BIOLOGIA PLANTARUM 46 (1): 101-105, 2003

Growth and enzyme activity in roots and calli of resistant and susceptible *Allium* lines as influenced by sterile culture filtrates of *Phoma terrestris*

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Abstract

Growth and activities of peroxidases, chitinases and glucanases were studied in order to evaluate the response of calli and roots of pink root-susceptible *Allium cepa* cvs. Valcatorce and T-412 and resistant *A. fistulosum* cv. Nogiwa Negi, to sterile culture filtrates of *Phoma terrestris*. Untreated calli and roots of *A. fistulosum* exhibited higher activity of peroxidases and glucanases than that of Valcatorce and T-412. Enzyme activities and growth of roots and calli were not affected in filtrate-treated *A. fistulosum*. The growth of calli and roots of *A. cepa* cultivars decreased significantly after exposure to *P. terrestris* filtrates while the peroxidase and glucanase activities increased. Peroxidase and glucanase activities were also enhanced in roots of Valcatorce bulbs grown in *P. terrestris*-inoculated soil as compared to healthy control plants. We conclude that a high constitutive activity of glucanases and perhaps chitinases might account for the resistance of *A. fistulosum*. The differential reaction (with respect to root growth) of pink root-susceptible and resistant materials to culture filtrates indicates that this *in vitro*-system might be useful for the screening of onion breeding lines.

Additional key words: onion, Japanese bunching onion, fungal toxins, *in vitro* screening.

Introduction

Pink root caused by the fungus *Phoma terrestris* Hansen (syn. *Pyrenochaeta terrestris* (Hansen) Gorenz, Walker and Larson) is a widespread onion disease, especially in warmer climates where yield reductions may be considerable. As with other soil-borne diseases, control is very difficult and not always efficient (Sumner 1995).

The use of resistant cultivars is a logical option, however, onion lines showing resistance in one region may behave as susceptible in another (Entwistle 1990, Piccolo and Galmarini 1994). Pink root resistance has been identified in *A. fistulosum* (Porter and Jones 1933), a species closely related to cultivated onion. Unfortunately, the incorporation of this resistance in onion is difficult as the F1 from the *A. cepa* × *A. fistulosum* crosses has been found to be highly infertile (Van der Valk *et al.* 1991). Recently, a major breakthrough in the *A. fistulosum*-based onion breeding was achieved when Peffley and Hou (2000) obtained male- and female-fertile backcross progeny with onion having a degree of pink root resistance comparable to that of the *A. fistulosum* parent.

P. terrestris is a necrotrophic fungus which destroys root tissue by the action of extracellular lytic enzymes allowing the mycelium to colonize the dead cells (Hess 1969). Specific and non-specific toxins have frequently been identified in the infection process of necrotrophic fungi. These toxins usually interact with specific cell proteins or membranes, suppressing or decreasing the defense capacity of the host cells (Johal *et al.* 1995). In *P. terrestris,* several toxins have been identified which facilitate invasion of the host tissues by generating a stress situation. The fungus releases these toxins (pyrenocines, secalonic acid) into the medium during its cultivation *in vitro* (Sparace *et al.* 1986). These authors described a positive correlation between the tolerance of some onion cultivars to pyrenocine A and their resistance to *P. terrestris*. Gourd *et al.* (1988) and Ludwig *et al.*

Received 25 July 2001, *accepted* 11 January 2002.

Abbreviations: 2,4-D - 2,4-dichlorophenoxyacetic acid; BAP - 6-benzylaminopurine.

Acknowledgements: The authors wish to thank M. Kiehr, R. Devalis and S. Delmastro for helpful technical assistance and critical comments. This work was supported by grants from the Universidad Nacional del Sur and CONICET (National Research Council for Science and Technology).

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(1992) studied the interaction between onion and *P. terrestris* in calli and observed that the response of different genotypes to fungal filtrates reflected the natural resistance of these plants to the pathogen.

When attacked by a fungus, specific plant enzymes play a crucial role in restricting the spread of the pathogen. Some of these enzymes are involved in the build-up of physical barriers consisting of lignin, suberin and callose on the cell wall, others may synthesize antimicrobial compounds of low molecular mass like phytoalexins. In addition, certain enzymes like chitinases or β-1,3-glucanases may directly attack the invading pathogens (Kombrink and Somssich 1995). Many of those enzymes occur in several isoforms.

The aim of the paper was *in vitro* studies of host-pathogen interactions which may be helpful in understanding the mechanisms involved in the defense response of the host tissue, and in finding out the kind of resistance mechanisms particularly involved. They also could provide useful information for *in vitro* selection of materials with increased resistance or tolerance to the pathogen (Daub 1986).

