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The pleiotropic phenotype of tomato cells selected for altered response to *Fusarium oxysporium* f. sp. *lycopersici* cell wall components

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Summary. With the aim of better understanding in vitro host-parasite interactions, tomato cell lines selected for altered response to *Fusarium oxysporum* f. sp. *lycopersici* cell wall components were further characterized. Particularly, their behaviour in dual culture in regard to both fungal inhibition and peroxidase activation was analysed and selected, and control cell clones were screened for esopolysaccharide content and toxin tolerance. Interclonal differences in growth response to 2,4-D and DMSO and the capacity to grow on a medium devoid of hormones (habituation) were taken as parameters representative of physiological variability not directly correlated with the response to pathogens. Significant differences between clones selected for increased (F+) and decreased (F–) response to fungal elicitors were found for pathogen inhibition, peroxidase and esopolysaccharide content, toxin tolerance being reduced in F– but not significantly different from the control in F+. As expected, clonal variability for the response to 2,4-D and DMSO, although significant, was not connected with host-parasite interactions. The data reported thus show that selection for a character (response to elicitors), probably critical for the response to pathogens, may lead to the recovery of genotypes showing a set of modifications suggestive of a cascade of events leading to active defense.

Key words: *Lycopersicon aesculentum* – *Fusarium oxysporum* – Peroxidases – Ion leakage – Esopolysaccharides – Dual cultures

Introduction

In vitro culture of plant cells is known to release a large amount of genetic variability, the spectrum of which (as shown by recent studies of its molecular nature) seems to differ from spontaneous or induced in vivo variation (Buiatti 1989). Particularly mutations affecting plant development seem to be more frequent in vitro than in vivo, with changes such as chromosomal rearrangements, DNA amplification, transposon activation, etc. probably more frequent than gene mutations as a cause of variation. One of the possible explanations of these peculiar features of so-called "somaclonal variation" may be the release of cultured plant cells from ontogenetic constraints, i.e. "diplontic selection" pressure (Gaul 1961), allowing variation spontaneously arising during development to be expressed. If this is true, plant cell cultures should offer good opportunities for the recovery of "novel" variability, provided a good selection system can be devised. Particularly, it should be possible to select variants (mutants) affecting complex developmental patterns, once selection pressure can be applied on key metabolic events at the top of cascades of reactions known to lead to the developmental switches under study.

As discussed by several authors, plant active resistance to pathogens can indeed be considered as the capacity of early switching on, after pathogen recognition, of a complex network of processes eventually leading to the limitation of parasite growth and/or toxic effects.

If, therefore, recognition is indeed one of the key events for active defense, selecting for increased interaction with the pathogen should lead to the obtaining of cells endowed with the capacity to activate, in the presence of the parasite, the metabolic pathways needed for its inhibition.

In a previous paper, we reported on the isolation of tomato cell clones altered in their response to *Fusarium oxysporum* f. sp. *lycopersici* heat-released cell wall components (from now on called "elicitors") (Buiatti et al. 1987). These cell clones were also shown to produce more

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phytoalexins than callus from the parental cultivar when treated with elicitors.

The aim of the present work was to analyse the "physiological phenotype" of the selected clones, with particular attention to structural changes and metabolic processes possibly connected with active defense. For this purpose, a dual culture system was set up (Storti et al. 1988), and high (F+) and low (F–) phytoalexin producer clones were checked for fungal inhibition, esopolysaccharide content, peroxidase induction in the presence of the pathogen and ion leakage in the presence of fusaric acid. As a control experiment, designed to check the existence in culture of unselected spontaneous physiological variability not correlated with defense processes, the capacity of cell clones to grow in the absence of phytohormones in the culture medium (habituation) or in the presence of 2,4-D or DMSO, a substance known to induce habituation (Durante et al. 1984), was tested.

Materials and methods

Plant and fungus cultures. The tomato (*Lycopersicon aesculentum*) cv Red River is a commercial cultivar susceptible to *Fusarium oxysporum* f. sp. *lycopersici*. Establishment and growth of callus cultures as well as the isolation of hypersensitivity plus (F+) and minus (F–) cell lines are described in a previous paper (Buiatti et al. 1987). *Fusarium oxysporum* f. sp. *lycopersici* race 1 was maintained on Czapek Dox broth DIFCO medium.

