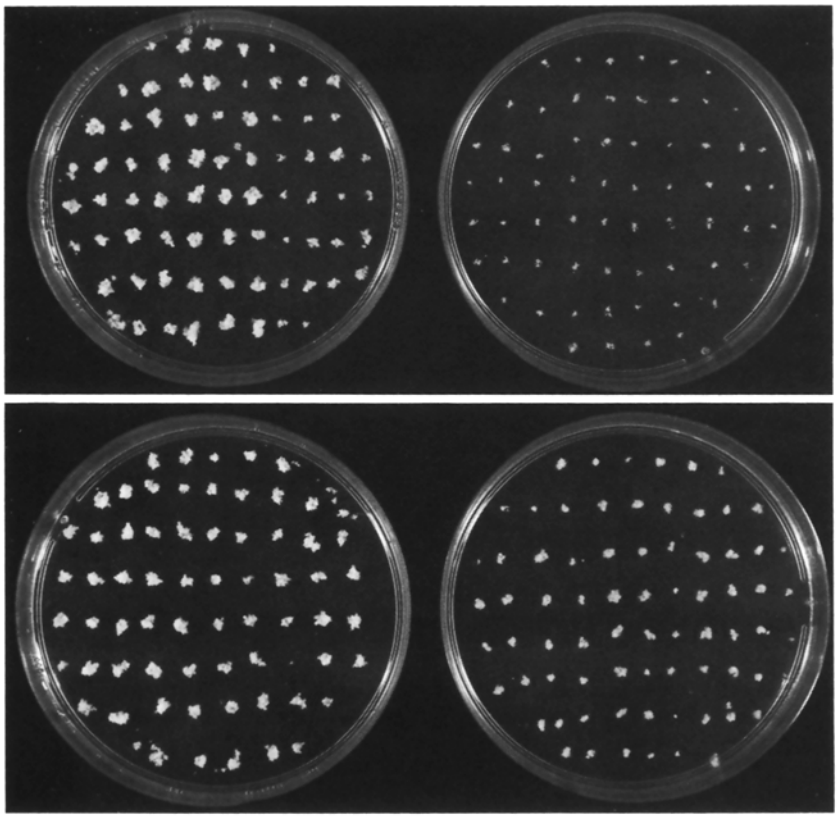

Fig. 2. Growth of nucellar callus of Citrus of medium with (*left*) and without culture filtrate (*right*). *Top* 'Shamouti' orange; *bottom:* 'Sour orange'

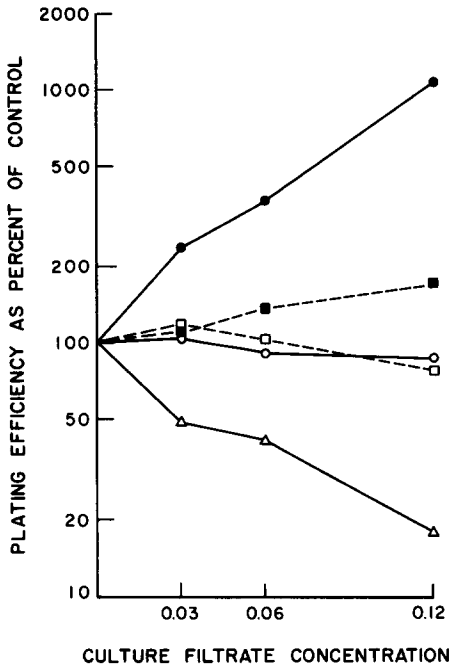

Fig. 3. Plating efficiency of protoplasts derived from nonselected calli of 'Murcott' mandarin (△), 'Shamouti' orange (□) and 'Villafranca' lemon (○) and from selected calli of 'Shamouti' (■) and 'Villafranca' (●) on media containing different concentrations of CF. Data are percent relative to control (= 100%)

the ability of protoplasts from selected calli to divide and form colonies becomes highly dependent on the presence of CF in the media.

The effect of 2,4-dichlorophenoxyacetic acid on plating efficiency

The vast increase in the plating efficiency of protoplasts derived from calli which were selected for CF-tolerance was rather unexpected. Furthermore, the dependence of selected cells on the selective factor was strikingly similar to the observation of Spiegel-Roy et al. (1983), who found that Shamouti calli selected for 2,4-D tolerance became dependent on this hormone. It should also be noted that all the protoplasts derived from the tested nucellar calli (non-selected) of *Citrus* do not require auxin in their culture medium (Vardi et al. 1982) and that the addition of auxin to the medium actually inhibits cell division (Vardi and Raveh 1976).

