COMPARISON OF THE EFFECTS OF *FUSARIUM SOLANI* FILTRATES *IN VITRO* AND *IN VIVO* ON THE MORPHOLOGICAL CHARACTERISTICS AND PEROXIDASE ACTIVITY IN PEA CULTIVARS WITH DIFFERENT SUSCEPTIBILITY

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SUMMARY

Intact plants and *in vitro* cultures of four pea (*Pisum sativum*) cultivars (Adept, Herold, Komet, Menhir) that vary in their degree of susceptibility/resistance to *Fusarium solani* were inoculated with this pathogen or treated with its culture filtrates to compare their reaction patterns at the phenotypic, histological and biochemical level. Changes in activity of three peroxidase forms, cytosolic, membrane- and ion-bound, and in peroxidase isozymes were studied in detail. In addition, the length and weight of roots of regenerating plantlets in explant cultures as well as symptom expression in intact plants were assessed. *In planta*, screening of the four pea cultivars revealed the highest degree of resistance in cv. Adept, and the highest level of susceptibility in cv. Menhir both to *F. solani* and its metabolites. Regarding *in vitro* cultures, the microfiltrated filtrates had stronger inhibitive effect than the autoclaved ones. In terms of peroxidase activity, the only significant difference among cultivars was found in its ionic form, for the most resistant cv. Adept versus the most sensitive cv. Menhir. Minor changes in activity of cytosolic peroxidase were noted *in planta* and *in vitro*, while the membrane- and ion-bound peroxidase significantly decreased in explants. The aim of our study was to compare reaction of pea plants and explants and to verify whether the application of fungal filtrates is able to mimic the *F. solani* pathogenesis. Several responses of explants to filtrates were found to be analogous to the plant reaction to pathogen infection both at morphological and physiological levels. These can be utilized in an early *in vitro* screening for plant tissue resistance to *F. solani*.

Key words: *in planta*, fungal metabolites, pathogenesis, selection for resistance, *Pisum sativum.*

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INTRODUCTION

In vitro methods of selection of plant genotypes with enhanced resistance to fungal diseases are primarily based on the utilization of explant cultures and their diversity, conditioned by somaclonal variation. *In vitro* selection proceeds with adapted pathogen-derived selective agents (Svábová and Lebeda, 2005; Lebeda and Svábová, 2010). Fungal culture filtrates or other pathogen-derived selective agents such as chitosan, fusaric acid, Fsph DNAse, and other elicitors are able to induce defence responses, including oxidative stress in plants (Hadwiger and Beckman, 1980; Hadwiger *et al.*, 1995; Kumar *et al.*, 2008; Kuzniak, 2001; Saikia *et al.*, 2006; Vidhyasekaran *et al.*, 2002). In optimal cases, mixtures of these fungal secondary metabolites elicit in plant tissues *in vitro* a range of physiological and biochemical changes analogous to *in vivo* pathogenesis. Resistant lines of various crops, e.g. banana, barley, grapevine, tobacco, tomato and wheat, were successfully developed via selection *in vitro* (Svábová and Lebeda, 2005; Lebeda and Svábová, 2010). A range of studies was focused on the principles of the interaction between selection agents *in vitro* and pathogenesis *in vivo* (Behnke, 1980; Buiatti *et al.*, 1985; Huang and Hartman, 1998; Hamid and Strange, 2000; Jayasankar *et al.*, 2000; Hollmann *et al.*, 2002), as well as on their biochemical basis, mainly the role of reactive oxygen species (ROS), and pathogenesis-related proteins (PR proteins), in plant-pathogen or explant-selective agent interactions (e.g. Djebali *et al.*, 2007; El-Gendy *et al.*, 2001; Kuzniak, 2001; Morkunas and Gmerek, 2007; Saikia *et al.*, 2006; Singh *et al.*, 2003; Unger *et al.*, 2005).

Expression of plant resistance to pathogens relates to many changes at the physiological, biochemical and molecular levels (Glazebrook, 2005; De Wit *et al.*, 2009). Peroxidases, a complex family of proteins that catalyze oxidoreduction of various substrates using H_2O_2, are omnipresent in plant tissues. They facilitate cell wall loosening and growth by elongation as well as cross-linking of cell wall components during structural de-

fence (Almagro *et al.*, 2009). The balance between cleavage and cross-linking of cell wall components is regulated by peroxidase (POX) activity and gene expression mainly through H_2O_2 and ascorbate concentrations (Passardi *et al.*, 2004).

Pea defence mechanisms are relatively well known at the intact plant level, but poorly understood in explant cultures *in vitro* (Lebeda *et al.*, 2001; Luhová *et al.*, 2002; Mlícková *et al.*, 2004). The interaction of pea and *Fusarium solani* is only partly understood at the physiological, biochemical and molecular level. The role of several traits of pea resistance to the pathogen has already been explained, e.g. phytoalexin pisatin, cuticle barrier, and activation of the non-host resistance response and PR proteins (Hadwiger, 2008). Several enzymes, mainly those participating in the metabolism of ROS, have been studied in relation to pea resistance against *F. solani* (Luhová *et al.*, 2002, 2003, 2006). However, the involvement of secondary metabolites in *F. solani* pathogenesis and their potential utilisation for resistance screening of pea is not well known (Svábová and Griga, 1997; Svábová *et al.*, 1998; Lebeda and Svábová, 2010; Lebeda *et al.*, 2010).

The major objective of this research was to develop an assay for *in vitro* selection of pea resistance to *Fusarium solani* using fungal culture filtrates. Application of this methodological approach depends primarily on the existence of a positive relationship between *in planta* resistance of pea to *F. solani* and tolerance of pea explants *in vitro* to fungal culture filtrates. These potential relationships were also studied from the viewpoint of phenotypic, histological and physiological (cytosolic, membrane- and ion-bound POX) changes in treated plants grown *in vivo* and *in vitro*.

MATERIAL AND METHODS

Plant material. Four dry seed pea (*Pisum sativum*) cvs Adept, Herold, Komet, and Menhir with different degrees of susceptibility to various fungal pathogens from the Pea Germplasm Collection of Agritec Plant Research Ltd. (Šumperk, Czech Republic) were used in these experiments (Table 1).