Dual culture. Dual culture experiments were carried out by sowing *Fusarium* conidia on filter paper disks (6.10^5 per disk), after which they were placed in petri dishes on LS medium supplemented with 0.4 ppm 2,4-D and 1 ppm kinetin at the same distances from calluses of the parental cultivar and of the clone to be tested. Fungal growth in the two directions was then measured after 3 days and the values were compared.

Electrolyte leakage. For ion leakage measurements, 1 g of the tissue to be tested was incubated in 25 ml of a 37 g/l sucrose solution supplemented, or not, with 3 m*M* fusaric acid (Aldrich Chemical). Conductivity was measured with a digital conductivity meter (Top tronic ×74,174) at 2 min intervals for 40 min.

Fluorescence assay with Calcofluor. Callus pieces (0.2 g) from different clones were plated on petri dishes on LS medium containing 0.02% Calcofluor white (Fluorescent brightener, Sigma). Dishes were kept in a growth chamber in the dark at 24 °C for 2 h and then calluses were screened for fluorescence under 365 nm UV light.

Peroxidase visual assay. For visual peroxidase screening, petri dishes were prepared for callus-pathogen cultures, placing a stiff nylon mesh on the surface of the culture medium. Dual cultures were then set up as described above and after 3 h were transferred using the nylon mesh as a holder to LS medium supplemented with 500 mg/ml guaiacol. Dishes were then placed in a growth chamber in the dark at 24 °C for about 3 h. Oxidase activation was then observed by scoring the appearance of red color in calli.

Peroxidase extraction and assay. Soluble and covalently bound peroxidases were extracted from tissues collected after different periods of dual culture, according to Ampomah (1983). For the ionically bound fraction, pellets from the previous centrifugation step were treated three times with 1% Triton X-100. The extracts were desalted through a G-25 Sephadex column (Pharmacia PD-10) equilibrated with phosphate buffer 0.05 *M*, pH 6, before analysis. Protein content was determined by the Bradford reagent method (Bradford 1976). The method used for the spectrophotometrical assay of peroxidase was based on that developed by Haskins (1955).

Peroxidase electrophoresis. Peroxidase isoenzymes were revealed using 7.5% polyacrylamide gel slab electrophoresis with the high pH discontinuous buffer system of Davis (1964). The sample concentration was 10 µg of total proteins for the soluble and 30 µg for the ionically and the covalently bound enzymes, standard consisting of 2 units of horseradish peroxidase (Sigma). Peroxidase staining was performed with guaiacol and H_2O_2 according to Kay and Basile (1987).

Electron microscopy. Very small F+ callus fragments embedded in 4% agar, fixed for 1 h with 4% glutaraldehyde in 0.2 *M* phosphate buffer, pH 7.2, and washed with the same buffer solution, were post-fixed for 2 h in 2% OsO4 solution. Dehydration in ethanol and embedding in Epon-Araldite (FLUKA) were carried out by standard procedures. Sections were made with an LKB IV ultramicrotome, stained with lead citrate and uranyl acetate, and viewed and photographed with an EM 201C Philips working at 80 kV.

Results

As shown in Fig. 1, when calluses from the unselected parental cultivar Red River and F+ cell clones were challenged with a growing *Fusarium*, fungal growth was from 5.3% to 22.58%, less as regards the clone consid-

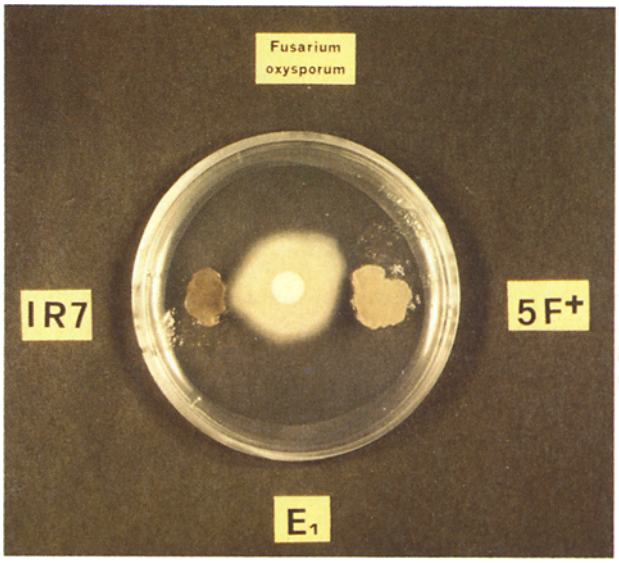

Fig. 1. An example of inhibition of fungal growth in dual culture by cells selected for improved fungal recognition (on the right). The callus to the left of the figure is from the parental unselected cultivar