To test the possible auxin-like effect of CF, we performed the following experiment: protoplasts from the four tested citrus varieties were plated on culture media with increasing amounts of 2,4-D. The results are presented in Table 1. It can be seen that 'Murcott' protoplast cell division is completely inhibited in the presence of the lowest 2,4-D concentration (0.5 ppm)

Table 1. The effect of 2,4-D on plating efficiency of protoplasts derived from 'Villafranca' lemon, 'Shamouti' orange, 'Murcott' mandarin and 'Sour orange' callus lines. 2,4-D concentrations are given in ppm. Data are presented as number of colonies per ml, scored 5 weeks after plating. Percent of control (= 100%) is given in parentheses

Callus line	Control	2,4-D (ppm)				
		0.5	1	2	4	8
'Villafranca' lemon	7,318	5,045 (68.93)	3,727 (50.93)	1,182 (16.15)	955 (13.05)	733 (10.56)
'Shamouti' orange	3,639	955 (26.24)	545 (14.97)	409 (11.24)	0	0
'Murcott' mandarin	5,082	0	0	0	0	0
'Sour orange'	7,545	1,272 (16.85)	0	0	0	0

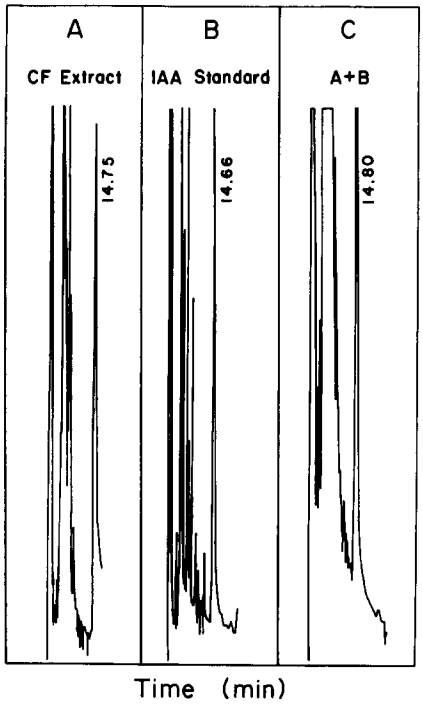

Fig. 4. GLC chromatograms of pentafluorobenzene of putative IAA from culture filtrate (*A*), standard IAA (*B*), and a mixture of the two (*C*)

used. The same effect was obtained with 'Sour orange' protoplasts at 1.0 ppm 2,4-D. 'Shamouti' orange protoplasts were found to be somewhat less sensitive; however, the least sensitive among the four systems were protoplasts derived from 'Villafranca' callus which still formed colonies even when the culture medium contained 8.0 ppm of 2,4-D.

These results are parallel to the response of the same calli to CF of *P. citrophthora* in the culture medium (Fig. 1).

The similarity in response to both CF and synthetic auxin led us to assume that an auxin-like substance may be present and active in the CF.

Detection of an auxin-like substance in CF of P. citrophthora

Extraction of putative IAA was as described in "Materials and methods". Thin layer chromatography with Kieselgel 60 F254 plates and chloroform : methanol : water (65 : 12 : 1) as a solvent showed that the isolated compound had the same R_f as IAA and fluoresced under U.V. light with blue color, as did authentic IAA.

The pentafluorotoluene derivative of the putative IAA is represented on the graph (Fig. 4) by a peak at about Rt = 14.75. This peak is identical to the Rt value of authentic IAA and was enhanced by the addition of known amounts of synthetic IAA which was derivatized the same way as the sample extracts (Fig. 4).