Cultures *in vitro*. Pea seeds were surface-sterilised with 96% ethanol for 30 sec, then in 10% Chloramin B (sodium N-chlorbenzensulphonamide, w/v) (Bochemie Ltd., CZ) for 20 min, and rinsed 3 times in sterile deionized water. Seeds were germinated in 250 ml Erlenmeyer flasks in the dark at 20°C for 3-5 days, on a layer of cellulose wadding covered with filter paper soaked with deionised water. Multiple shoot cultures were initiated from nodal segments of aseptically germinated seeds by stimulation of axillary meristems proliferation and cultured as described by Griga *et al.* (1986). Isolated nodal segments were placed on media containing macro- and microelements, B5 vitamins, 1 µM NAA, 20 µM BAP, 5.5% plant agar (DUCHEFA) and 3% sucrose, and supplemented with autoclaved or microfiltrated fungal filtrates at concentrations according to the particular experiment. The explants were cultured in a growth room at 20-22°C, 16/8h day/night photoperiod and light density 45 µE m^{-2} s^{-1} provided by daylight fluorescent tubes.

For *in vitro* experiments on roots, the shoots were excised from pre-cultured multiple shoots and transferred onto the same media as stated above, omitting 20 µM BAP. The application of filtrates and culture conditions were identical to those described for multiple shoot cultures.

Pathogen culture and preparation of filtrates. The highly virulent isolate FSVG of *Fusarium solani* f. sp. *pisi* (Jones) Snyder et Hansen (syn. = *F. martii* app. et Wu var. *pisi* Jones) (Ondrej *et al.*, 2008) was maintained on Czapek-Dox agar and stored at 4°C. Prior to the production of filtrates, the fungus was grown on pea agar media made from an extract of 200 g pea seeds

Table 1. Passport and description data (according to EVIGEZ) of pea cultivars (*Pisum sativum* L. convar. *sativum*) used in the experiments.

Pea cultivar	Accession No. (EVIGEZ)	Leaf type	Seed colour	Disease resistance to fungal pathogens in the field conditions (1-9 scale)	
				Anthracnoses	Complex of root diseases
Adept	05L0100762	leaf	yellow	5	7
Herold	05L0100964	afila	yellow	7	6
Komet	05L0100736	leaf	yellow	6	4
Menhir	05L0100768	afila	yellow	5.5	3

EVIGEZ – database of Plant Genetic Resources Documentation in the Czech Republic (http://genbank.vurv.cz/genetic/resources/asp2/default_a.htm).
Disease resistance in field conditions (according to Anonymous, 2000, 2008).
Anthracnoses (*Ascochyta pisi*, *Mycosphaerella pinodes*, *Phoma medicaginis* var. *pinodella*).
Complex of root diseases (*Pythium* spp., *Aphanomyces euteiches*, *Phoma medicaginis* var. *pinodella*, *Mycosphaerella pinodes, Thielaviopsis basicola, Fusarium oxysporum* f. sp. *pisi, F. solani, Rhizoctonia solani*).
Evaluation scale of resistance: 1-highly susceptible; 2,3-susceptible, 4,5-low resistance; 6,7-intermediate resistance; 8,9-resistant.

boiled in 1 litre of tap water, complemented with 15 g D-glucose and 5.5 g agar in Petri dishes (90 mm) and cultured at 20-22°C, 16/8 h day/night photoperiod, and light density 45 µE m^{-2} s^{-1} provided by daylight fluorescent tubes. Filtrates were prepared by culture of quadrants of agar with well-grown mycelium laid on the surface of liquid media (0.8% w/v KNO_3, 0.05% w/v KH_2PO_4, 2% w/v sucrose per 1 l of tap water). After 4 weeks the surface mycelium and spores were removed by straining through sieves and cellulose wading. Autoclaved filtrate (A) was produced by autoclaving (120°C, 240 kPa, 15 min), whilst a variant of microfiltrated filtrate (F) was prepared by sieving through a 0.22 µm Millipore filter. Both variants were stored in darkness at 4°C before use.

Screening of plants for resistance to *Fusarium solani* and filtrates. A modified test by Ondrej *et al*. (2008) for evaluation of tolerance/sensitivity to *F. solani* and two types of filtrates were utilised in a series of experiments. Seeds were sterilised in a 5% v/v solution of SAVO [5% NaClO (w/v), Bochemie Ltd., CZ], and imbibed in water for 24 h. Seeds were then sown in plastic pots in wet perlite (type EP AGRO, Perlit Ltd. CZ). For the inoculation variant (I) the *F. solani* spore suspension (concentration 10^5-$10^6$$ml^{-1}$) was poured over the seed (100 ml per row of 20 seeds), whilst for the variants with adjusted selective agents, solutions of A and F at concentrations of 50 and 100% (v/v) were poured over the seed. Plants were grown at 20-22°C, 16/8 h day/night photoperiod and light intensity 220 µE m^{-2} s^{-1} provided by 400W sodium lamps. Disease symptoms were scored using a 0-4 scale (Lebeda and Buczkowski, 1986): 0 = no visible symptoms; 1= mild chlorosis (1-25%); 2 = chlorosis and growth depression (26-50%); 3 = severe chlorosis, wilting, root necrosis (51-75%); 4 = complete collapse of the plant (76-100%). Assessments were performed 10 and 20 days post inoculation (dpi) or treatment by filtrates.

Disease index was determined with the following formula:

$$DI = \Sigma(0 \times A + 1 \times B + 2 \times C + 3 \times D + 4 \times E)/N$$

where: DI = the total degree of infection; A, B, C, D, E = number of plants in each assessed category (0-4); N = total number of assessed plants.

Evaluation of filtrate influence on morphology of *in vitro* explants. The effect of different variants of fungal metabolites on *in vitro* explants was evaluated as percentage of surviving explants, weight (g) and length of roots (mm).

