Theor Appl Genet (1992) 84:123–128

THEORETICAL AND APPLIED GENETICS

The in vitro physiological phenotype of tomato resistance to *Fusarium oxysporum* f. sp. *lycopersici*

E. Storti*, C. Latil, S. Salti, P. Bettini, P. Bogani, M.G. Pellegrini, C. Simeti, A. Molnar, and M. Buiatti

Department of Animal Biology and Genetics, Via Romana 17, I-50125 Firenze, Italy

Received April 17, 1991; Accepted October 17, 1991
Communicated by G. Wenzel

Summary. With the aim of dissecting host-parasite interaction processes in the system *Lycopersicon aesculentum-Fusarium oxysporum* f. sp. *lycopersici* we have isolated plant cell mutants having single-step alterations in their defense response. A previous analysis of the physiological phenotypes of mutant cell clones suggested that recognition is the crucial event for active defence, and that polysaccharide content, fungal growth inhibition, peroxidase induction in in vitro dual culture and ion leakage induced by cultural filtrates of the pathogen can be markers of resistance. In this paper we present the results of a similar analysis carried out on cell cultures from one susceptible ('Red River'), one tolerant ('UC 105') and three resistant ('Davis UC 82', 'Heinz', 'UC 90') tomato cultivars. Our data confirm that the differences in the parameters considered are correlated with resistance versus susceptibility in vivo. Therefore, these parameters can be used for early screening in selection programmes. These data, together with those obtained on isolated cell mutants, suggest that the selection in vitro for altered fungal recognition and/or polysaccharide or callose content may lead to in vivo – resistant genotypes. The data are thoroughly discussed with particular attention paid to the importance of polysaccharides in active defense initiation.

Key words: Recognition – Resistance – In vitro selection – Tomato – *Fusarium*

Introduction

The resistance of tomato to *Fusarium oxysporum* f. sp. *lycopersici* has been shown to be correlated with callose and lignin deposition (Matta et al 1970; Friend 1976; Beckmann 1987; Mueller 1988), synthesis of phytoalexins and phenolic compounds (Bell and Mace 1981; Scala et al. 1985; Kroon et al. 1991; Vandermolen et al. 1977), peroxidase induction (Grzelinska et al. 1976), toxin tolerance (Shahin and Spivey 1986) and restriction of fungal growth on callus (Kroon et al. 1991). However, few and conflicting data are available on the involvement of these processes in resistance and, eventually, in defense. As a result no conclusion can be drawn on the role of single mechanisms in the hierarchy of the defense reaction, which seems, in this system as in others, to be under the control of single, dominant, alleles (Beckman 1987). In vitro cultures could be a good system by which to tackle this problem. In order to study the *Fusarium*-tomato interaction a standardized dual culture method has been developed. Techniques for the in vitro screening of most of the forementioned parameters have been already defined (Buiatti et al. 1987; Storti et al. 1990), and this system has been successfully used for the isolation of cell mutants with increased or decreased hypersensitive reaction, high and low polysaccharide, callose and peroxidase content (Buiatti et al. 1987; Storti et al. 1990; Storti in preparation). These mutants would then be used for a dissection of in vitro host-parasite interactions with a rationale similar to that used in classical biochemical genetics to dissect metabolic patterns-with mutants impaired in single steps of the pathway to be studied (Storti et al. 1989, 1990).

Preliminary data suggest that in vitro recognition and polysaccharide content are the limiting factors for defense. In fact, when both parameter increase mutants display the whole spectrum of defense reactions. Correlations between active defense, peroxidase synthesis and toxin tolerance have also been found. Nevertheless, these events can be necessary but not sufficient for resistance,

* Correspondence to: E. Storti

because high peroxidase and toxin-tolerant cell mutants do not inhibit the pathogen and do not show any improvement in other parameters indicative of the hypersensitive reaction (Storti et al. 1989; 1990).

Our data suggest that resistance should occur in high recognition and high in vitro polysaccharide-producing genotypes. These two characters should induce the whole cascade of events leading to active defense, and their action should be favoured by toxin tolerance (Buiatti and Ingram 1991).

