Differential Responses of Potato Cell Suspensions to a Culture Filtrate of *Phytophthora infestans*

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General (polygenic) resistance of plant hosts to an attack by a range of pathogens is an important feature of plant defense responses against the infection. In search of biochemical markers defining this resistance, cell suspensions derived from leaves of potato (*Solanum tuberosum* L.) cvs. Tarpan and Bzura that are polygenically susceptible and resistant to *Phytophthora infestans*, respectively, were inoculated with culture filtrate (CF) of the fungus. Cell suspension of Tarpan responded to CF treatment by a higher extracellular alkalinization and more significant reduction in their viability and growth than those of the Bzura cultivar. The stimulation of phenylalanine ammonia-lyase (PAL) activity, but not of β-1,3-glucanase, was significantly higher in CF treated Bzura cells than in Tarpan ones. The obtained results suggest that sensitivity to the fungal toxins and variation of PAL activity may represent useful markers for the evaluation of polygenic resistance in potato.

Introduction

Interactions between plant hosts and their pathogens or pathogen cell free extracts or specific metabolites, known as elicitors, trigger several plant defense responses. Very early responses, considered to be involved in recognition and initial signalling events, include changes in plasma membrane potential and ion fluxes (Atkinson *et al.*, 1990; Baker *et al.*, 1993; Kombrink and Somssich, 1995). Subsequently, various biochemical pathways are activated, i.e. phenylpropanoid pathways [where phenylalanine ammonia-lyase (PAL) is the key enzyme], synthesis of antimicrobial substances, "phytoalexins" and accumulation of pathogenesis-related (PR) proteins comprising a wide range of different plant defense proteins, including hydrolytic enzymes, β-1,3-glucanase and chitinase (Kombrink and Somssich, 1995).

Cell-free extracts as well as metabolites of plant pathogens, commonly known as "elicitors", trigger the same biochemical responses in plants and cultured cells as does infection by pathogens (Ebel and Cosio, 1994; Boller, 1995). In studies on defense responses in potato tissues, culture filtrate (CF) and mycelial homogenate (MH) of *P. infestans* as well as arachidonic acid (AA) have been used as biotic elicitors (Friend, 1991). The CF contains a number of fungal metabolites, including polysaccharides and glycoproteins (Stolle and Schuber, 1985; Davidse *et al.* 1986; Huet *et al.*, 1994), MH is a mixture of hyphal components, whereas AA is an unsaturated fatty acid present in the cell wall of *P. infestans* but absent in potato tissues (Bostock *et al.*, 1981). In our early studies, detached leaflets of potato cvs. Tarpan and Bzura that are polygenically susceptible and resistant to *P. infestans*, respectively (Swiezynski *et al.*, 1993), were treated with AA, CF and MH. The leaflets of Bzura cv. developed earlier and showed more necrosis than those of Tarpan after these treatments (Awan *et al.*, 1995). These results were in agreement with the observations that leaves of potato cvs. with a high level of general resistance were highly sensitive to the components of CF from *P. infestans* (Davidse *et al.*, 1986).

To investigate further the general potato resistance to *P. infestans*, in the present study cell sus-

Abbreviations: AA, arachidonic acid; CF, culture filtrate; MH, mycelial homogenate; MTT, 3-(4,5 dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide; PAL, phenylalanine ammonia-lyase; PR, pathogenesis-related.

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pension cultures of both cvs., Tarpan and Bzura, established from leaf-derived calli, were used. Cultured cell suspensions with uniform cell type, defined growth stage and synchronous response to stimulation are frequently used as models for the characterization of defense responses at biochemical and molecular levels (Kombrink and Somssich, 1995). We report here the kinetics and changes in extracellular pH, cell viability, cell growth and enzymatic activities in cell suspensions treated with CF of *P. infestans*. In preliminary studies, we had found that the response of cell suspensions from both cvs. to the treatments with AA and MH was either less or similar to that with CF (Maciejewska *et al.*, 1995).

Materials and Methods

Potato cell suspensions

Cell suspension cultures of both cvs. Tarpan and Bzura, established from leaf-derived calli, were maintained on MS medium (Murashige and Skoog, 1962) supplemented with vitamins (Gamborg *et al.*, 1968), glycine (2 mg l^{-1}), hydrolysed casein (500 mg l^{-1}), sucrose (3%) and phytohormones: 2,4-D (2 mg l^{-1}), NAA (2 mg l^{-1}) and BAP (0.1 mg l^{-1}). The cultures were kept on a rotary shaker (120 r min^{-1}) at 26 °C in the dark and subculturing in the fresh medium was done every 8th day. Cell cultures in the log growth phase, 7–8 days after subculturing, were used for all experiments, except the determination of cell growth, where cell suspensions were used just after subculturing.