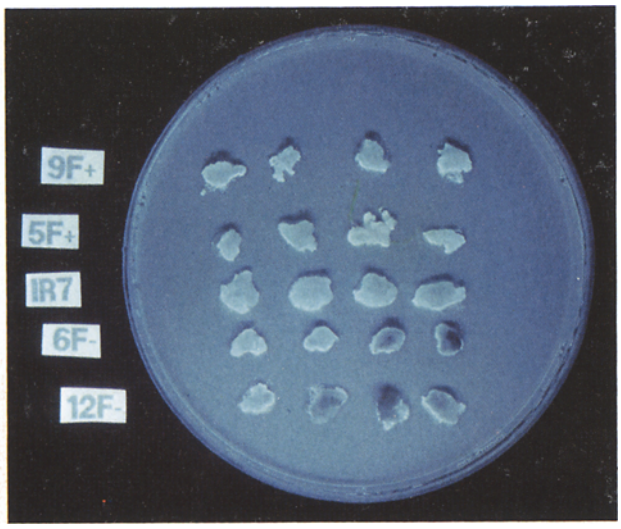

Fig. 2. Fluorescence of callus grown on Calcofluor. 9 F^+ and 5 F+ are cultures selected for better fungal recognition, IR7 the parental cultivar, 12 F− and 6 F− cells selected for resistance to the elicitor

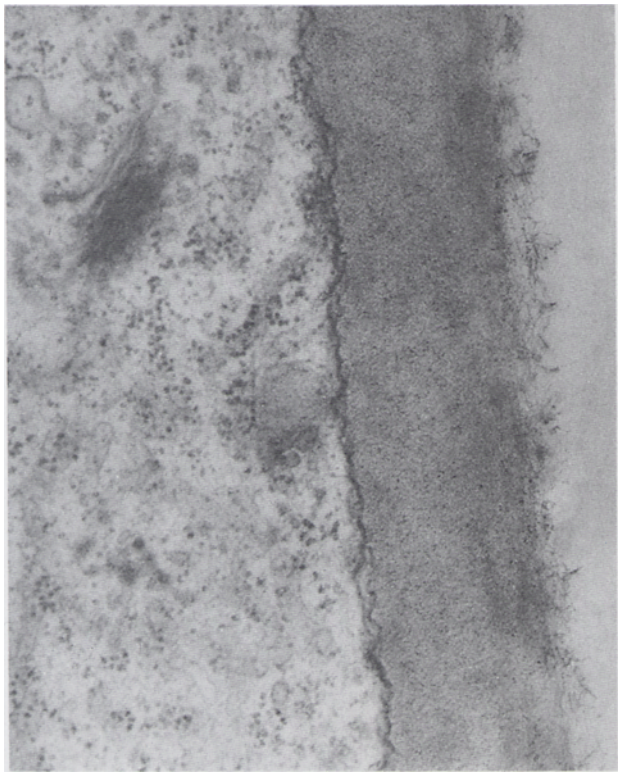

Fig. 3. Electron mycroscopy of an F+ cell (78,000 ×). A fibrillar component on the cell wall is apparent

ered on the side of F+ calluses than on that of Red River inhibition, occurring even in absence of direct contact between plant and fungal cells. A similar experiment in which F− clones were compared to Red River showed a reduction in inhibitory capacity from 2.56% to 11.20% in the presence of cells selected for reduced recognition. Pretreatment of calluses with *Fusarium* elicitor was shown not to increase significantly the inhibitory capacity of tomato cells, thus suggesting the release by the fungus in a dual culture of "eliciting" substances through the medium.