In vivo-resistant genotypes should show the whole spectrum of in vitro reactions such as high recognition and high polysaccharide-producing mutants. The aim of the investigation reported in this paper was therefore to test this hypothesis with in vitro cultures of tomato genotypes known to be resistant to *F. oxysporum* f. sp. *lycopersici* in vivo. For this purpose, in vitro cultures from one moderately tolerant ('UC 105') and one susceptible ('Red River'), three resistant ('Heinz 2274', 'Davis UC 82', 'UC 90'), tomato cultivars were challenged in dual culture with the pathogen and screened for polysaccharide and callose content, fungal inhibition, peroxidase induction and ion leakage in the presence of fusaric acid.

Materials and methods

Plant and fungus cultures

The commercial tomato (*Lycopersicon aesculentum*) cultivars used were 'UC 90', 'Davis UC 82' and 'Heinz 2274', resistant to *Fusarium oxysporum* f. sp. *lycopersici* race 1; 'UC 105', tolerant; and 'Red River', susceptible.

The establishment and growth of the callus cultures have been previously described (Buiatti et al. 1987). *F. oxysporum* f. sp. *lycopersici* race 1 was maintained on a Czapek Dox broth Difco medium.

In vivo staining of polysaccharides with calcofluor

Callus explants from the different tomato cultivars were plated on petri dishes containing LS medium supplemented with 0.02% (w/v) calcofluor white (Fluorescent Brightener, Sigma). The dishes were incubated in a growth chamber in the dark at 24 °C for 2 h, and then the calluses were screened for fluorescence under UV light at 365 nm.

In vivo staining of callose with aniline blue

Callus pieces from the different tomato cultivars were plated on petri dishes containing medium consisting of 0.1 *M*, Na_2PO_4 buffer pH 8.5, supplemented with 0.1% (w/v) aniline blue and 7 g/l Difco agar. Dishes were incubated in a growth chamber in the dark at 24 °C for 10 min, and calluses were then screened for fluorescence under UV light at 365 nm.

Dual culture

Dual culture experiments were carried out by sowing *F. oxysporum* conidia on filter paper disks (3.10^5 conidia/disk). The discs were then plated on petri dishes containing medium supplemented with 0.4 ppm 2,4-D and 1 ppm Kinetin at the same distance from one explant of each of two cultivars to be tested; one resistant, the other susceptible. Fungal growth in the two directions was measured after 3 days, and values were compared.

Preparation of heat-released mycelial cell-wall components (elicitors) and callus treatment

F. oxysporum f. sp. *lycopersici* race 1 was surface cultured in 150 ml Czapek Dox Broth Difco in 500-cc conical flasks at 24 °C for 21 days. Heat-released cell-wall components (elicitors) were isolated as described previously (Buiatti et al. 1985). The concentration of an elicitor was measured with the phenol-sulfuric acid method (Hodge and Hofreiter 1962) and expressed as glucose equivalents. The elicitor was then concentrated to 1 g fresh-weight (f.w.) mycelium/1 ml distilled water and filtered through a 0.45-μm Millipore. Seven- to ten-day-old callus pieces (about 1 g f.w.) of the different cultivars were treated with 100 μl of elicitor solution. Controls were treated with distilled water in the same manner. Cultures were incubated in a growth chamber at 24 °C under continuous light, and browning caused by elicitor treatment was visually evaluated after 24 h.

Electrolyte leakage

For ion leakage measurements, 1 g of the callus 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 X74174) every 2 min for 40 min.

Peroxidase extraction and assay

Soluble, ionically bound and covalently bound peroxidases were extracted from calluses of the different cultivars as previously described (Storti et al. 1989). Protein content was determined by the Bradford reagent method (Bradford 1976). The method used for the spectrophotometrical assay of peroxidase activity was based on that developed by Haskins (1955). Each experiment was independently repeated twice.