Fungal growth and preparation of culture filtrate

P. infestans (MP 245, complex race) obtained from Potato Research Institute Mlochów (Poland) was maintained on rye agar medium supplemented with cholesterol (50 μg ml^{-1}) (Hohl, 1991) at 15 °C in the dark. After every three months, the fungus was passed through tuber slices of susceptible potato cv. Tarpan to maintain the virulence. Concentrated CF was prepared from fungi grown for six weeks in the liquid Henniger medium (Henniger, 1959). The medium was separated from the fungus by filtration, the filtrate dialyzed for 48 h against water at 4 °C, the dialysate lyophilized, the residue taken up in one-tenth of the initial volume and the resulting solution filter-sterilized. The CF was quantified as μg glucose equivalents per ml. Carbohydrate content was determined by colorimetric method using glucose as standard (Dubois *et al.*, 1956).

Extracellular pH

Cells from the cultures were collected by vacuum filtration, washed with the fresh growth medium, sieved to remove the bigger aggregates, again filtered and resuspended (0.1 g fresh wt. ml^{-1}) in the fresh medium. Aliquots of 20 ml were taken in 100 ml Erlenmeyer flasks and preincubated for 2 h on a shaker (120 r min^{-1}) at 26 °C. These cell suspensions were inoculated with CF (10 μg glucose equivalents ml^{-1}). The controls were treated with distilled water. Extracellular pH was recorded by glass electrode fitted with digital monitor (Pharmacia).

Cell viability

Cells were prepared and treated with CF as described above. The viability of cell suspensions was determined by MTT [3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (Aldrich) colorimetric assay (Sanchez *et al.*, 1994). A 50 μl of MTT solution (5 mg ml^{-1}) was added to 500 μl of cell suspension. After 2 h incubation at 26 °C, the mixture was supplemented with 500 μl isopropanol containing 0.04 N HCl and centrifuged at 2000 r min^{-1} for 10 min. The absorbance (test wavelength 570 nm – reference wavelength 630 nm) in control was taken as 100% viability and cell viability (%) was calculated from the ratio of the value for the treated cells to that in control.

Cell growth

After subculturing, 70 ml cell suspensions were taken in 300 ml Erlenmeyer flasks. Two days later, CF (5 μg glucose equivalents ml^{-1}) or sterile distilled water was added to the cell cultures. Cell growth was determined in 10 ml aliquots from zero to eight days after subculturing with two days intervals. For determination of dry weight, cells obtained by vacuum filtration were incubated at 60 °C until the constant weight was achieved.

Cell suspension treatment, extraction and enzyme assay

Aliquots (20 ml) of cell suspension in the log growth phase were treated with CF (10 µg glucose equivalents ml^{-1}); to the controls sterile distilled water was added. After specific time period of the treatment, cells were collected by vacuum filtration, washed with distilled water, freezed in liquid nitrogen and stored at -80 °C.

PAL and β-1,3-glucanase assays

For PAL assay, frozen cells (0.5 g) were ground in liquid nitrogen and homogenized in 2 ml of 0.2 M borate (Na^+) buffer, pH 8.8, containing 14 mM 2-mercaptoethanol. The extract was centrifuged at 14,000 x *g* for 20 min. PAL activity in the supernatant was determined using colorimetric method (Havir and Hanson, 1968). The reaction mixture of 4 ml containing the enzyme extract (200–300 µg proteins), 0.12 M borate buffer, pH 8.8 and 22 mM L-phenylalanine was incubated at 30 °C for 60 min. The content of trans-cinnamic acid was determined at 290 nm and calculated using molar coefficient (Havir and Hanson, 1968).

For β-1,3-glucanase assay, frozen cells (1.0 g) were ground in liquid nitrogen and homogenized in 3 ml of 0.5 M sodium acetate buffer, pH 5.2. The extract was centrifuged at 14,000 x *g* for 20 min and supernatant desalted on Sephadex G-25 column. β-1,3-glucanase activity in the pooled elution fractions containing protein was determined essentially as described by Kombrink and Hahlbrock (1986), except that reducing sugars were determined with 4-hydroxybenzoic acid hydrazide (Lever, 1972).