Materials and methods

Plants and cultivation: *Allium cepa* L. susceptible cvs. Valcatorce (INTA, Argentina) and T-412 (*Takki Seed*, Kyoto, Japan) and *A. fistulosum* L. resistant cv. Nogiwa Negi were used in these experiments (Porter and Jones 1933, Piccolo and Galmarini 1994, Rabinowitch 1997, A. Azpilicueta, personal communication).

Callus was induced following the technique of Van der Valk *et al.* (1992); embryos were extracted and cultivated in BDS medium (Dunstan and Short 1977) containing 1 mg dm^{-3} 2,4-D, 0.1 mg dm^{-3} BAP and 7 g dm^{-3} agar; in darkness at 25 ± 3 °C. After 2-month induction period and two monthly subcultures in the same medium, the calli were ready to be used in the assays. To obtain plantlets, embryos extracted from seeds were cultivated *in vitro* in BDS medium without plant growth regulators. After two monthly subcultures in the same medium plantlets were ready to use. Roots and most of the leaves were eliminated when subcultured.

Highly aggressive isolates of *P. terrestris* were obtained from roots of naturally infected onion plants. The fungus was cultivated at room temperature either in potato-dextrose-agar medium or in liquid Czapek-Dox medium (*Merck*, Darmstadt, Germany) with horizontal orbital agitation at 90 rpm.

***In vivo* inoculations:** For *in vivo* inoculations, healthy and uninjured bulbs of a homogeneous size (*ca.* 4 cm equatorial diameter), were selected from a bulk of bulbs of cv. Valcatorce which had been grown in a field with no previous history of any *Allium* crop. Sterile soil (0.25 dm^3 per pot) was mixed with seven disks extracted with a 7 mm-cork borer from actively growing cultures of *P. terrestris* in potato dextrose agar in Petri dishes. Three bulbs were planted in each pot and cultivated 4 weeks at 25 ± 2 °C, watered as needed. After three weeks, symptomatic roots of inoculated plants and healthy roots from control plants were chosen for the assays.

Exposure to pathogen culture filtrates: Calli and plantlets were exposed to fungus liquid culture filtrates as follows. 9- to 10-d-old liquid cultures of *P. terrestris* were sequentially filtered through *Whatman No. 5* and 0.2 μm *Millipore®* microfilters. The sterile filtrates obtained were incorporated into BDS medium to a final concentration of 10 % (v/v). This concentration had been found to be adequate in preliminary tests. Since incorporation of 10 % of uninoculated culture medium enhanced growth, BDS medium was added in the control. Each treatment contained 25 calli or 25 plantlets. After 30 d cultivation of calli and 20 d cultivation of plantlets, growth and activity of peroxidases, chitinases and β-1,3-glucanases in calli and roots were evaluated.

Evaluation of peroxidase, chitinase and glucanase activities: Calli and roots were frozen in liquid air and separately homogenized at 4 °C in 0.1 M phosphate buffer pH 7, containing 0.001 % (v/v) Triton X-100, 0.25 mM leupeptin and 0.03 mM phenylmethylsulfonyl fluoride, using a 2:1 (v/m) buffer to fresh mass ratio. The homogenate was kept at 4 °C for 30 min and then centrifuged at 13 000 *g* for 10 min at 4 °C. Supernatants were stored at -20 °C until use. Three samples per treatment were extracted. Protein concentration in the supernatant was determined by the method of Bradford (1976). The peroxidase activity of the extracts was determined by a colorimetric assay using guaiacol as substrate in a *Hitachi* (Tokyo, Japan) spectrophotometer (Koike and Shimada 1992). Horseradish peroxidase Type I (HRPx; *Sigma*, St. Louis, USA) was used as reference for the enzymatic activity. Chitinase activity was obtained following the protocol of Spindler (1997), using 0.6 % *p*-nitrophenyl-N-acetyl-β-D-glucosaminide (*Sigma*) as substrate. The reaction product *p*-nitrophenol was measured at 410 nm in a *Shimadzu* (Kyoto, Japan) spectrophotometer. One mM solution of *p*-nitrophenol (PNP) had an absorbance of 6.367 (dal Soglio *et al.* 1998). Activity of β-1,3-glucanases was determined following the protocol of Mutasftschiev *et al.* (1997).

