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Changes in phenolic acids and stilbenes induced in embryogenic cell cultures of Norway spruce by two fractions of *Sirococcus strobilinus* mycelia

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ABSTRACT: We examined defence responses in embryogenic cell suspension cultures of Norway spruce (*Picea abies* [L.] Karst) elicited by intracellular protein and cell wall fractions (PF and WF, respectively) prepared from mycelia of the fungus *Sirococcus strobilinus* Preuss focusing on changes in (soluble and cell wall-bound) phenolic and stilbene concentrations. Treatment with both preparations induced an increase in the total contents of phenolic acids in Norway spruce cells and variations in the levels of stilbene glycosides. More rapid and intense induction of defence response was observed in cells after WF application. The contents of soluble phenolic acids (especially benzoic acid derivatives) and cell wall-bound phenolic acids (especially ferulic acid) started to increase (relative to controls) within 4 h after the addition of the WF preparation and remained high in elicited cells for 8–12 h. A moderate increase in phenolic acids in cells exposed to the PF preparation was observed within 8 h after application. However, after 24 h of WF treatment a decline of total phenolics was observed, while in PF elicited Norway spruce cells the phenolic content continued to increase. Significantly decreased concentrations of stilbene glycosides, isorhapontin, astringin and piceid, were determined in PF and WF treated Norway spruce cell cultures. The total content of stilbene glycosides decreased within 8 h after WF application to 68% of the amount determined in the control and within 12 h to 73% of the control in PF-treated cells. These results demonstrate that both PF and WF prepared from the Sirococcus strobilinus mycelium elicit changes in the metabolism of phenylpropanoids, which are involved in the defence responses of plants to pathogens.

Keywords: defence response; Norway spruce; phenylpropanoids; stilbenoids

The decline of the forest tree population caused by fungal diseases is a long-term factor influencing the stability of forest ecosystem. The recent widespread dieback of Norway spruces in the Orlické hory Mountains, Czech Republic, was caused by the combined effects of air pollution, climatic conditions and attacks by the potentially pathogenic fungi *Sirococcus strobilinus, Phoma* spp., and *Ascocalyx abietina*. Plants respond to a pathogen challenge by activating the range of defence mechanisms that can be local or result in systemic acquired resistance (Bonello, Blodgett 2003). A universal feature of plant responses to pathogens or other elicitors is the activation of phenylpropanoid synthesis. An important manifestation of defence is the accumulation of polyphenols in the cell walls, which is accompanied by an increase in lignification and suberization (De Ascensao, Dubery 2003). The induction of phenylpropanoid biosynthesis and consequent increase in bark polyphenols in Norway spruce trees following wounding or fungal infection were documented histo- and immunochemically (Franceschi et al. 1998). An increase in cell wall phenolics in the bark of Norway spruce

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branches infected by *Ascocalyx abietina* was also reported in our previous paper (Cvikrová et al. 2006). Stilbenoids are an important group of phenolics, specifically linked with resistance to fungal attack. Stilbenes occur as glycosides in the healthy phloem of Norway spruce (Bringolas et al. 1995). The main constitutive stilbene glycosides in *Picea* species are astringin and isorhapontin (Lindberg et al. 1992). Rapid accumulation of stilbene aglycones in response to injury or fungal infection is considered to be an active defence response of Norway spruce (Nicholson, Hammerschmidt 1992).

The use of tissue cultures facilitates detailed studies of early response to challenge with pathogen or elicitor preparations (Groten, Barz 2000). The aim of the study was to characterize changes in (soluble and cell wall-bound) phenolic and stilbene concentrations during defence response in Norway spruce embryogenic cell suspension cultures induced by intracellular protein and wall preparations from *Sirococcus strobilinus* mycelia.

MATERIAL AND METHODS

Plant material

The embryogenic tissue derived from zygotic embryos of mature seeds of Norway spruce was initiated on modified AE medium (Arnold, Eriksson 1979). Embryogenic cultures were grown on gelrite-solidified medium (2 g·l^{-1}) supplemented with 6-benzylaminopurine and 6-furfurylaminopurine (both 0.5 mg·l^{-1}), 2,4-dichlorophenoxyacetic acid (1 mg·l^{-1}), glutamine (400 mg·l^{-1}), casein hydrolyzate (400 mg·l^{-1}), $FeSO_4$·7 H_2O (27.8 mg·l^{-1}) and sucrose (20 mg·l^{-1}), pH of the media was adjusted to 5.8. Cultures were cultivated under controlled conditions (24°C) in the dark and subcultured every 3 weeks (Malá 1991). For establishment of suspension cultures, approximately 3 g fresh weight of embryogenic tissue were inoculated to 100 ml of liquid medium of the same composition as mentioned above in 250 ml Erlenmeyer flasks and grown at 24°C in the dark on an orbital incubator (IOC.400.XX2.C SANYO-Gallenkamp, Leicester, UK) at 110 rpm. Five-day-old cell suspension cultures were used for the experiments.