In vivo staining experiments with Calcofluor white (Fig. 2) showed significant differences between F+, F− and Red River cells, suggesting accumulation of beta-linked polysaccharides (Matthysse et al. 1981). Electron microscopy observations show the presence of an unusual fibrillar component spreading out of the cell wall in F+ cells (Fig. 3).

Dual culture experiments and transfer on guaiacol-containing media (see "Materials and methods") showed early intensive staining only in the case of F+ calluses (Fig. 4). Peroxidase activation was confirmed by the analysis of enzymatic activity. Higher levels of soluble, covalently bound and ionically bound peroxidases were shown to occur in F+ strains than in Red River, F− cells activity being drastically reduced also in comparison with the parental cultivar (Figs. 5–7). Particularly in the case of soluble peroxidases, significant activity increases in the presence of the pathogen were shown to occur, with a peak after 16 h of dual culture, a decrease occurring only in F+ clones in the parental cultivar and in 7F−, while values for 6F− remained constant. Increases, as shown by electrophoretic patterns, were more evident for a particular band. A similar situation was observed for ionically bound peroxidases. In this case again an activity peak was apparent, particularly in 5F+ and, to a lower extent, in the parental cultivar. 1F+ activity remained constant but, at higher levels, both F− clones showing a strikingly reduced activity. Electrophoretic analysis revealed a specific increase in one band and the lack of several isozymes in F− clones. Earlier activity peaks (8 h of dual culture) were observed for covalently bound peroxidases in 5F+, the parental genotype 1F+ showing an increase at 16 h and F− remaining at constant, lower levels. Electrophoretic analysis showed the presence of several additional bands in F+ in comparison with Red River, one high molecular weight isozyme being strikingly activated by the presence of the pathogen.

As shown in Fig. 8, where ion leakage from F+, F− clones and a sample of unselected Red River cell clones in the presence of fusaric acid is reported, F− cell clones show a higher susceptibility to the toxic effect, and F+ and Red River are not significantly different.

Finally, Table 1 shows the effects of different hormone balances in the medium, and of the treatment with two concentrations of dimethylsulfoxide (DMSO) on the growth of F+, F− and Red River calluses. Six strains, including Red River (four F+ and one F−), were shown to be able to grow also in the absence of auxins and