Peroxidase electrophoresis

Peroxidase isoenzymes were analysed as previously described (Storti et al. 1989). Peroxidase staining was performed with guaiacol and H_2O_2 according to Kay and Basile (1987).

Results

As a preliminary step prior to biochemical analysis, calli from the different cultivars were tested for fungal recognition capacity by scoring for hypersensitive browning after treatment with fungal elicitor. The susceptible cv 'Red River' and the tolerant cv 'UC 105' lacked the hypersensitive reaction, while the resistant cvs 'Heinz', 'Davis UC 82' and 'UC 90' showed intense browning (Fig. 1 a).

To investigate the possibility that calli from the resistant cultivars possess a higher content of polysaccharides and callose than the susceptible ones, in vivo staining experiments were performed by using, respectively, cal-

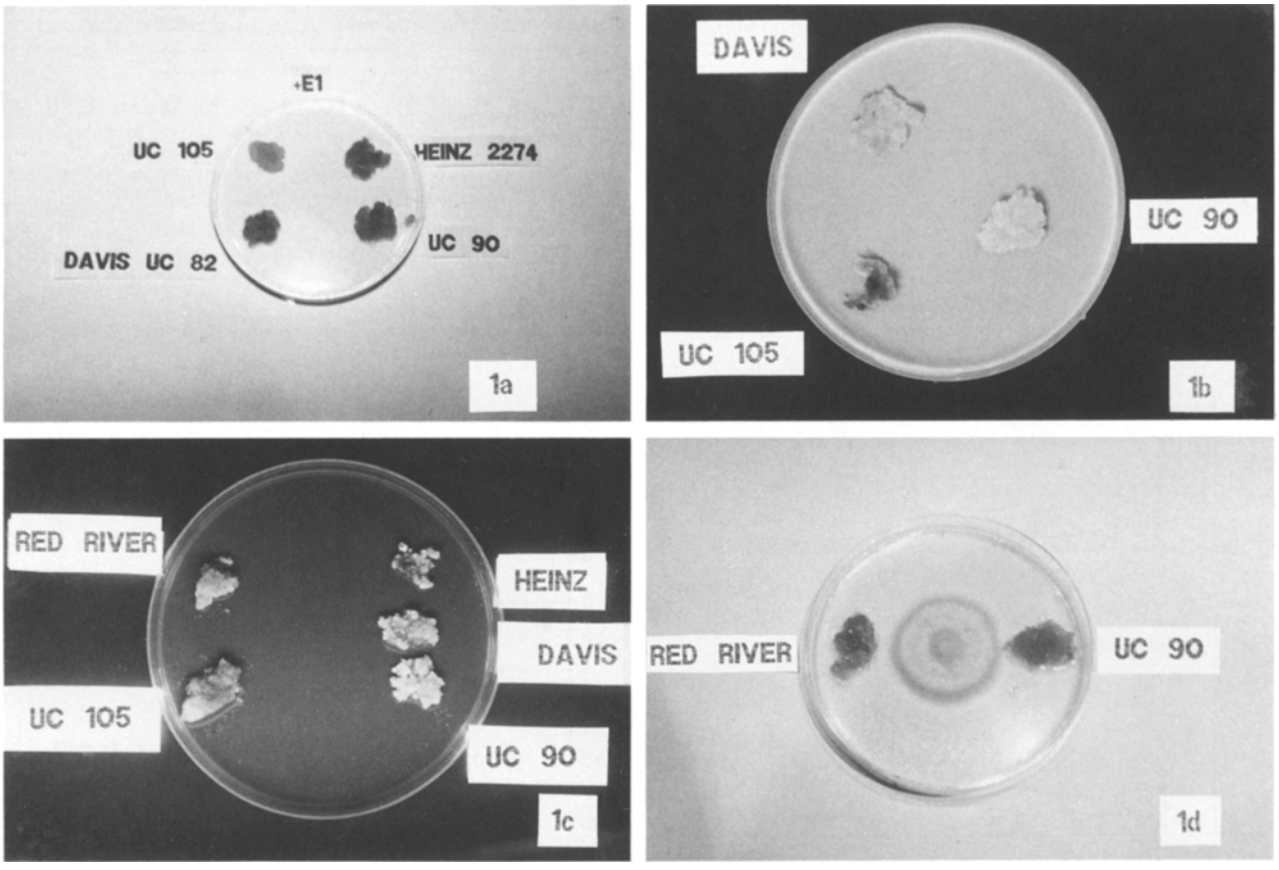

Fig. 1. **a** Browning of calluses of resistant ('Heinz', 'Davis UC 82', 'UC 90') and moderately tolerant ('UC 105') cultivars of tomato after treatment with *Fusarium oxysporum* cell-wall components. **b** Fluorescence of growing calluses on calcofluor white. **c** Fluorescence of growing calluses on aniline blue. **d** Inhibition of fungal growth in dual culture by callus of the resistant cv 'UC 90'. The callus on the *left* is from the susceptible cv 'Red River'

cofluor white and aniline blue. Figures 1 b and c show that 'Heinz', 'UC 90' and 'Davis UC 82' calluses had a higher UV fluorescence intensity on dye-containing media than the susceptible 'Red River' and 'UC 105'.