Discussion

Apparently, the availability of a cell-culture system having the capability to regenerate functional plants and of an extract from a pathogenic fungus, with toxin characteristics and activity on the cellular level should, when combined, constitute an efficient means to select for pathogen-resistant variants. Thus, the efficient protoplast system of *Citrus* (Vardi et al. 1982) and 3–4-week-old culture filtrate of *P. citrophthora,* containing high and low molecular weight phytotoxin components capable of inducing typical disease symptoms on lemon seedlings (Breiman and Barash 1981), seemed rather favorable for such a selection experiment. However, on the basis of results obtained in the present study, the use of *P. citrophthora* CF as a reliable selection tool in cell culture seems doubtful. The response of the four

calli tested to CF is in reverse order to what is known of the response of those *Citrus* cultivars to natural and artificial inoculation with *Phytophthora* spp. (Klotz 1978). As can be seen from Figs. 1 and 2, the calli derived from the more resistant varieties appeared to be highly susceptible to CF, while the susceptible varieties underwent the whole cycle of selection procedure. Moreover, protoplasts derived from CF-selected calli of 'Shamouti' orange and 'Villafranca' lemon showed higher plating efficencies with increasing CF in the protoplast culture medium (Fig. 3).

All nucellar calli and protoplast systems of *Citrus* are habituated and do not require the addition of plant hormones for cell division. The addition of auxin inhibits cell division (Vardi and Raveh 1976; Kochba et al. 1980). Selected lines resistant to 2,4-D become 2,4-D dependent (Spiegel-Roy et al. 1983). Comparison of results presented in Fig. 1 and Table 1 show a similarity in response of calli to CF and of protoplasts derived from the same calli to 2,4-D. The characterization of an auxin-like substance by GLC (Fig. 4) in the CF could explain the results obtained.

Culture filtrates of fungi are rich in secondary metabolites, growth-inhibiting and -stimulating substances (Pegg 1976; Yoder 1980). Most toxins produced by plant pathogenic fungi and bacteria have not yet been rigorously evaluated for their role in disease. The fact that 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 in the culture filtrates, as well as several amino acids and high and low molecular weight phytotoxins in the CF of *P. citrophthora* (Breiman and Barash 1981), supports Yoder's (1983) conclusion that: "Crude filtrates would not necessarily be expected to select plants resistant to disease since growth media that have been colonized by microorganisms (whether pathogenic or not) contain many secondary metabolites which, in combination, can be phytotoxic but have nothing to do with disease development."

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References

Behnke M (1979) Selection of potato callus for resistance to culture filtrates of *Phytophthora infestans* and regeneration of resistant plants. Theor Appl Genet 55:69–71

Behnke M (1980a) General resistance to late blight of *Solanum tuberosum* plants regenerated from callus resistant to culture filtrate of *Phytophthora infestans.* Theor Appl Genet 56:151–152

Behnke M (1980b) Selection of dihaploid potato callus for resistance to the culture filtate of *Fusarium oxysporum.* Z Pflanzenzücht 85:254–258

Breiman A, Barash I (1981) Partial characterization of phytotoxic compounds in culture filtrates of *Phytophthora citrophthora.* Phytopathol Z 102:1–9

Breiman A, Galun E (1981) Plant protoplasts as tools in quantitative assays of phytotoxic compounds from culture filtrates of *Phytophthora citrophthora.* Physiol Plant Pathol 19:181–191

Brettell IRS, Thoma E, Ingram DS (1980) Reversion of Texas male-sterile cytoplasm maize in culture to give fertile T-toxin resistant plants. Theor Appl Genet 58:55–58

Brian PW, Elson GW, Hemming HG, Badley M (1954) The plant growth promoting properties of gibberellic acid, a metabolic product of the fungus *Gibberella fujikuroi.* J Sci Food Agric 5:601–612

Deaton WR, Keyes GJ, Collins GB (1982) Expressed resistance to black shank among tobacco callus cultures. Theor Appl Genet 63:65–70

Durbin RD (1981) Applications. In: Durbin RD (ed) Toxins in plant diseases. Academic Press, New York, pp 495–503

Epstein E, Cohen JD (1981) Microscale preparation of pentafluorobenzyl esters. Electron capture gas chromatographic detection of indole-3-acetic acid from plants. J Chromatogr 209:413–420

Epstein E, Lavee S (1984) Conversion of indole-3-butyric acid to indole-3-acetic acid by cutting of grapevine (*Vitis vinifera*) and olive (*Olea europea*). Plant Cell Physiol 25:697–703

Epstein E, Miles PG (1967) Identification of indole-3-acetic acid in the basidiomycete *Schizophyllum commune.* Plant Physiol 10:405–440