Images of plant samples were captured with a colour digital camera (Nikon 5 Mpi) with controller Nikon DSU-1 (Nikon Instruments, CS-Optoteam, CZ) placed approximately 350 mm above the sample. Images of explants were taken with a macrolens Pentax Cosmicar (Nikon Instruments, CS-Optoteam, CZ), illumination provided by inferior light box Kaiser Prolite Basic (Kaiser Fototechnik, Germany) and superior tubular light Schott (Schott Glass, Germany). NIS Elements AR 2.30 software (Laboratory Imaging Prague, CZ) was used for subsequent image analysis. Measurement of the root length was based on stamen model.

Rootage of seedlings grown *in vitro* was weighted 20 days following treatment by *F. solani* filtrates (30 and 40% autoclaved, 10 and 20% microfiltrated) using a laboratory scale Mettler PE1600. Data are presented as a mean of cultivars (n = 20-50).

Statistical analyses. Each experimental variant was set up with a minimum of 20 explants or plants. Data were analysed by ANOVA and the means were compared with the Fischer LSD test. Data of POX activity were further processed to calculate correlation coefficients using STATISTICA 8 CZ software (StatSoft, USA).

Enzyme extraction procedures. For determining POX activity a three-step extraction protocol was used (Sedlárová *et al.*, 2007). Cytosolic POX was extracted by 1% polyvinylpyrrolidone (w/v) in 50 mM phosphate buffer (pH 7.0), membrane-bound POX by 1% Triton X-100 in phosphate buffer, and ionic POX by 0.1 M KCl in phosphate buffer. For peroxidase isozyme analysis, *ca.* 0.5 g of plant tissues (leaves and stems) were homogenized in three volumes of 0.1 M potassium phosphate extraction buffer (pH 7.0) using mortar and pestle. Extracts were centrifuged (12,000 *g*, 4°C, 15 min) and the supernatant stored in aliquots at -80°C until assayed.

Peroxidase activity. Activity of cytosolic (cPOX), membrane (mPOX) and ionic-bound (iPOX) peroxidases were assayed spectrophotometrically on a microplate reader (Synergy HT, Biotek, USA). POX activity was measured by a modified method with guaiacol (Angelini *et al.*, 1990). The reaction mixture contained plant extract, 0.1 M potassium phosphate buffer (pH 7.0), and 5 mM guaiacol. The reaction was started by pipetting 35 mM hydrogen peroxide (final concentration of 4.4 mM). Time-dependent increases in absorption due to formation of tetraguaiacol at λ = 436 nm (ε = 4.5 nM^{-1} cm^{-1}) were detected continuously for 1 min at 30°C.

Isozymes and antioxidative enzymes. Electrophoretic separation of POX isoforms was performed by non-denaturing PAGE (30 µl of sample, 4% stacking gel and 8% resolving gel with 0.025 M Tris and 0.19 M glycine buffer pH 8.3, at 4°C). There were no significant differences in protein concentration in sample extracts (data not shown). Specific visualizations of POX isozymes were performed using SigmaFast™ 3,3´-di-

aminobenzidine (DAB) tablets (Sigma-Aldrich, USA). Changes in number, intensity and position of bands were assessed visually.

Histochemical localization of POX. POX activity within plant tissues was visualised by formation of an intense dark brown precipitate due to DAB oxidation. Approximately 5 mm long fragments of pea hypocotyl, adjacent parts of root and stem (i.e. 3 fragments per plant were stained), or from explants were vacuum-infiltrated with DAB-urea hydrogen peroxide solution (SigmaFast™, Sigma-Aldrich, USA). After staining (3 h), the samples were boiled in ethanol for 10 min, rinsed in deionised water and immersed in 70% glycerol until studied. Transverse sections of tissue segments (40-60 μm thick) were cut with a hand slicing microtome and observed with an Olympus BX60 light microscope equipped with a CCD camera DP70 (Olympus Czech Group, CZ).

RESULTS

***In planta* screening of pea cultivars for resistance to *F. solani* and fungal metabolites.** As to the effect of *F. solani* culture filtrates, the only significant differences in disease index (DI) among genotypes were found in cvs Adept and Menhir, both 10 and 20 dpi (Fig. 1). Disease symptoms (Fig. 2) were recorded in all variants of inoculation and filtrate treatment, although the exhibited effects differed with the cultivar and filtrate variants. In both types of filtrates a positive relationship was found between filtrate concentration and intensity of disease symptoms. No substantial differences were found between autoclaved and microfiltrated filtrates applied at identical concentrations. In general, cv. Adept was the most tolerant to both types of filtrates and cv. Menhir the most susceptible. Nevertheless, no direct relationship between the level of pea cultivar susceptibility to fungal infection and filtrate treatment was detected. Tolerant plants of cv. Adept did not show growth depression after inoculation, the roots were attacked only