Results

Some of the calli of cv. Valcatorce, but not of T-412 and *A. fistulosum*, developed a superficial red pigmentation 3 to 5 d after the exposure to culture filtrates of *P. terrestris*. After 30 d of treatment with culture filtrates, callus growth of the pink root susceptible cvs. Valcatorce and T-412, but not of the resistant *A. fistulosum*, was inhibited significantly ($P < 0.001$) when compared with untreated controls (Fig. 1*A*). Likewise, the root growth of small plants of cv. Valcatorce, but not of T-412 or *A. fistulosum*, was found to be decreased significantly by the fungal culture filtrates (Fig. 1*B*).

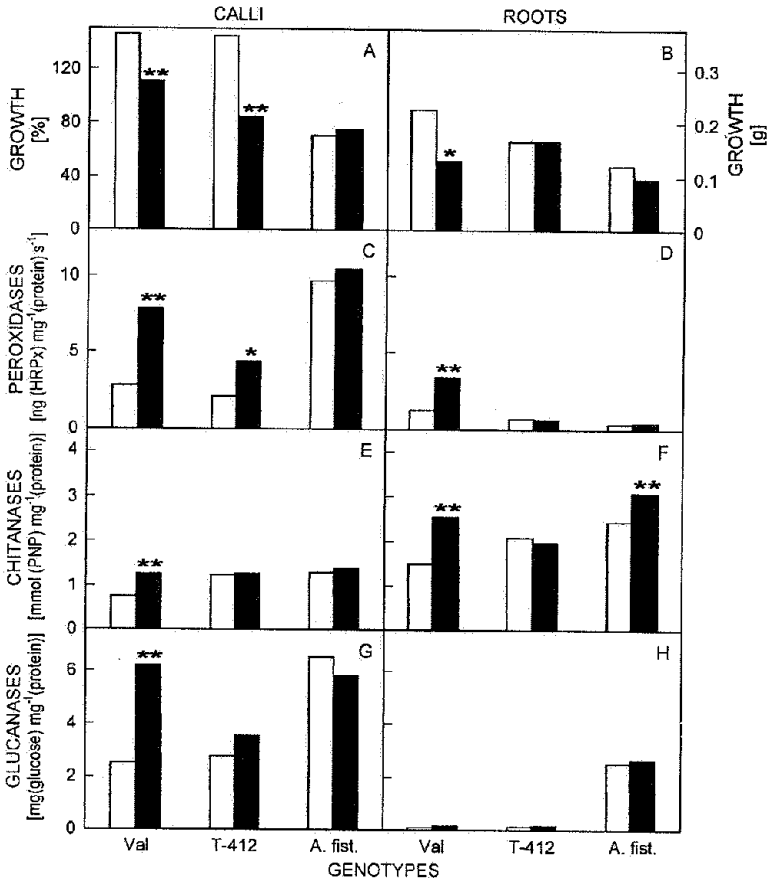

Fig. 1. Effect of culture filtrates of *Phoma terrestris* on the growth and the activity of peroxidases, chitinases and β-1,3-glucanases in calli (30 d of exposure) and roots (20 d of exposure). *Open columns* - calli or roots non-exposed, *closed columns* - calli or roots exposed to culture filtrates; Val - *A. cepa* cv. Valcatorce, T-412 - *A. cepa* cv. T-412, A. fist. - *A. fistulosum* cv. Nogiwa Negi. Difference significant at * - $P < 0.05$; ** - $P < 0.01$.

Total peroxidase activity, on a protein basis, was higher in calli than in roots. There was a very high activity of these enzymes in untreated *A. fistulosum* calli which remained unaltered after filtrate-treatments. On the contrary, Valcatorce and T-412 showed a low peroxidase activity in untreated calli which increased significantly when treated with filtrates (Fig. 1*C*). In the roots, only Valcatorce showed enhanced peroxidase activity in response to fungal culture filtrates (Fig. 1*D*).

The activity of chitinases was found to be higher in the roots than in the calli. In untreated conditions, the activity was slightly higher in T-412 and *A. fistulosum* than in Valcatorce in both calli and roots. However, there was a significant increase in chitinase activity in treated calli and roots of Valcatorce, as well as in roots of *A. fistulosum* (Fig. 1*E,F*).

The activity of β-1,3-glucanases was found to be much higher in calli than in roots. There was a significant difference ($P < 0.01$) between control and filtrate-treated calli in Valcatorce. In calli and roots of *A. fistulosum*, the glucanase activity in the control was much higher than in the susceptible onion lines, and there was no further increase after treatments with fungal culture filtrates (Fig. 1*G,H*).