The activities of both enzymes were calculated in katals, i.e. mole product released per s, and expressed as relative to controls. Protein was determined by the method of Lowry *et al.* (1951).

Results and Discussion

Suspension cultured cells of potato cultivars possessing different levels of polygenic resistance varied in their responses to CF of *P. infestans*. The CF treatment caused an increase in extracellular pH of both cell suspensions as compared to the controls, where pH of Tarpan cell culture remained constant during the whole experiment, while in Bzura cell suspension it increased slightly after 1 h (Fig. 1). In CF treated Tarpan cell suspension, the increase in pH lasted for up to 6 h of the treatment, reaching at maximum about one unit. In Bzura cells, the pH increase was less pronounced and lasted up to 2 h (Fig. 1). There were no further changes in the response of both cell suspensions treated with twice higher concentration of CF (data not shown).

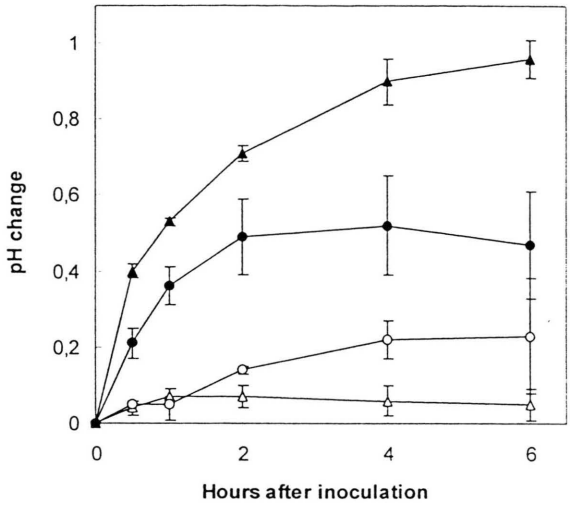

Fig. 1. Time-course of changes in extracellular pH of Tarpan (△, ▲) and Bzura (○, ●) cell suspensions treated with CF (10 µg glucose equivalents ml^{-1}) of *P. infestans* (▲, ●) or distilled water as control (△, ○). Initial pH of both cell suspensions was 4.7–4.8. The interaction of CF with the medium (0.06 unit increase in the pH) has been subtracted from the data. Results are means of two independent experiments; bars indicate SE.

The viability of Tarpan cells was significantly reduced by CF treatment and only 33% cells exhibited viability at 6 h after treatment. In contrast, Bzura cells were slightly affected as 81% cells remained viable at the same time (Table I). The viability of both cells did not decrease further in response to twice higher concentration of CF (data not shown).

An increase in extracellular pH as well as cell death are attributed to recognition of incompatibility in pathogen/non-host and race/cultivar interactions (Atkinson *et al.*, 1990; Baker *et al.*, 1993; Baker and Mock, 1994). In this light, one might expect that the response of Bzura cells to CF treatment should be more pronounced than that of Tarpan ones. However, we should keep in mind that CF of *P. infestans* contains substances that are

Table I. The effect of culture filtrate (CF) of P. infestans *on the viability of cell suspensions of potato cvs. Bzura and Tarpan.* Cells were prepared and treated with CF (10 μg glucose equivalents ml^{-1}) as described in the Materials and Methods. Cell viability was determined by MTT colorimetric method (Sanchez *et al.*, 1994). The value of controls (treated with distilled water) was considered as 100% viability and cell viability (%) was calculated from the ratio of the value for the treated cells to that in controls. Results are means of three independent experiments each consisting of four replicates (± SE).

Time [h]	Cell viability (%)	
	Bzura	Tarpan
0	100	100
0.5	100	71 ± 8
1	100	61 ± 2
2	90	53 ± 3
4	90	40 ± 2
6	81 ± 6	33 ± 1

toxic to potato root cap cells (Galal *et al.*, 1991), leaf tissues (Galal *et al.*, 1991; Huet *et al.*, 1994) as well as to mesophyll protoplasts and anther microspores (Möller *et al.*, 1992). It has also been stated that plasma membrane is the main target for a number of fungal toxins (Kohmoto and Otani, 1991). Therefore, it is conceivable that the changes observed in Tarpan cells are mostly due to damages caused by the fungal toxins and their effects are predominant over possible defense reactions.