Pathogen culture

Stock culture of the non-lyophilized mycelium of *Sirococcus strobilinus* was obtained from Dr. A. Lilja (METLA, Finnish Forest Research Institute, Vantaa Research Unit, FI-01301 Vantaa, Finland). Pieces of the stock fungus were plated onto the Malt Extract Agar (MA: 12 g·l^{-1} Difco Maltose Extract, 12 g·l^{-1} Difco Agar, Detroit, Michigan, USA) and incubated at 24°C (Lilja et al. 2005). After multiplication, the mycelium was transferred into 100 ml Erlenmeyer flasks containing 50 ml of 12 g·l^{-1} Difco Maltose Extract and incubated at 24°C in an orbital incubator (IOC.400.XX2.C SANYO-Gallenkamp, Leicester, UK) at 120 rpm. Approximately 5 g of fresh mycelium was transferred into 50 ml of fresh liquid medium (Difco Maltose extract 12 g·l^{-1}) and cultured under the above mentioned conditions for 4 weeks.

Preparation of mycelial intracellular protein fraction

The *Sirococcus strobilinus* mycelium was washed three times with distilled water, harvested by filtration through Whatman No. 1 filter paper and the mycelial mass was then ground in liquid nitrogen and homogenized with 0.1 M Tris-HCl buffer pH 7.2 containing 2mM β-mercaptoethanol, 500 μg·ml^{-1} amoxicillinum, and 100 μg·ml^{-1} acidum clavulanicum (Augmentin 600, SmithKlineBeechcham Pharmaceuticals, Worthing, UK). The resulting homogenate was centrifuged at 14,000 g for 20 min at 4°C to obtain a supernatant containing intracellular proteins – 12.20 mg protein g^{-1} mycelium fresh weight, according to assays following the method of Bradford (1976) using bovine serum albumin as a standard. The final protein content of the intracellular fraction in 100 ml of liquid medium was 4 mg.

Preparation of mycelial wall fraction

The mycelial cell wall fraction (WF) was prepared according to the method described by Momany et al. (2004), with slight modifications, as follows. Cultures of *Sirococcus strobilinus* were filtered through Whatman No. 1 filter and washed with distilled H_2O. The resulting mycelial mass was ground in liquid nitrogen and homogenized with 0.1M Tris-HCl buffer (pH 7.2) containing 2mM β-mercaptoethanol. The cell walls were separated by centrifugation at 3,000 g for 10 min and the pellet was repeatedly washed with distilled water. To determine the amount of ionically bound protein in the mycelial walls a part of the mycelial wall preparation was resuspended in 0.1M Tris-HCl buffer

(pH 7.2) containing 0.1M KCl and stirred for 1 h at 20°C. The extract was centrifuged (3,000 g for 10 min) and the protein content of the supernatant was determined (Bradford 1976) using bovine serum albumin as a standard. The results indicated that the mycelial cell walls contained 19.40 mg of ionically bound protein per gram of the cell wall preparation (fresh weight) and 77.6 mg per gram of the pellet material dried at 40°C for 24 h. The dried mycelial wall powder was suspended in distilled water (pH 5.2) and autoclaved for 5 min. The concentration of the WF used in the experiment was 30 mg of mycelium powder per 100 ml of liquid medium.