Secondary roots produced on bulbs of Valcatorce planted in *P. terrestris*-infested soil developed typical

pink root-symptoms, while those developed in uninfested soil remained healthy. Infected and healthy roots showed a total activity of peroxidases of 5.1 and 2.3 [ng(HRPx) mg^{-1}(protein) s^{-1}], respectively; of glucanases of 6.0 and 3.8 [mg(glucose) mg^{-1}(protein)], respectively, and of chitinases of 7.3 and 5.5 [mM(PNP) mg^{-1}(protein)], respectively. Thus, the total activities of peroxidases, glucanases and chitinases were higher in the infected than in the healthy roots, with differences being significant (Tukey) in peroxidases ($P < 0.01$) and glucanases ($P < 0.05$), but not in chitinases.

Discussion

Roots of the susceptible onion cv. Valcatorce grown in *P. terrestris*-infested soil, developed typical pink root symptoms and showed an enhanced activity of peroxidases, glucanases and, less so, chitinases. Small plants of the same cultivar, exposed to sterile culture filtrates of the same fungus, responded with a reduction in secondary root growth and an increase in the activity of peroxidases, glucanases and chitinases. Likewise, calli of cv. Valcatorce, treated with sterile culture filtrates, reacted with a reduction in growth and an enhancement of the activity of the three enzymes. This would indicate that both secondary roots on small plants and calli, when exposed to culture filtrates, partly reflect the situation in naturally infected soil-grown plants and might be used as model system for the study of the pathogenic processes of pink root in onion.

Root and callus growth, as well as enzyme activities, in the pink root resistant *A. fistulosum* cv. Nogiwa Negi were not affected by culture filtrates of *P. terrestris* indicating that the experimental model is able to differentiate between susceptible and resistant genotypes of *Allium* and might therefore be employed in the selection of tolerant or resistant breeding lines.

Culture filtrates of *P. terrestris* affected callus mass and produced visible damage of susceptible but not of resistant lines of *Allium* species; however, the reaction of shoots and plantlets was not as consistent (Gourd *et al.* 1988). Our results indicate that the reaction of secondary roots developed on small plants, rather than whole plantlets as used by Gourd *et al.* (1988), may give a reliable response. In our experiments, the filtrates applied included only the liquid fraction of the pathogen culture in Czapek-Dox medium and were filtered, not autoclaved, while Gourd *et al.* (1988) and Ludwig *et al.* (1992) used the extracts obtained from the blended mycelial mat which were autoclaved before use.

Roots are the natural target site of the pink root fungus in the field, thus the response of this organ may be more trustworthy than callus resistance. Moreover, small plants are faster, easier and cheaper to grow than calli, making this system attractive for the screening of large numbers of genotypes.

In non-treated calli of pink root resistant *A. fistulosum* the activity of peroxidases, chitinases and glucanases was significantly higher than in the susceptible cv. Valcatorce. The activity of those enzymes was found to increase in the latter, but not in *A. fistulosum*, after treatment of calli with fungal culture filtrates. In the root system, the constitutive activity of peroxidases was higher, of chitinase slightly lower and of glucanases much lower in Valcatorce than in *A. fistulosum*. From these results it can be inferred that the resistance of *A. fistulosum*, as the root system is concerned, might be related to a high constitutive level of glucanases and perhaps chitinases. Both enzymes are involved in degrading cell wall polymers of fungi and have been shown to control the advance of infection in many pathosystems (Kombrink and Somssich 1995). The increased activity of these enzymes after exposure to culture filtrates observed in the susceptible Valcatorce but not in the resistant *A. fistulosum*, apparently does not contribute to the defense reaction, at least in the late stage of pathogenesis.

Peroxidases are known to play an important role in the defense response against fungal pathogens (Tyagi *et al.* 2000). In the onion-*P. terrestris* system, the increase of peroxidase activity observed in calli and roots of susceptible Valcatorce, but not of resistant *A. fistulosum* exposed to fungal filtrates was correlated with susceptibility rather than with resistance; thus it seems possible that this enzyme might be involved in the destructive effect of pink root infection.

A reddish coloration was observed in filtrate-treated calli of Valcatorce but not of *A. fistulosum*. Gourd *et al.* (1988) related this kind of discoloration to nutritional stress. The intensity of this red pigmentation was also used as a measure of the damage caused by culture filtrates on calli (Gourd *et al.* 1988, Ludwig *et al.* 1992). In our case, the red compounds only appeared in calli exposed to pathogen culture filtrate but not in the control; hence, it could not be related to nutritional stress. Reddish phenolic compounds have been reported to accumulate after bacterial infection of onion (Omidiji and Ehimidu 1990).

We conclude that a high constitutive activity of glucanases and perhaps chitinases might account for the resistance reaction of *A. fistulosum*. The differential reaction with respect to root growth of pink root-susceptible and resistant materials to culture filtrates indicates that this *in vitro*-system might be useful for the screening of onion breeding lines.

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