The inhibition of fungal growth was analysed in dual culture experiments by challenging calluses from resistant and susceptible genotypes with the pathogen. A typical result is shown in Fig. 1 d: the shape of the mycelium indicates that only the resistant cv 'UC 90' was able to inhibit fungal growth; the same result was obtained with 'Heinz' and 'Davis UC 82'. A quantitative analysis of *F. oxysporum* inhibition was also performed by evaluating the distance between the mycelium and the callus border. The results (Table 1) show that *Fusarium* growth inhibition occurred only in the presence of calli from resistant genotypes. On the contrary, tolerant 'UC 105' and susceptible 'Red River' were unable to influence fungal growth.

In order to analyse the peroxidase content, calli from different cultivars were challenged with the pathogen in dual culture for 0, 8, 16 and 32 h. Samples corresponding to soluble, ionically bound and covalently bound peroxidases were used both for the spectrophotometrical assay of enzyme activity and for the electrophoretic analysis of isoenzymatic patterns.

Table 1. Measurement of inhibition of fungal growth in dual culture experiments of resistant ('Heinz', 'Davis UC 82', 'UC 90'), moderately tolerant ('UC 105') and susceptible ('Red River') cultivars

Cultivars		$\frac{D-R}{D}$	
A	B	A	B
Heinz	UC 105	0.650	0.590
Davis	UC 105	0.665	0.620
UC 90	UC 105	0.610	0.560
Heinz	Red River	0.640	0.590
Davis	Red River	0.645	0.600
UC 90	Red River	0.650	0.580

R, Radius of mycelium; D, distance between the center of the filter disc and the border of the callus

The results for enzyme activity are reported in Fig. 2 a (soluble peroxidases), b (ionically bound peroxidases)