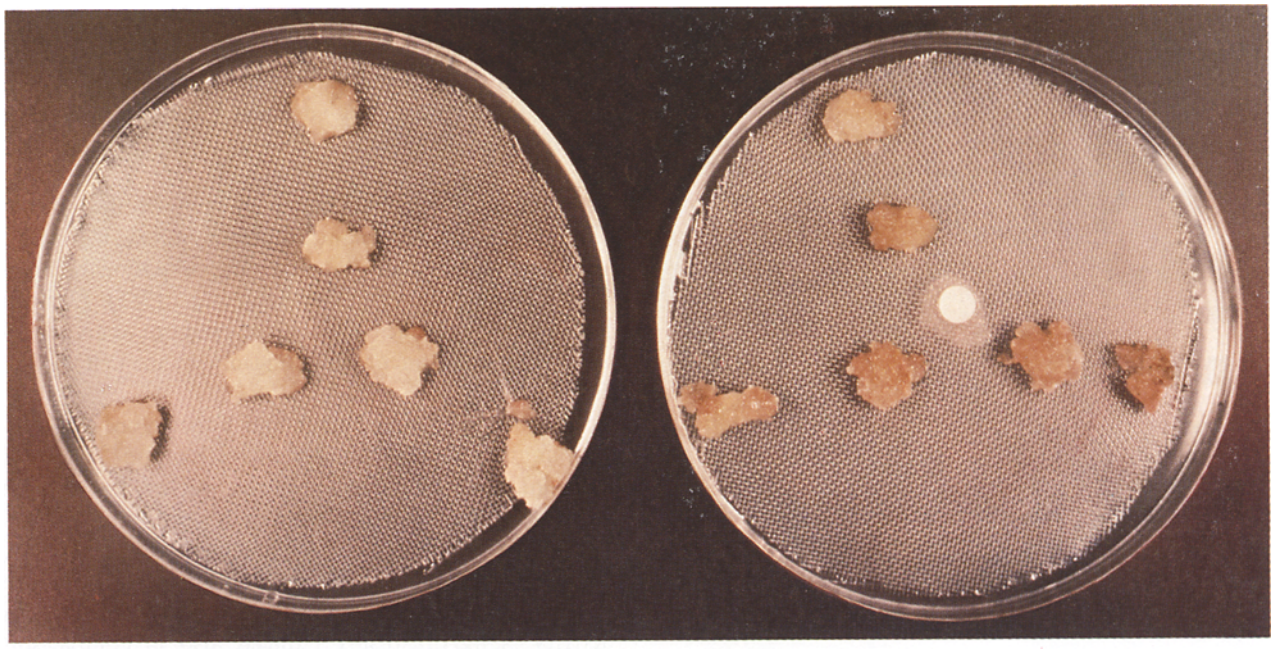

Fig. 4. In vivo peroxidase staining with guaiacol of a culture selected for improved fungal recognition (1 F+) in the presence of the pathogen. To the left, callus of the same strain grown without the fungus

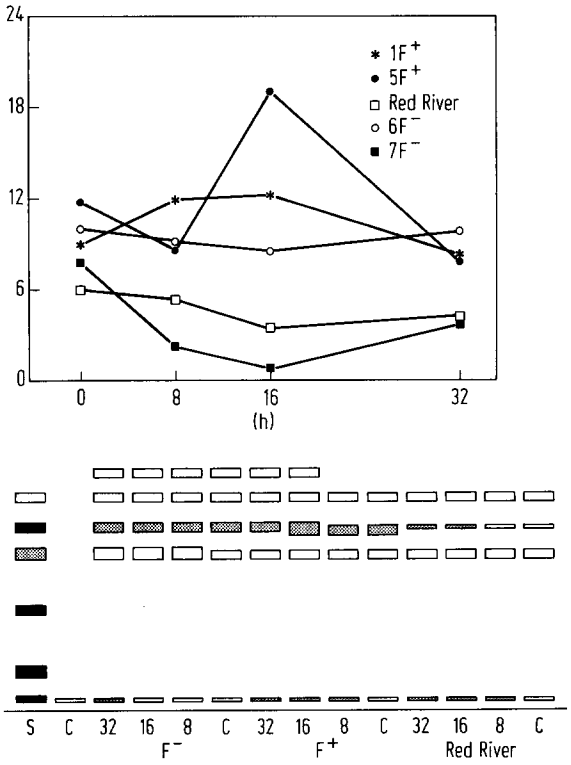

Fig. 5. Soluble peroxidases in cells from two F+, two F− and the parental cultivar grown for different times in the presence of the pathogen

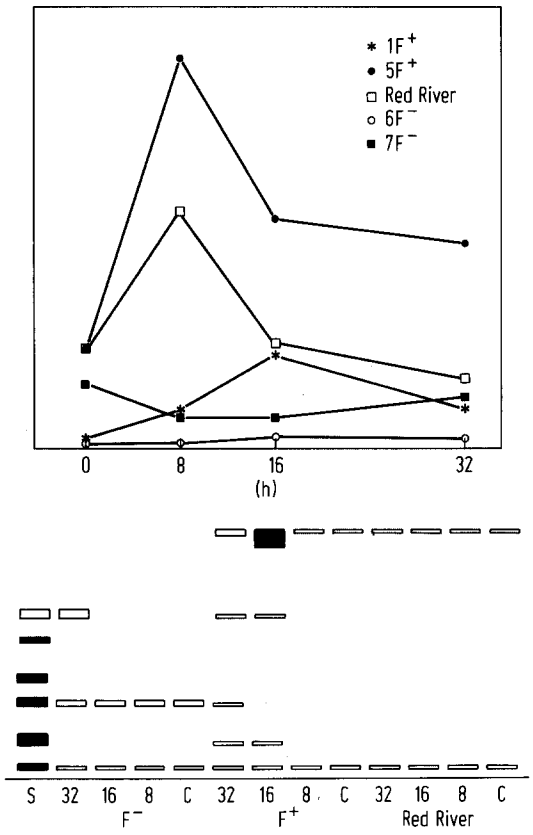

Fig. 6. Covalently bound peroxidases in cells from two F+, two F− and the parental cultivar grown for different times in the presence of the pathogen