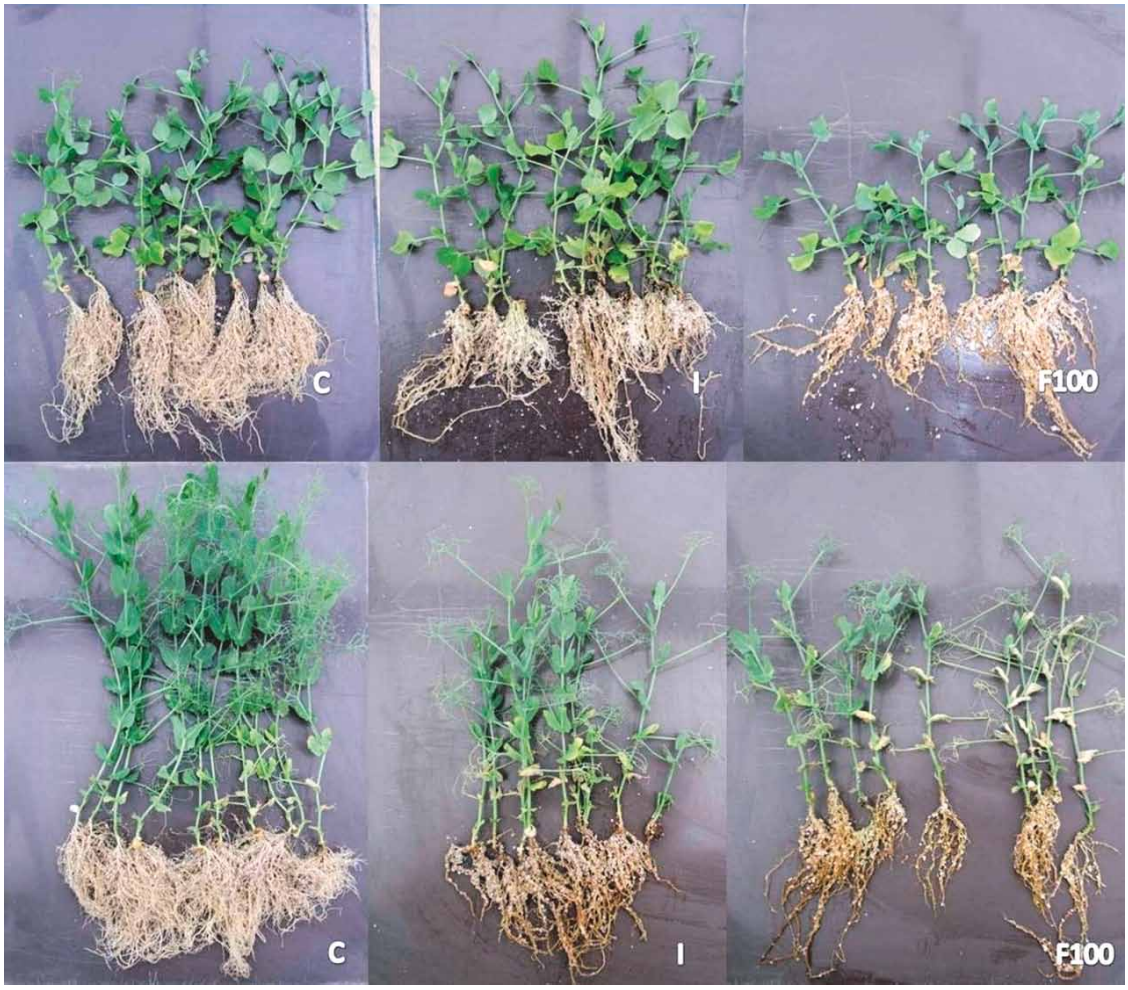

Fig. 1. Symptoms observed in cvs Adept (upper row) and Menhir (lower row), after inoculation by *Fusarium solani* (I), and application of microfiltrated culture filtrate (100% concentration, F100) of *F. solani* after 20 days. C = control.

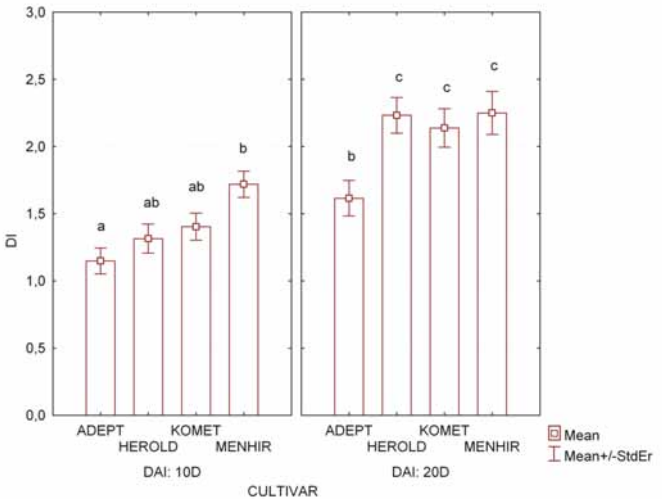

Fig. 2. Differences in susceptibility of four pea cultivars to *Fusarium solani* 10 and 20 days after inoculation, evaluated by disease index (DI); different letters mean statistically significant differences.

slightly, but after treatment with 100% filtrate the growth depression was visible. Plants of cv. Menhir exhibited growth depression, wilting of leaves, and browning of roots both following inoculation and 100% filtrate treatment.

Effect of autoclaved and microfiltrated filtrates on explants morphology. Four pea cultivars grown in multiple shoot culture were treated with both types of *F. solani* filtrates in concentrations of 25 and 50% (v/v). Generally, the microfiltrated variant of filtrate (F) expressed more inhibitive effect on *in vitro* cultures than the autoclaved (A) variant (Fig. 3). Both 25 and 50% concentrations (v/v) of microfiltrated filtrate had a severe inhibitory effect on pea growth and development *in vitro*, suppressing shoot development four weeks after treatment. The autoclaved filtrate at 25% (v/v) had only a minor effect on *in vitro* explants, but at 50% (v/v) it reduced the development of cvs Herold and Komet but not those of either the most tolerant cv. Adept or the most sensitive cv. Menhir. Moreover, cv. Menhir displayed swelling of the basal parts of explants (Fig. 3). Due to the effect observed in this series of preliminary experiments, the concentrations of fungal filtrates were changed in subsequent experiments aimed at assessing explant survival, root weight and length. The concentrations were decreased to 10 and 20% for