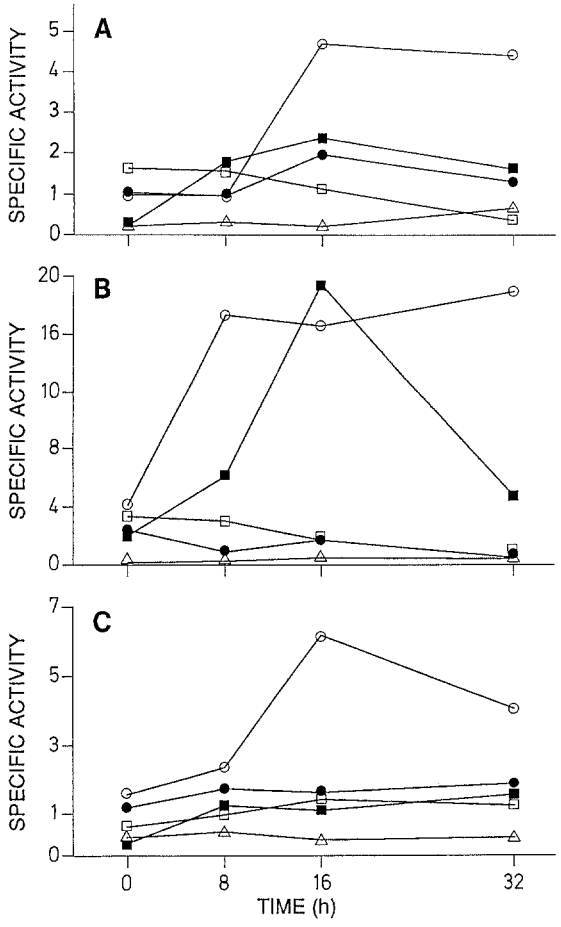

Fig. 2. Activity of soluble (**A**), ionically bound (**B**), covalently bound (**c**) peroxidases from calli of resistant ('Heinz', 'Davis UC 82', 'UC 90'), moderately tolerant ('UC 105') and susceptible ('Red River') cultivars grown for different times in the presence of the pathogen

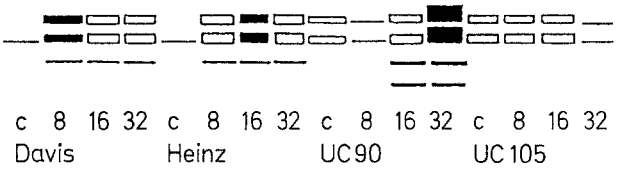

Fig. 3. Electrophoretic pattern of covalently bound peroxidases from calli of resistant ('Heinz', 'Davis UC 82', 'UC 90') and moderately tolerant ('UC 105') cultivars grown for different times in the presence of the pathogen

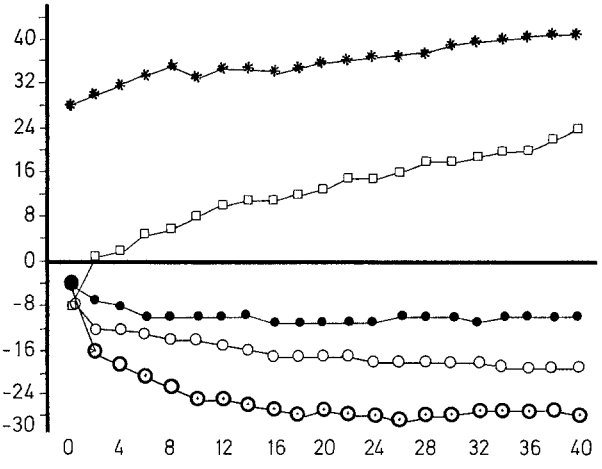

Fig. 4. Ion leakage from calli of resistant ('Heinz', 'Davis UC 82', 'UC 90'), moderately tolerant ('UC 105') and susceptible ('Red River') cultivars in the presence of fusaric acid

and c (covalently bound peroxidases). In all cases constitutive values were not significantly different and did not correlate with resistance. The resistant cv 'UC 90' showed a high level of induction for all classes of enzymes. The induction of ionically bound peroxidases occurred after 8 h of dual culture, while both the soluble and covalently bound peroxidase fractions were induced at 16 h. The resistant cv 'Davis UC 82' also showed a peak of enzyme activity for the ionically bound peroxidase fraction. While the level of induction was slightly lower for the other resistant cultivars examined, it was higher than that obtained for the susceptible cv 'Red River'.

The electrophoretic analysis of peroxidase isoenzymes was carried out on the three resistant cultivars and tolerant 'UC 105'. No difference was detected between cultivars in their isoenzyme electrophoretic patterns for the soluble and ionically bound fractions (data not shown). The differential staining of electrophoretic bands also confirmed the abovementioned quantitative differences.

Dual culture appeared to induce the synthesis of one additional isoenzyme in the covalently bound fraction of all the resistant cultivars examined (Fig. 3.). Moreover, only 'UC 90' showed a band that could be detected after 16 h of dual culture, the time of the induction of enzyme activity (Fig. 3). The supernumerary bands observed in 'UC 90' were always absent in 'UC 105'.