Extraction and analysis of phenolic acids

Phenolic acids were extracted as described by Cvikrová et al. (1991). Briefly, free, ester-bound (released after alkaline hydrolysis) and glycoside-bound (released after acid hydrolysis) phenolic acids were obtained from a methanol extract of the tissue ground in liquid nitrogen. The fraction of cell wall-bound phenolic acids was obtained after alkaline hydrolysis of the residual material following the methanol extraction. 2,6-Di-tert-butyl β-cresol was used as an antioxidant to minimize the oxidation of phenolic acids during alkaline hydrolysis and nitrogen was immediately bubbled through the sample after NaOH addition. Phenolic acids were analysed by means of HPLC using a Dionex liquid chromatograph (P660-HPLC pump, ASI-100 automated sample injector, TCC-100 thermostated column compartment, PDA-100 photodiode array detector, Chromeleon software 6.5) with C18 Spherisorb 5 ODS column (250 × 4.6 mm). Acetonitrile and acetic acid gradient was used for elution. Phenolic acids were detected at their absorption maximum. λ_{max} was detected from the authentic compounds (Sigma-Aldrich, Prague, Czech Republic) that were used as references for quantitative analyses.

Extraction and analysis of stilbenes

For the extraction of stilbenes the procedure described by Viiri et al. (2001) was followed with slight modifications. Briefly, samples of cell culture (0.5 g fresh weight) were frozen in liquid nitrogen, homogenized with 5.0 ml of 80% (v/v) methanol in mortar, stirred on an orbital shaker for 60 min at room temperature and then centrifuged (5,000 × g for 20 min). The supernatant was evaporated in the vacuum to dryness. Aliquots of methanol-soluble material were analyzed by means of HPLC using a Dionex liquid chromatograph (P660-HPLC pump, ASI-100 automated sample injector, TCC-100 thermostated column compartment, PDA-100 photodiode array detector, Chromeleon software 6.5) with C18 Spherisorb 5 ODS 2 column (250 × 4.6 mm). Acetonitrile and acetic acid gradient was used for elution. Stilbenes were detected at 303 nm. Authentic samples of stilbenes (Polyphenol Laboratories AS, Sandnes, Norway) were used for qualitative and quantitative determinations.

RESULTS

Phenolic acid contents

Variations in the total contents of phenolic acids (represented by the sum of free, methanol soluble conjugated forms, i.e. ester- and glycoside-bound phenolics, and methanol-insoluble cell wall-bound phenolic esters) induced by both elicitor preparations are presented in Fig. 1. In the control Norway spruce cells the soluble glycoside-bound forms of phenolic acids (SG) accounted for most of the total content (about 85%), followed by the methanol-insoluble cell wall-bound phenolic esters (CWE; 7–8%). The amounts of methanol soluble esters (SE) and free phenolic acids (F) were low in control cells, accounting for ca 2 and 4–5% of total phenolic contents, respectively. Responses to challenges with both elicitors were manifested most clearly by marked increases (compared with controls) in SG contents. During the WF treatment, SG levels significantly increased after 4 h and almost doubled after 8 h. In addition, increases in CWE contents by 50% and doubled content of F after 12 h were observed in WF-elicited cells (Fig. 1). In PF-elicited cells, the level of SG increased by about 40% after 8 h. After 12 h of treatment with PF the amount of SG was still at its 8-h level, but levels of the other forms of phenolic acids increased; levels of F by 70% and levels of CWE by 25% (Fig. 1). After 24 h of WF treatment a decline in the contents of total phenolics was observed, while in PF-elicited cells a further significant rise in SG and CWE was determined.

The HPLC analyses indicated the presence of two cinnamic acid derivatives, *p*-coumaric and ferulic acids and of five benzoic acid derivatives (anisic, *p*-hydroxybenzoic, vanillic and syringic acids) in the Norway spruce cells, and there were no qualitative differences in the phenolic acid com-

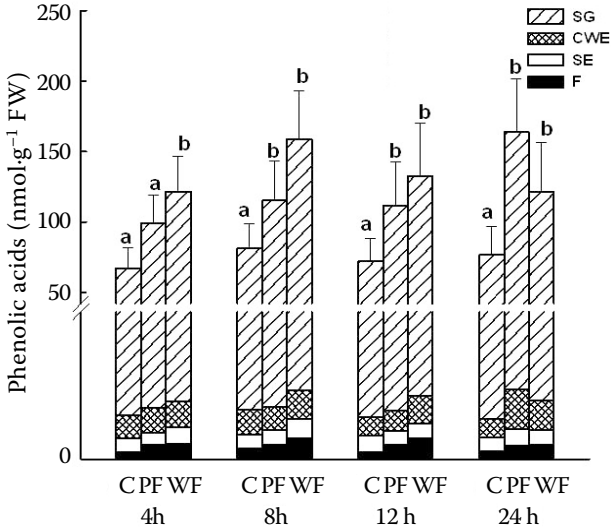

Fig. 1. Changes in the contents of free (F), soluble ester-bound (SE), cell wall ester-bound (SWE) and soluble glycoside-bound (SG) phenolic acids in control cells of embryogenic cultures of Norway spruce (C) and in the cells elicited by mycelium protein fraction (PF) and mycelium wall fraction (WF) in the course of 24 h. Means ± Standard Error of two independent experiments with two replicates. Different letters above the bars indicate significant differences in SG contents from the controls ($P < 0.05$)