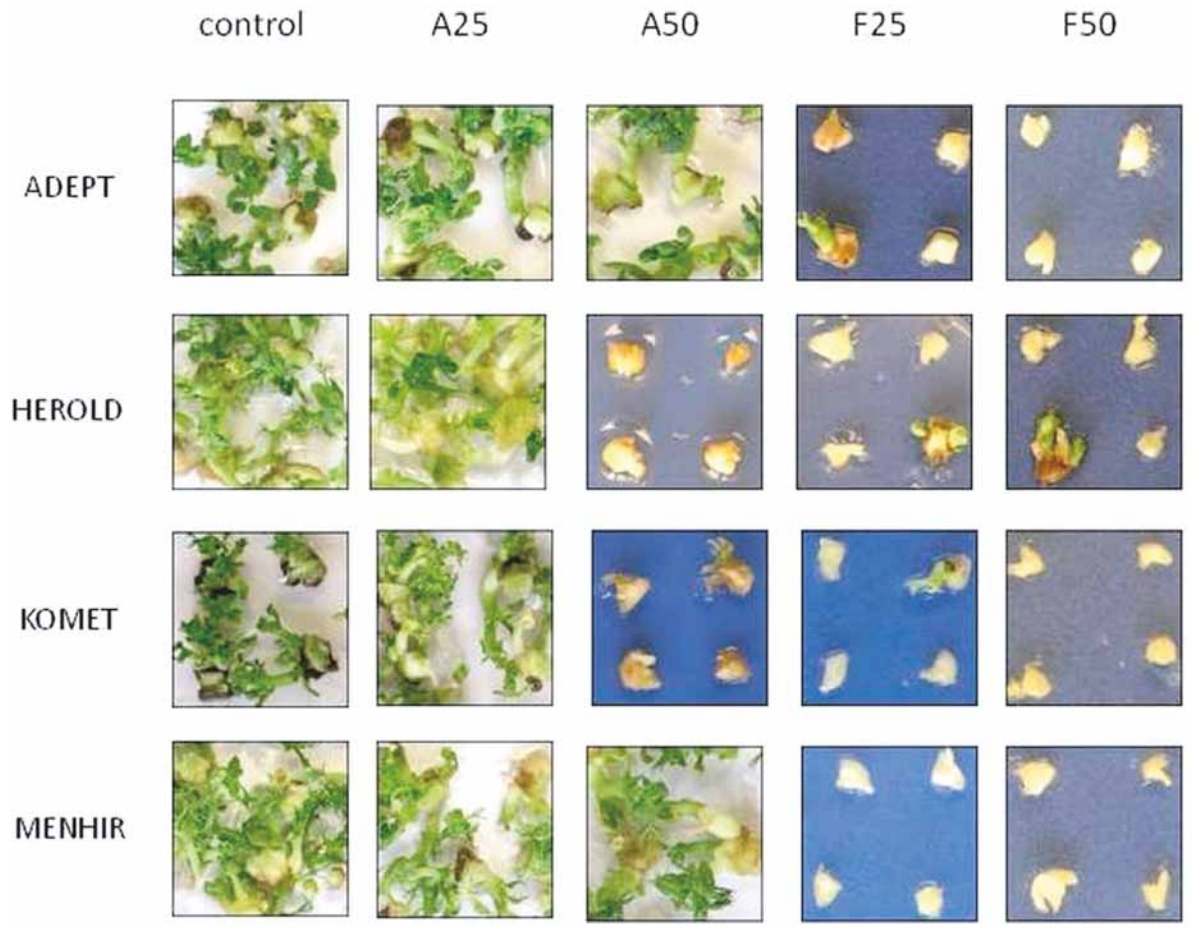

Fig. 3. Effect of treatment by *F. solani* filtrates (A-autoclaved, F-microfiltrated) in two concentrations (25 and 50% v/v) on multiple shoot culture of four pea cultivars, 20 days after treatment compared to untreated control.

microfiltrated variants (10F, 20F), and 30 and 40% for autoclaved ones (30A, 40A).

Effect of filtrates on *in vitro* explant survival, length and weight of roots. When the percentage of surviving explants treated with *F. solani* A and F filtrates was compared, resistant cv. Adept showed a significantly higher percentage of explant survival (84%), whereas the other cultivars did not differ from each other. Similarly, all variants of filtrate treatments differed from the control (mean 91%), but did not differ between each other (Fig. 4).

Length of roots assessed by image analysis was used for evaluation of the relative tolerance/sensitivity of cultivars to fungal filtrates. The mean value of total root length was 37 mm. However, all treatment variants (control, 10F, 30A and 40A), except for 20F, did not differ statistically from one other.

Significant differences in pea cultivar response to application of fungal filtrates were recorded in the weight of roots. Resistant cv. Adept exhibited the largest and heaviest root system, whereas differences among the remaining pea cultivars were insignificant (Fig. 5). Individual filtrate variants differed in their influence on plant root weight, i.e. F variants significantly impacted plant root weight at 10 and 20% concentration, whereas A variants were not effective at both 30 and 40% concentration.

Isoenzymes of peroxidase. The changes of POX isoenzyme spectra were studied to detect possible biochemical responses of plant tissue to infection and to pathogen-derived selective agents at the level of intact plants and *in vitro* cultures. Electrophoretograms of POX showed no differences in position and intensity of bands (both *in planta* and *in vitro* cultures). The only exception was an increase in the intensity of slow-migrating bands in the upper part of electrophoretograms (samples from 7th and 14th dpi), which might be related to the growth of plants and cultures *in vitro* rather than