Finally, the resistant cultivars were also found to be tolerant to fusaric acid, as shown by the lower ion release observed in these genotyes in comparison with 'UC 105' and 'Red River' (Fig. 4).

Discussion

Previous work carried out in our laboratory had shown that tomato cell mutants selected for a high hypersensitivity response to *Fusarium oxysporum* f. sp. *lycopersici* had a pleiotropic phenotype, i.e. high polysaccharide and callose content, peroxidase induction, phytoalexin synthesis and pathogen inhibition in dual culture (Buiatti et al. 1987; Storti et al. 1989).

Furthermore, the same phenotype was also observed in cell mutants selected for high polysaccharide content, which were also found to be more tolerant to toxins. On the other hand, cell variants selected for high constitutive and inducible peroxidase activity were modified just in this specific character (Storti et al. 1989, 1990).

The data reported in this paper show that the highly resistant cvs 'Heinz 2274', 'UC 90' and 'Davis UC 82' display in vitro the same physiological phenotype as cell mutants selected for improved pathogen recognition and high polysaccharide content. On the contrary, both the moderately tolerant cv 'UC 105' and susceptible 'Red River' had a low polysaccharide content and did not show any hypersensitive reaction when treated with fungal elicitor.

In our system all the parameters analysed seem to be good markers of resistance versus susceptibility in vivo. Moreover, being easily scored, they can be useful in breeding programmes as early screening criteria in vitro.

The data also suggest that single genes controlling resistance to *Fusarium* in tomato (Beckman 1987) may act through recognition events someway favoured by high in vitro polysaccharide content. This leads to a complex hypersensitive phenotype including phytoalexin synthesis and increased peroxidase activity. Fusaric acid tolerance has been shown to occur only in in vivo resistant cultivars, but not necessarily in isolated cell mutants.

Therefore, peroxidase induction may be a consequence of recognition, while toxin tolerance may be important in vivo to protect the plant from the inhibition of active defense processes by the pathogen as suggested by Buiatti and Ingram (1991). The mechanism through which high polysaccharide (callose?) content or their de novo synthesis may play such an important role in triggering defense processes still remains rather obscure. However this feature seems to be common to other systems, like *Diantus caryophillus – Fusarium oxysporum* f. sp. *dianthi* and *Solanum tuberosum – Alternaria alternata*, which have been studied in our laboratory (unpublished results).

Callose and polysaccharide have been found to be at higher levels in incompatible interactions and to accumulate early after infection (Matta et al. 1970; Beckman et al. 1982; Mueller et al. 1988). In our case the difference between resistant and susceptible cultivars is constitutive in vitro, i.e. independent from the presence of the pathogen. This may be due to a differential in vitro synthetic ability because the leaves and internodes of non-infected resistant and susceptible plants do not show any difference in this character (data not shown).

It should be stressed, however, that the presence of high levels of polysaccharides in incompatible interactions should be generally considered as evidence for a direct inhibition of the fungus by these compounds. The polysaccharides may act through the formation of physical barriers, thus denying any real role in active defense. On the other hand, since the initial work by Darvill and Albersheim (1984), vast evidence has been collected suggesting that oligosaccharins derived from cell-wall degradation may act as secondary messengers for the hypersensitive response to pathogens. Whether there is any connection between these independent results and our work remains to be ascertained.

Finally, it should be noted that none of the parameters studied suggest any difference in the behavior of the tolerant cv 'UC 105' from the fully susceptible cv 'Red River'.

'UC 105' has been reported alternatively as resistant or susceptible, and it is considered to show some degree of tolerance. The biochemical basis of this behaviour is, to our knowledge, obscure.

Acknowledgements. The authors are grateful to Dr. E. Castigli (Harvard Medical School, Boston, Mass.) for revising the English of the manuscript, to R. Innocenti and S. Bambi for the photographic work, and to C. Giuliani for graphical work.