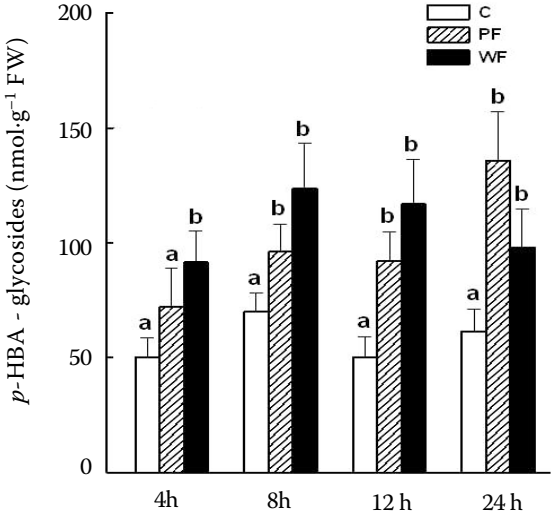

Fig. 2. Changes in the contents of soluble glycoside-bound *p*-hydroxybenzoic (*p*-HBA) acid in control cells of embryogenic cultures of Norway spruce (C) and in the cells elicited by mycelium protein fraction (PF) and mycelium wall fraction (WF) in the course of 24 h. Means ± Standard Error of two independent experiments with two replicates. Different letters above the bars indicate significant differences from the controls ($P < 0.05$)

plements between the control and elicited cells. The enhancement of phenolic contents in treated cells was mainly due to increases in SG forms of *p*-hydroxybenzoic and vanillic acids. We focused predominately on changes in the contents of *p*-hydroxybenzoic acid. Marked increases in *p*-hydroxybenzoic acid glycosides were detected in WF-elicited cells after 8 h and remained high after 12 h. The level of glycosides of the above-mentioned phenolic acid was maximal after 24 h in PF-treated cells (Fig. 2). Both elicitor treatments induced increases in CWE forms of *p*-hydroxybenzoic acid and both cinnamic acid derivatives, *p*-coumaric and ferulic acids.

Contents of stilbenes

Significantly decreased concentrations of stilbene glycosides, isorhapontin, astringin and piceid, were determined in WF and PF treated Norway spruce cell cultures (Fig. 3). Within 8 h after WF application the total content of stilbene glycosides decreased to 68% of the amount determined in the control. The level of isorhapontin, the stilbene

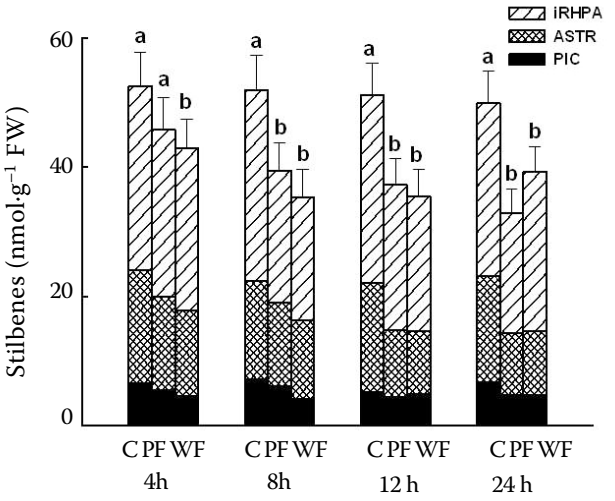

Fig. 3. Contents of stilbene glycosides, isorhapontin (iRHAP), astringin (ASTR) and piceid (PIC), determined in Norway spruce cell cultures treated with 5% and 20% *A. abietina* culture filtrate and from the control cells (C). Means of two independent experiments with two replicates. Bars represent the sum of SE of isorhaportin, astringin and piceid. Different letters above the bars indicate significant differences from the controls ($P < 0.05$)

which occurred in the highest concentration in embryogenic cell cultures, decreased to less than 63% (compared with the control) within 8 h after WF application. The content of piceid, which was present in the lowest amount in cells, did not change markedly. The stilbene glycoside levels in WF-elicited cultures remained more or less stable till the end of 24 h treatment. During 12 h of treatment PF evoked the decline of total stilbene glycosides to 73% (compared with the control). The decline in the levels of isorhapontin and astringin continued 24 h after PF application representing less than 68% and 50%, respectively, of the contents determined in the control cells (Fig. 3).