To verify further the results obtained for cells' viability after CF treatment, we tested the effect of CF on the cell growth. The CF (5 μg glucose equivalent ml^{-1}) was added to both cell suspensions after two days of subculturing. Using such young cells allowed us to follow their response to CF treatment until reaching the log growth phase. The growth of Tarpan cells was completely blocked just after two days (Fig. 2a), whereas the growth of Bzura cells was not affected as compared to the control (Fig. 2b). A visible browning of Tarpan cells was noted within one day after treatment, while Bzura cells became brown 4–6 days after inoculation, when they reached the log phase of growth. Thus, the cells of both cultivars varied in this response in respect of their age.

Browning of cells is most frequently attributed to activation of phenylpropanoid pathways, including PAL activity (Fritzmeier *et al.*, 1987), processes that are stimulated by different stress conditions,

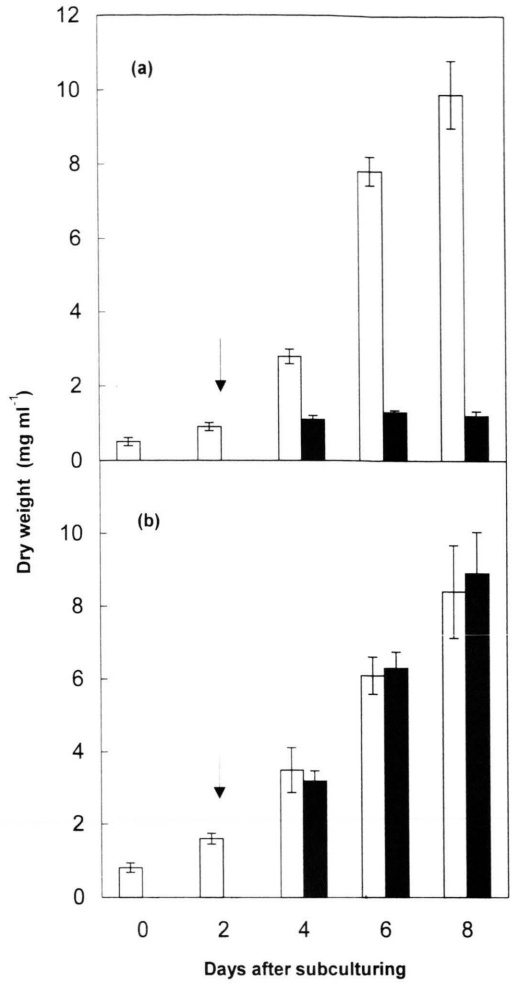

Fig. 2. The growth of (a) Tarpan and (b) Bzura cell suspensions in the presence (black boxes) of CF (5 μg glucose equivalents ml^{-1}) of *P. infestans* that was added after two days of subculturing (↓). Controls (white boxes) were treated with sterile distilled water. Results are means of two independent experiments; bars indicate SE.

including pathogenesis (Dixon and Paiva, 1995). Accordingly, we have measured the activity of PAL in both Tarpan and Bzura cells (Fig. 3a). Although basic PAL activity was almost similar in both cultivars, after CF treatment stimulation of the enzyme activity was higher and more prolonged in resistant Bzura cells than that in susceptible Tarpan ones. The results confirm earlier studies on PAL role in defence response (Fritzmeier *et al.*, 1987; Mozzetti *et al.*, 1995).