Reference

Beckman CH (1987) Genetic variation in the host and pathogen. In: The nature of wilt disease of plants. Academic Press New York, pp 103–116

Beckman CH, Mueller WC, Tessiar BJ, Harrison NA (1982) Recognition and callose deposition in response to vascular infection in *Fusarium* wilt resistant or susceptible tomato plants. Physiol Plant Pathol 20:1–10

Bell DA, Mace ME (1981) Biochemistry and physiology of resistance. In: Mace ME, Bell AA, Beckman CH (eds) Fungal wilt diseases of plants. Academic Press, New York, pp 431–486

Bradford M (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein using the principles of protein-dye binding. Anal Biochem 72:248–254

Buiatti M, Ingram DS (1991) Phytotoxins as tools in breeding and selection of disease resistant plants. Experientia 47:811–819

Buiatti M, Scala A, Bettini P, Nascari G, Morpurgo R, Bogani P, Pellegrini MG, Gimelli F, Venturo R (1985) Correlation between in vivo resistance to *Fusarium* and in vitro response to fungal elicitors and toxic substances in carnation. Theor Appl Genet 70:42–47

Buiatti M, Simeti C, Marcheschi G, Scala A, Bettini P, Bogani P, Pellegrini MG (1987) Isolation of tomato cell lines with altered response to *Fusarium* cell wall components. Theor Appl Genet 75:37–40

Darvill AG, Albersheim P (1984) Phytoalexins and their elicitors – a defense against microbial infection in plant. Annu Rev Plant Physiol 35:243–298

Friend J (1976) Lignification in infected tissue. In: Friend J, Threlfall DR (eds) Biochemical aspects of plant parasite relationship. Academic Press, New York, pp 291–303

Grzelinska A (1976) Peroxidase isoenzyme in *Fusarium* infected tomato plant. 73:154–159

Haskins F (1955) Changes in the activities of several enzymes during germination and seedling development in corn (*Zea mays* L.). Plant Physiol 30:74–79

Hodge JE, Hofreiter BT (1962) Determination of reducing sugars and carbohydrates. In: Methods in carbohydrates chemistry ed. by Whister RL, Wolfram ML. Vol. 1, Academic Press New York, pp 380–394

Kay LE, Basile DV (1987) Specific peroxidase isoenzymes are correlated with organogenesis. Plant Physiol 84:99–105

Kroon BAM, Scheffer RJ, Elgersma DM (1991) Interactions between *Fusarium oxysporum* f. sp. *lycopersici* and callus of susceptible and resistant tomato lines: fungal growth and phytoalexin accumulation (in press)

Matta A, Gentile I, Giai I (1970) Differential inhibition and activation in healthy and *Fusarium*-infected tomato plants. Phytopathol Mediterr 9:168–175

Mueller WC, Beckman CH (1988) Correlated light and electron microscope studies of callose deposits in vascular parenchyma cells of tomato plants inoculated with *Fusarium oxysporum* f. sp. *lycopersici*. Physiol Mol Plant Pathol 33:201–209

Scala A, Bettini P, Bogani P, Pellegrini MG, Tognoni F (1985) Tomato-*Fusarium* interaction: in vitro analysis of several possible pathogenic factors. Phytopathol Z 113:90–94

Shahin EA, Spivey R (1986) A single dominant gene for *Fusarium* wilt resistance in protoplast-derived tomato plants. Theor Appl Genet 73:154–159

Storti E, Bogani P, Bettini P, Bonzi Morassi L, Pellegrini MG, Matteo M, Simeti C, Buiatti M (1989) The pleiotropic phenotype of tomato cells selected for altered response to *Fusarium oxysporum* f. sp. *lycopersici* cell wall components. Theor Appl Genet 78:689–695

Storti E, Buiatti M, Bettini P, Pellegrini MG, Bogani P, Matteo M, Simeti C (1990) Active defense processes and in vitro selection for resistance to pathogens. Acta Hortic 280:361–366

Vandermolen GE, Beckman CH, Rodehorst E (1977) Vascular gelation a general response phenomenon following infection. Physiol Plant Pathol 11:95–100