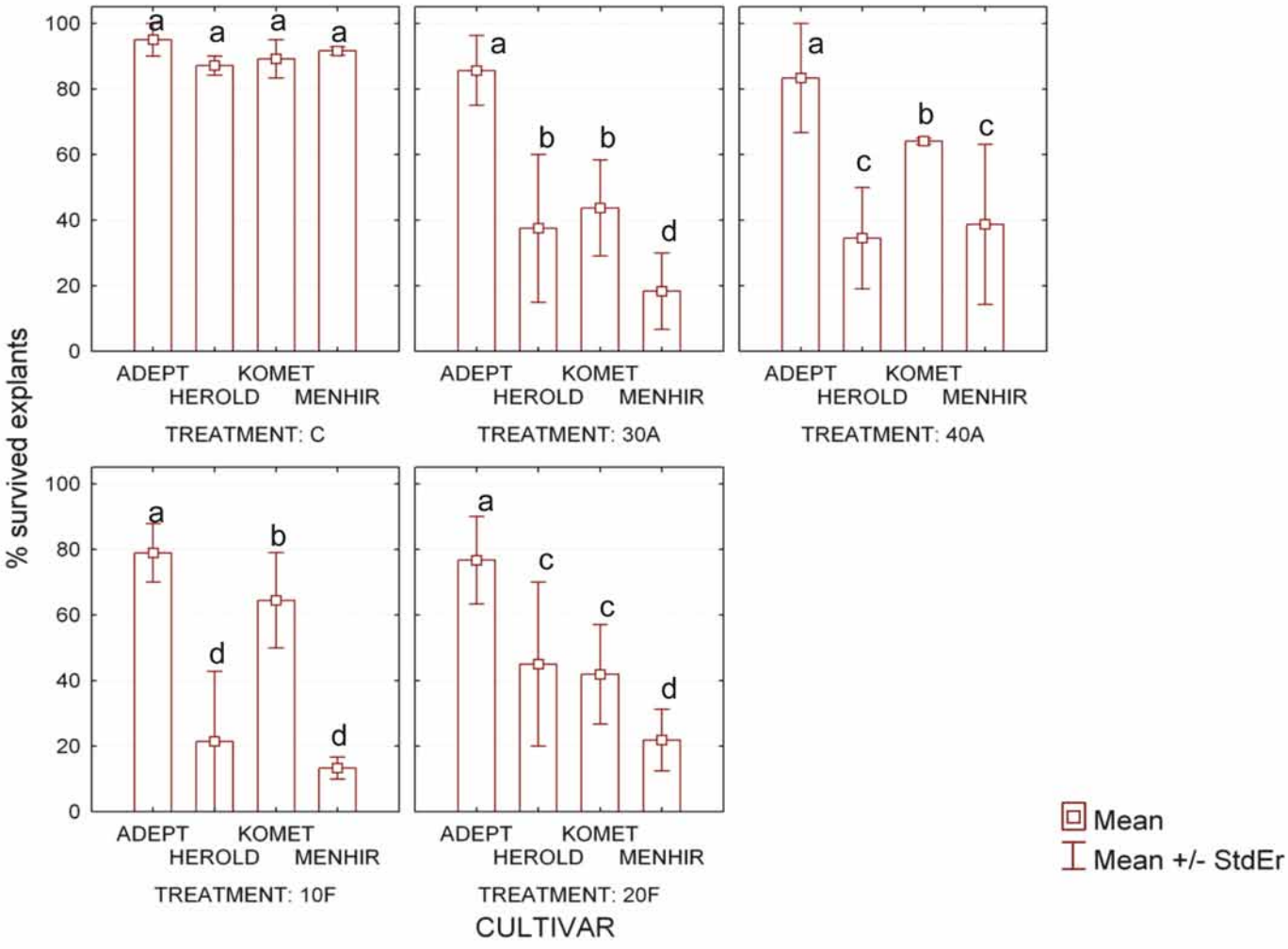

Fig. 4. Effect of *F. solani* filtrates on percentage of survived explants of pea *in vitro* explants (C- control; 30A, 40A-30, 40% v/v autoclaved filtrate; 10F, 20F-10, 20% v/v microfiltrated filtrate); different letters mean statistically significant differences.

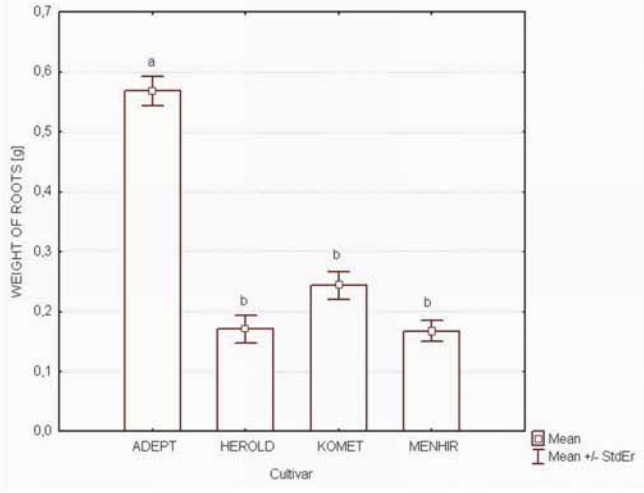

Fig. 5. Weight of roots in *in vitro* cultures after 20 days of treatment by *F. solani* filtrates (C, 30A, 40A, 10F, 20F) expressed as mean of cultivars; different letters mean statistically significant differences.

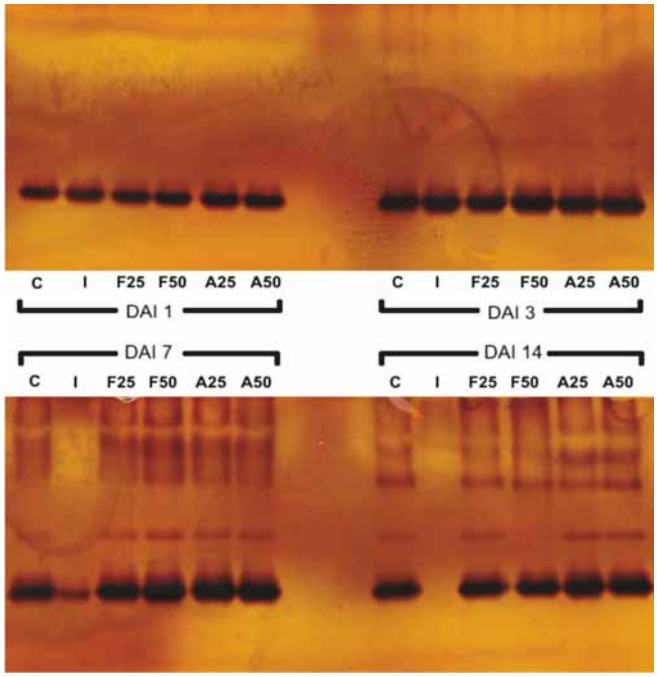

Fig. 6. Changes in peroxidase isoforms in cv. Menhir during 1, 3, 7 and 14 days after treatment (C-untreated control; I-inoculation; A-autoclaved and F-microfiltrated treatment, both 25 and 50% v/v).

to fungal infection. The only significant changes in band intensity were observed in plant samples I (inoculation) as a decrease of intensity at 7 dpi and nearly invisible band at 14 dpi (Fig. 6).