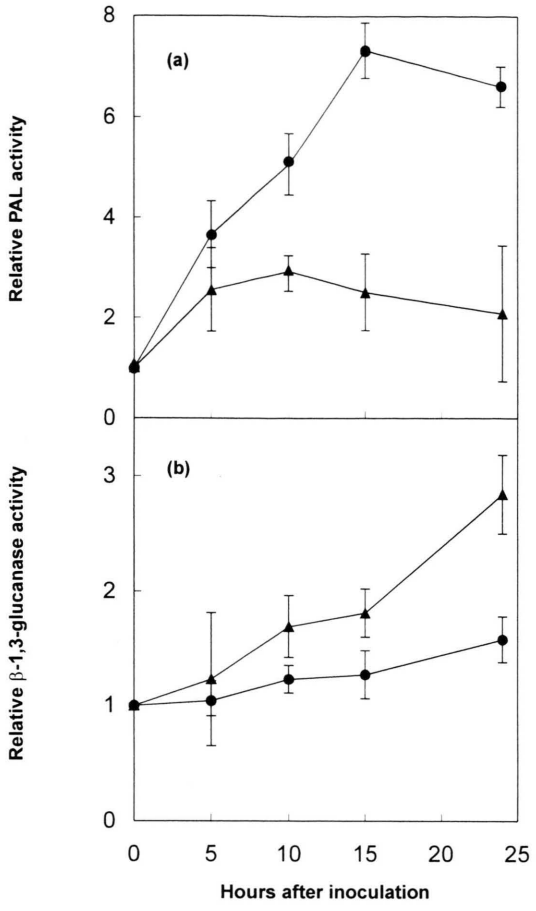

Fig. 3. Time-course of changes in PAL (a) and β-1,3-glucanase (b) activities in Tarpan (▲) and Bzura (●) cell suspensions treated with CF (10 μg glucose equivalents ml^{-1}) of *P. infestans*. The constitutive PAL activity in both controls (treated with sterile distilled water) was 10 pkat mg^{-1} protein and has been equalized to one. The constitutive β-1,3-glucanase activity in control cells (treated with sterile distilled water) of Tarpan and Bzura was 30.7 and 21.3 nkat mg^{-1} protein, respectively and has been equalized to one. Results are means of two independent experiments; bars indicate SE.

In contrast to PAL activity, the constitutive level of β-1,3-glucanase activity as well as the degree of its stimulation after CF treatment were higher in Tarpan than those in Bzura cells (Fig. 3b). This was unexpected, since β-1,3-glucanase, among other PR proteins, is rapidly and markedly induced in potato leaves after CF treatment or infection by *P. infestans* (Schröeder *et al.*, 1992; Kombrink *et al.*, 1988). Whatever the reason for this discrepancy, it appears that the induction of β-1,3-glucanase activity following CF treatment is not the major marker of pathogen resistance in the Bzura (resistant) cultivar.

In summary, our results indicate that potato cultivar with lower level of polygenic resistance is more sensitive to CF containing the fungal toxins. It was also found that CF treatment induced defense reactions in cells of both resistant and susceptible cvs. In the cells of resistant cultivar, no toxic effects were noticeable and even if they were, the cells were able to cope with them and to activate defense strategies efficiently. Based on the obtained results, we propose that sensitivity to the fungal toxins and variation of PAL activity may be used as useful markers for the evaluation of polygenic resistance in potato.

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Atkinson M. M., Keppler L. D., Orlandi E. W., Baker C. J. and Mischke C. F. (1990), Involvement of plasma membrane calcium influx in bacterial induction of the K^+/H^+ and hypersensitive response in tobacco. Plant Physiol. **92**, 215–221.

Awan M. F. M., Zarzycka H., Szczerbakowa A., Wielgat B. and Kleczkowski K. (1995), Response of potato tissues to *Phytophthora infestans*. In: Environmental Biotic Factors in Integrated Plant Disease Control (M. Manka, ed.). The Polish Phytopathological Society, Poznan, pp 167–173.

Baker C. J. and Mock N. M. (1994), An improved method for monitoring cell death in cell suspension and leaf discs assay using Evans blue. Plant Cell Tissue Organ Culture **39**, 7–12.

Baker C. J., Mock N., Glazener J. and Orlandi E. (1993), Recognition responses in pathogen/non-host and race/cultivar interactions involving soybean (*Glycine max*) and *Pseudomonas syringae* pathovars. Physiol. Mol. Plant Pathol. **43**, 81–94.

Boller T. (1995), Chemoperception of microbial signals in plant cells. Annu. Rev. Plant Physiol. Plant Mol. Biol. **46**, 189–214.

Bostock R. M., Kuc J. A. and Laine R. A. (1981), Eicosapentaenoic and arachidonic acids from *Phytophthora infestans* elicit fungitoxic sesquiterpenes in the potato. Science **212**, 67–69.

Davidse L. C., Boekeloo M. and van Eggermond A. J. M. (1986), Elicitation and suppression of necrosis in potato leaves by culture filtrate components of *Phytophthora infestans*. In: NATO ASI Series, Vol. **III.** Biology and Molecular Biology of Plant-Pathogen Interactions (J. Bailey, ed.). Springer Verlag, Berlin/Heidelberg, pp 205–206.

Dixon R. A. and Paiva N. L. (1995), Stress-induced phenylpropanoid metabolism. Plant Cell **7**, 1085- 1097.

Dubois M., Gilles K. A., Hamilton J. K., Rebers P. A. and Smith F. (1956), Colorimetric method of the determination of sugars and related substances. Anal. Chem. **68**, 350–356.

Ebel J. and Cosio E. G. (1994), Elicitors of plant defense responses. Internat. Rev. Cytol. **148**, 1–36.