Table 1. Growth of F^+, F^- and parental cultivar cells on different media supplement or not with DMSO, 2,4-D, IAA, kinetin in percent of growth on the medium routinely used (see "Materials and methods")

Cell clones	Culture Medium							
	A1 (%)	L.S.o. (%)	DMSO 0.02% (%)	DMSO 0.20% (%)	2,4-D/KIN 1/2 (%)	2,4-D/KIN 2/1 (%)	IAA/KIN 1/4 (%)	IAA/KIN 4/1 (%)
1 F^+	100.00	0.00	19.56	16.48	48.35	43.40	54.90	−9.80
5 F^+	100.00	43.70	28.50	14.55	170.30	232.20	−3.70	37.03
9 F^+	100.00	17.10	11.52	18.20	117.40	113.60	51.10	27.30
12 F^+	100.00	101.30	14.80	25.70	−40.70	−40.70	−7.50	92.50
14 F^+	100.00	10.48	38.40	70.50	−1.80	14.48	49.60	55.86
Red River	100.00	9.25	5.89	29.22	135.60	153.70	47.90	26.60
6 F^-	100.00	83.90	18.90	18.20	84.67	37.90	10.90	11.60
7 F^-	100.00	2.48	−2.66	14.36	−9.45	52.70	20.60	22.40
12 F^-	100.00	−8.70	26.50	−8.30	27.70	13.40	57.10	26.90
21 F^+	100.00	−4.71	−4.81	11.88	−4.50	0.79	31.40	16.20

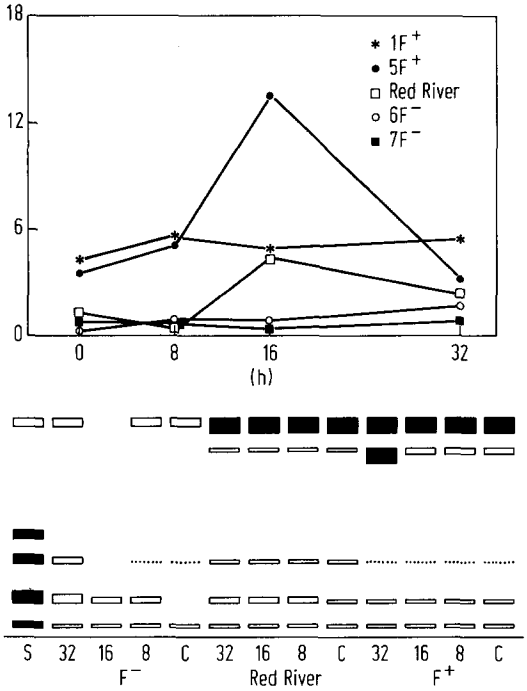

Fig. 7. Ionically bound peroxidases in cells from two F+, two F− and the parental cultivar grown for different times in the presence of the pathogen

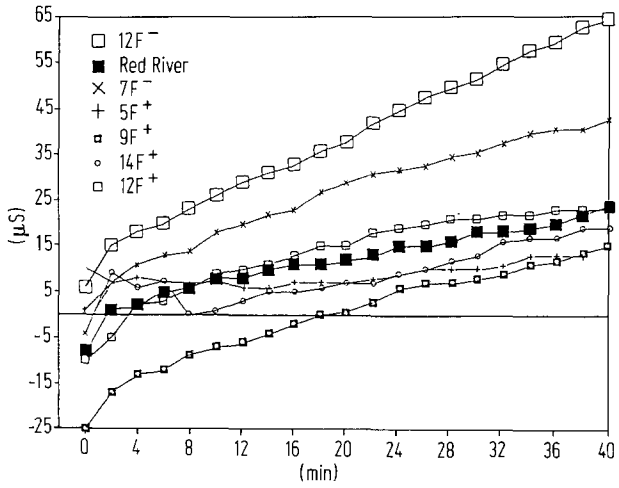

Fig. 8. Ionic leakage from cells selected for increased (F+) and decreased (F−) elicitor recognition in the presence of fusaric acid, compared with that of cells from the parental cultivar (IR7)

cytokinins, with the best hormone balance 0.4 ppm 2,4-D and 1 ppm kinetin in the case of all F− clones, 1F+, 14F+; 0.4 ppm 2,4-D plus 2 ppm kinetin in 9F+; 0.8 ppm 2,4-D and 1 ppm kinetin for the parental cultivar and 5F+. DMSO, moreover, was found to inhibit growth of autotrophic (habituated) strains (5F+, 12 F+, 9F+, 6F−), being inhibitory only at the higher concentration in Red River and inducing habituation in 14F+, 1F+, 7F−, 12F−.