Peroxidase activity. The first experiment with all four pea cultivars was performed 20 dpi, but the inoculated variant had to be removed 10 dpi due to complete necrotization of explants *in vitro*. Before the beginning of the experiment very low activity of all three POX types, with a mean value of 0.80 μkat g^{-1} FW, was recorded in control variants (C1). After 20 days there was a strong increase of POX activity in control samples (C2) to a mean value 12.86 μkat g^{-1} FW, but with low activity of mPOX 4.73 μkat g^{-1} FW. With the exception of filtrate treatment 50F, all other treatments (25F, 25A, 50A) did not differ significantly from the control (C2). All cultivars, except for cv. Komet, possessed the expected activity values of the three POX types, with respect to their tolerance or sensitivity to *F. solani*. Cvs Adept, Herold and Menhir were characterized by mean values over all three POXes of 9.84, 7.21, and 5.42 μkat g^{-1} FW, respectively, i.e. POX (especially iPOX) activity decreased with cultivar sensitivity to *F. solani*. However, the highest average value of POX activity, 10.49 μkat g^{-1} FW, was recorded in the moderately sensitive cv. Komet.

Detailed experiments with the most susceptible cv. Menhir focused on differences between the two variants *in planta* and *in vitro*. The activities of all three forms of peroxidases were assayed 1, 3, 7 and 14 dpi in *in vitro* and *in planta* samples treated with *F. solani* filtrates. The mean values of activity for all POX forms increased with time interval both within *in vitro* and *in planta* samples. Two main differences between *in vitro* and *in planta* samples were observed: (i) increased activity of cytosolic POX in *in vitro* conditions versus gradual increase of ionic POX in *in planta* samples during the experimental period (Fig. 7, 8); (ii) in general, 9- to 10-fold higher POX activity was detected in *in planta* samples. Mean of cytosolic POX activity in plants was slightly higher (0.95 μkat g^{-1} FW) than in samples from *in vitro* cultures (0.67 μkat g^{-1} FW). The membrane-bound POX was 30-fold higher in plants than *in vitro* (2.13 μkat g^{-1} FW vs. 0.07 μkat g^{-1} FW), and ionic POX was 33-fold higher in plants compared to *in vitro* cultures (5.27 μkat g^{-1} FW vs. 0.16 μkat g^{-1} FW).

In planta mPOX activity slightly correlated with mPOX activity *in vitro* (r = 0.56671). In contrast, iPOX activities did not correlate (r = 0.09314), and there was a negative correlation (r = - 0.5361) between *in planta* and *in vitro* response of cPOX activities (data not shown).

Localization of POX within plant tissues. Histochemical detection of POX in intact plants and explants of pea treated with *F. solani* or with individual variants of filtrates (25F, 50F, 25A, 50A) showed no significant differences in the effects of filtrate concentration on POX accumulation (data not shown). The only difference in POX accumulation was found between untreated controls and samples co-cultivated with the pathogen, where growing mycelium induced necrosis of host tissues (Fig. 9). A strong signal for POX activity was detected both in pathogen's hyphae and in plant cells in intimate contact with *F. solani*. No variation in

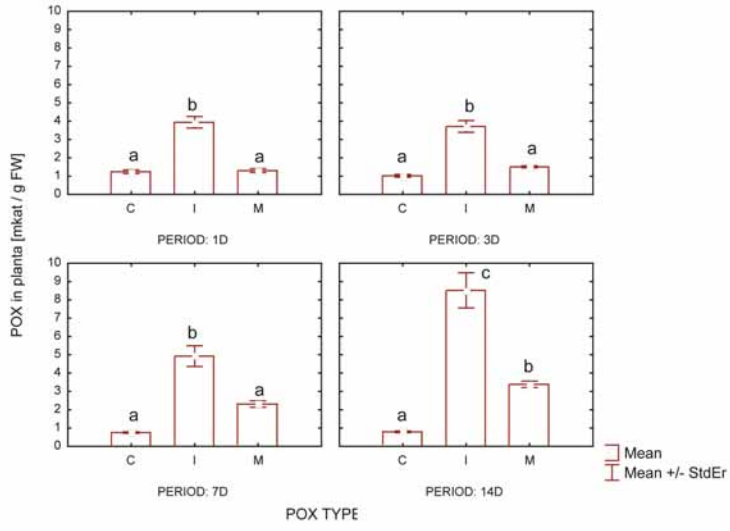

Fig. 7. POX activity during 14 days in cv. Menhir, differences in activities of cytosolic (C), ionic (I), membrane-bound (M) POXes between *in planta*.

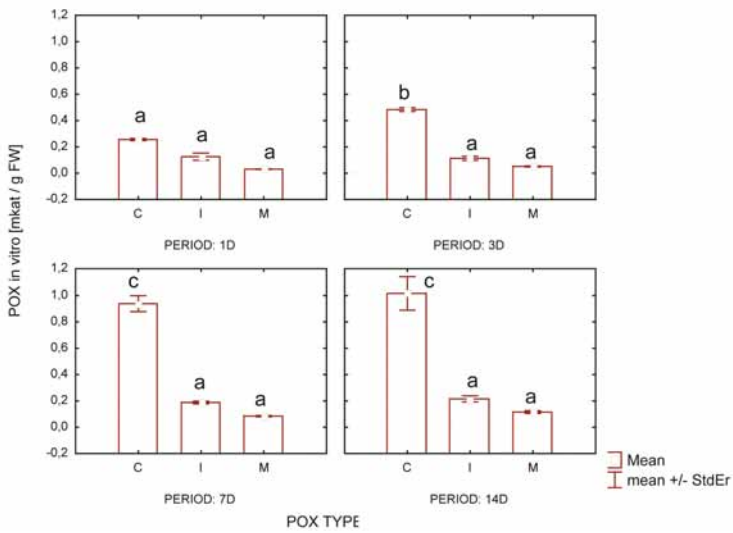

Fig. 8. POX activity during 14 days in cv. Menhir, differences in activities of cytosolic (C), ionic (I), membrane-bound (M) POXes between *in vitro* cultures.