Friend J. (1991), The biochemistry and cell biology of interaction. Adv. Plant Pathol. **7**, 85–129.

Fritzmeier K.-H., Cretin C., Kombrink E., Rohwer F., Taylor J., Scheel D. and Hahlbrock K. (1987), Transient induction of phenylalanine ammonia-lyase and 4-coumarate: CoA ligase mRNA in potato leaves infected with virulent or avirulent races of *Phytophthora infestans*. Plant Physiol. **85**, 34–41.

Galal A. A., Barna B. and Ersek T. (1991), Use of toxic substances from *Phytophthora infestans* (Mont.) de Bary in screening for late blight resistant potatoes. Acta Phytopathol. Entomol. Hung. **26**, 263–270.

Gamborg O. L., Miller R. A. and Ojima K. (1968), Nutrient requirements of suspension cultures of soybean root cells. Exp. Cell Res. **50**, 151–158.

Havir E. A. and Hanson K. R. (1968), L-Phenylalanine ammonia-lyase. I. Purification and molecular size of the enzyme from potato tubers. Biochemistry **7**, 1896–1903.

Henniger H. (1959), Ver Suche zur Kulture verschiedener Rassen von *Phytophthora infestans* (Mont.) de Bary aufkiinstlichen Nährboden. Phytopathologische Z. **34**, 285–306.

Hohl H. R. (1991), Nutrition. Adv. Plant Pathol. **7**, 53–83.

Huet J.-C., Sallé-Tourne M. and Pernollet J.-C. (1994), Amino acid sequence and toxicity of the elicitin secreted with ubiquitin by *Phytophthora infestans*. Mol. Plant Microbe Inter. **7**, 302–304.

Kohmoto K. and Otani H. (1991), Host recognition by toxigenic plant pathogens. Experientia **47**, 755- 764.

Kombrink E. and Hahlbrock K. (1986), Responses of cultured parsley cells to elicitors from phytopathogenic fungi. Plant Physiol. **81**, 216–221.

Kombrink E. and Somssich I. E. (1995), Defense responses of plants to pathogens. Adv. Bot. Res. **21**, 1–34.

Kombrink E., Schröder M. and Hahlbrock K. (1988), Several "pathogenesis-related" proteins in potato are 1,3-β-glucanases and chitinases. Proc. Natl. Acad. Sci. USA. **85**, 782–786.

Lever M. (1972), A new reaction for colorimetric determination of carbohydrates. Anal. Biochem. **47**, 273–279.

Lowry J. O. H., Rosenbrough N. J., Farr A. L. and Randall R. J. (1951), Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**, 265–275.

Maciejewska U., Awan M. F. M., Klos B. and Wielgat B. (1995), Pathogenesis-related proteins in cell suspension culture of potato in response to elicitors from *Phytophthora infestans*. In: Environmental Biotic Factors in Integrated Plant Disease Control (M. Manka, ed.). The Polish Phytopathological Society, Poznan, pp 373–378.

Möller C., Zitzlsperger J. and Wenzel G. (1992), The effects of a toxin preparation from *Phytophthora infestans* on potato protoplasts and microspores. Physiol. Mol. Plant Pathol. **41**, 427–435.

Mozzetti C., Ferraris L., Tamietti G. and Matta A. (1995), Variation in enzyme activities in leaves and cell suspensions as markers of incompatibility in different *Phytophthora* – pepper interactions. Physiol. Mol. Plant Pathol. **46**, 95–107.

Murashige T. and Skoog F. (1962), A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol. Plant. **15**, 473–497.

Sanchez L. M., Doke N., Ban Y. and Kawakita K. (1994), Involvement of suppressor-glucans and plant epidermal cells in host-selective pathogenesis of *Phytophthora capsici*. J. Phytopathol. **140**, 153–164.

Schröder M., Hahlbrock K. and Kombrink E. (1992), Temporal and spatial patterns of 1,3-β-glucanase and chitinase induction in potato leaves infected with *Phytophthora infestans*. Plant J. **2**, 161–172.

Stolle K. and Schuber B. (1985), Wirkung eines Toxins von *Phytophthora infestans* (Mont.) de Bary auf Kartoffelblätter. Potato Res. **28**, 389–402.

Swiezynski K. M., Domanski L., Sieczka M. T. and Zarzycka H. (1993), Specific resistance to *Phytophthora infestans* in the potato. Genet. Polon. **34**, 327–336.