Gengenbach BG, Green CE, Donovan CM (1977) Inheritance of selected pathotoxin resistance in maize plants regenerated from cell culture. Proc Natl Acad Sci USA 74: 5113–5117

Gregory P, Earle ED, Gracen VE (1980) Effects of purified *Helminthosporium maydis* Race T toxin on the structure and function of corn mitochondria and protoplasts. Plant Physiol 66:477–481

Gruen H (1959) Auxins and fungi. Annu Rev Plant Physiol 10:405–440

Johnston JC, Trione EJ (1974) Cytokinin production by the fungi *Taphrina cerasi* and *Taphrina deformans.* Can J Bot 52:1583–1589

Klotz LJ (1978) Fungal, bacterial, and nonparasitic diseases and injuries originating in the seedbed, nursery, and orchard. In: Reuther W, Clair Calavan E, Carman GE (eds) The citrus industry, vol IV. Division of Agricultural Sciences, University of California, Berkeley CA, pp 1–66

Kochba J, Spiegel-Roy P, Saad S (1980) Selection for tolerance to sodium chloride (NaCl) and 2,4-dichlorophenoxyacetic acid (2,4-D) in ovular callus lines of *Citrus sinensis.* In: Sala F, Parisi B, Cella R, Ciferri O (eds) Plant cell cultures: results and perspectives. Elsevier/North-Holland Biochemical Press, Amsterdam pp 187–192

Kono Y, Knoche HW, Daly JM (1981) Structure: fungal host specific. In: Durbin RD (ed) Toxins in plant disease. Academic Press, New York, NY, pp 221–257

Ling DH, Vidhyaseharan P, Borromeo ES, Zapata FJ, Mew TW (1985) In vitro screening of rice germplasm for resistance to brown spot disease using phytotoxin. Theor Appl Genet 71:133–135

Matern V, Strobel G, Shepard J (1978) Reaction to phytotoxins in a potato population derived from mesophyll protoplasts. Proc Natl Acad Sci USA 75:4935–4939

Murashige T, Tucker DPH (1969) Growth factor requirements of *Citrus* tissue culture. in: Chapman HD (ed) Proc 1st Int Citrus Symp, vol 3. Riverside CA, pp 1155–1161

Pegg GF (1976) Endogenous auxins in healthy and diseased plants. In: Heitfuss R, Williams PH (eds) Encyclopedia of plant physiology, new series, vol 4. Springer, Berlin, pp 560–581

Plich M, Rudnicki RM (1979) Studies of the toxins of *Phytophthora cactorum* pathogenic to apple trees. 1. Isolation, some of the properties and activities of a toxin produced by the fungus cultured in vitro. Phytopathol Z 94:270–278

Rines HW, Luke HH (1985) Selection and regeneration of toxin-insensitive plants from tissue cultures of oats (*Avena Sativa*) susceptile to *Helminthosporium victoriae.* Theor Appl Genet 71:16–21

Sacristan MD (1982) Resistance response to *Phoma lingam* of plants regenerated from selected cell and embryogenic cultures of haploid *Brassica napus.* Theor Appl Genet 61:193–200

Shepard J, Bidney D, Shahin E (1980) Potato protoplasts in crop improvement. Science 208:17–24

Spiegel-Roy P, Kochba J, Saad S (1983) Selection for tolerance to 2,4-dichlorophenoxyacetic acid in ovular callus of orange (*Citrus sinensis*). Z Pflanzenphysiol 109:41–48

Vardi A, Raveh D (1976) Cross-feeder experiments between tobacco and orange protoplasts. Z Pflanzenphysiol 78: 350–359

Vardi A, Spiegel-Roy P, Galun E (1982) Plant regeneration from *Citrus* protoplasts: variability in methodological requirements among cultivars and species. Theor Appl Genet 62:171–176

Walton JD, Earle ED (1984) Characterization of the host-specific phytotoxin victorin by high pressure liquid chromatography. Plant Sci Lett 34:231–238

Yoder OC (1980) Toxins in pathogenesis. Annu Rev Phytopathol 18:103–129

Yoder OC (1983) Use of pathogen-produced toxin in genetic engineering of plants and pathogens In: Kosuge T, Meredith CP, Holaende A (eds) Genetic engineering of plants. Basic life sciences, vol 26. Plenum Press, New York, pp 335–353