DISCUSSION

We showed that the Norway spruce cells responded more rapidly to the mycelial WF preparation than to the mycelial PF preparation. The contents of soluble (especially benzoic acid derivatives) and cell wall-bound phenolic acids (especially ferulic acid) started to increase within 4 h after the addition of the WF preparation. The response of cells to the PF fraction was slower; a significant increase was first detected after 8 h (Fig. 1). It is in agreement with findings reported by Plazek et al. (2003, 2005) in winter oilseed rape calli. These authors found out that pectinase (PF is a rich source of soluble, hydrolytic enzymes) activated the phenylpropanoid pathway in the calli less strongly than chitosan (major polysaccharide components of fungal cell walls). In a study examining possible correlations between the synthesis of hydroxycinnamic amides and the formation of wall-bound phenolic polymers it was also shown that the phenylpropanoid pathway could be induced by pectinase and pronase in tobacco cell suspension cultures (Negrel, Javelle 1995).

The fungal cell wall has a highly complex structure. It forms a network of polysaccharides in which various proteins are embedded (Saikia et al. 2006). It could be supposed that the defence response of Norway spruce cells was induced by mycelial wall polysaccharides. This is in agreement with the accepted knowledge that major polysaccharide components of fungal cell walls, glucans and chitin act as general elicitors of defence responses (Yamaguchi et al. 2000). Similarly, induction of phenylpropanoid biosynthesis and accumulation of phenolics were observed in soybean leaves following the exposure to chitin and chitosan (Khan et al. 2003). An increase in the levels of soluble glycosides of *p*-hydroxybenzoic acid culminated in WF-elicited cells after 12 h, while in PF-treated cells their levels were maximal at the end of 24-h treatment (Fig. 2). It corresponds to results obtained in callus cultures of *Pinus sylvestris* treated with mycelial extracts of *Fusarium nivale* which were reported by Shein et al. (2003), who concluded that the accumulation of *p*-hydroxybenzoic acid plays an important role in the protection of conifer cells by acting as a fungicidal agent when fungi penetrate into the cytosol. Furthermore, plant glycosides are often hydrolysed by vacuolar glycosidases following the pathogen invasion, releasing aglycones that may be quite toxic to the invader (Keen 1999).

Stilbenes are generally described as phytoalexin-like compounds or phytoanticipins in conifers as they are often present in certain tissues constitutively rather than appearing *de novo* following the infection (Mansfield 2000). Because of their strong antimicrobial properties *in vitro* they are implicated in the defence of conifers against pathogens (Lindberg et al. 1992; Celimene et al. 2001). The present Norway spruce cell cultures responded to treatment with both elicitor preparations by a decrease in concentrations of stilbene glycosides. The decrease in isorhapontin (occurring in the highest concentrations in embryogenic cell cultures), astringin and piceid levels was observed in WF-elicited cells after 12 h, while in PF-treated cells at the end of the 24 h treatment (Fig. 3). Our results agree with those of Lindberg et al. (1992), who concluded that the bark of Norway spruce contains more isorhapontin than astringin (Fig. 3). The rapid decline of the levels of glycosides was described in *in vitro* maintained excised bark discs of Sitka spruce following the fungal challenge (Woodward, Pearce 1988).

It is known that β-glycosidase enzymes are able to metabolize stilbene glycosides to the respective aglycones (Woodward, Pearce 1988). Since the β-glycosidase activities were not measured in this experiment, we can only speculate that the significant decrease in isorhapontin, astringin and piceid contents in Norway spruce cells after treatment with both WF and PF preparations might result from the activities of β-glycosidase enzymes. The decrease in stilbene levels in treated cells might also be partly explained by their incorporation into the cell walls (Lange et al. 1994).

Thus, our results show that although the components of the pathogen cell walls and intracellular protein preparations of *Sirococcus strobilinus* mycelium differed substantially, the responses of treated cells to them (characterized by variations in

contents of phenolics and stilbenes) were similar, although there were differences in the kinetics of these responses.

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