These results, then, confirm the existence of between-clone somaclonal variation for phytohormone requirement, but do not show any correlation of this character with host-parasite interactions.

Discussion

The results of these and earlier experiments (Buiatti et al. 1987) strongly support the hypothesis that the use of *F. oxysporum* cell wall components as selective agents allows the isolation of cell clones modified in a complex network of responses to the pathogen. Cells selected for resistance to the elicitor (F−) show a decreased tolerance to fusaric acid and, both in dual culture and after elicitor

treatment, fail to show active defense reactions. On the other hand F+ cells, besides showing the accumulation of polysaccharidic material on cell walls, react to the pathogen with the induction of peroxidases, the synthesis of phytoalexins and the inhibition of fungal growth.

Variability for hormone requirements and response to DMSO, two characters taken as representative of physiological variation, on the other hand, seem to be present both in clones belonging to the F+ and F– groups.

The synthesis of pathogen-inhibitory substances is known to take part in active defense in vivo and the same is true in some cases such as the systems *Erisiphe polygoni-Pisum* (Kalia and Sharma 1988), *Helminthosporium avenae-Phalaris arundinacea* (Vance et al. 1976) and *Colletotrichum lagenarium-Cucurbitaceae* (Smith and Hammerschmidt 1988) for peroxidase activation. Oxidative enzmyes are certainly only a part of a complex set of proteins which are synthesized after plant-fungal recognition and, thereby, may be a good marker of active defense. In our case, peroxidase activation shows a definite peak at 8 or 16 h of dual culture and seems to be largely due to the specific increase of a few isozymes. Work is in progress for a further characterization of this activity for a better understanding of its possible role in in vitro fungal inhibition. Cell wall polysaccharides may be involved in defense processes (Aist 1983; Mueller and Beckman 1988), while a correlation between toxin tolerance and resistance to pathogens has been shown in many systems (Buiatti and Scala 1984). Calcofluor differential staining of F+ and F– cells and electron mycroscopy data, along with similar differences observed between calli of resistant and susceptible cultivars of potato and carnation (Buiatti et al. 1988), although not yet substantiated by the biochemical characterization of the polysaccharides involved, suggests that elicitor recognition may be somehow connected with beta-linked polysaccharide apposition. This hypothesis is supported by the recent finding that selection for high hexopolysaccharide content also leads to the isolation of clones which exert an inhibitory action on fungal growth in dual cultures (E. Storti et al., unpublished results).

On the other hand, although endogenous phytohormone levels may play a role in host-parasite interactions, variation for the response to auxin/cytokinin balance does not seem to be correlated with the capacity to respond to fungal cell wall components.

Our results, then, suggest that selecting for fungal recognition may result in selection for a series of pathogen-inducible reactions, active defense being a hierarchically controlled complex developmental process elicited by recognition. Furthermore, they also confirm the existence in in vitro cultures of a wide variation for major physiological parameters such as the response to hormone equilibria. Particularly, habituation has been shown to be frequent in tomato cell clones, DMSO inhibitory action on habituated cells being coherent with the hypothesis of "threshold hormone balances" for autonomous growth proposed in an earlier paper (Nacmias et al. 1987).

All the data reported, hence, point to the possibility of devising new, powerful in vitro selection methods for modifications of major developmental processes and of gaining in this way information on such processes, provided unwanted somaclonal variation is discarded. Finally, they open up the possibility of obtaining a series of variants (mutants) modified each at a specific level of a developmental pattern, as active defense may be considered, and using them for the definition of the "priorities" in the cascade of events which characterize that particular pattern.

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