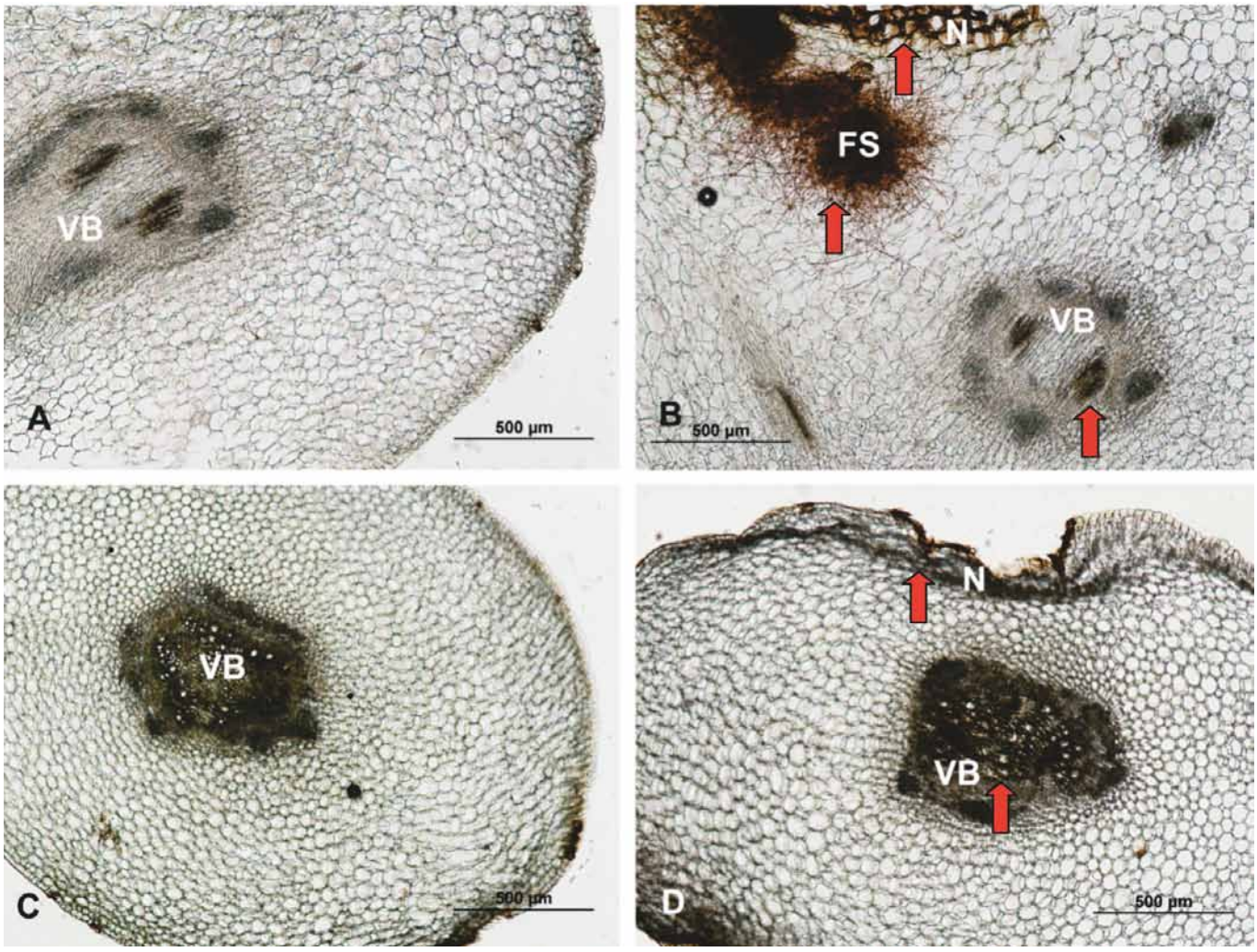

Fig. 9. Localization of POX in tissues of explants (A,B) and stems closely above hypocotyl (C, D) in *Pisum sativum* cv. Adept without treatment (A,C) and 7 dai by *Fusarium solani* (B,D). Dark staining for POX was localized in vascular bundles (VB), hyphae of *F. solani* (FS) and necrotizing cells (N).

POX presence among roots, hypocotyls and stems was revealed in intact plants. Localization of POX changes in explants was influenced by a strong accumulation in tissues due to previous mechanical injury of plant tissues during preparation of explants.

DISCUSSION

Culture filtrates of some plant pathogenic fungi are able to produce disease symptoms and thus can be used for resistance selection (Svábová and Lebeda, 2005; Lebeda and Svábová, 2010). This paper expands our previous studies focused on defence mechanisms of *P. sativum* against *Fusarium* spp. (Svábová and Griga, 1997; Svábová *et al*., 1998; Luhová *et al.*, 2002, 2003, 2006) and contributes to the general understanding of disease resistance in this pathosystem (Hadwiger, 2008), and to the possibility of using fungal culture filtrates for resistance selection *in vitro* (Lebeda and Svábová, 2010).

The host-pathogen interaction between pea and *F. solani* is very variable. Previous data on the variation in resistance/susceptibility of peas to *Fusarium* spp. have been reviewed (Hagedorn, 1984; Jacobsen, 1992; Lebeda *et al.*, 2010) and Ali *et al.* (1994) have summarised the available information on valuable sources of resistance to soil-borne root diseases of peas. It is evident that sources of resistance in peas are rather limited. Our *in planta* screening of four pea cultivars with *F. solani* showed genotypic differences in resistance. Twenty days after inoculation or treatment with filtrates, cv. Adept expressed a higher level of resistance compared with cvs Herold, Komet and Menhir (Fig. 2). Treatment of the same set of intact pea plants by inoculation and with filtrates resulted in a similar reaction pattern. However, significant differences were recorded between two tested concentrations (50 and 100%) of filtrates. In general, the most similar reaction pattern to *in planta* inoculation was recorded with the 100% microfiltrated fungal suspension. These data disclose a relationship between pathogenicity of *F. solani* and symptoms expression after treatment of pea plants with the fungus and fungal filtrates in the studied pathosystem. A similar relationship was reported between plant response to inoculation by pathogen and treatment by filtrates of *F. oxysporum* in chickpea (Hamid and Strange, 2000) and pineapple with *F. subglutinans* (Borrás *et al.*, 2001). Moreover, a positive correlation between the area under disease progress curve of inoculated plants and the cut seedling test was observed in soybean using culture filtrates of *F. solani* (Huang and Hartman, 1998).

Inactivated, i.e. autoclaved or microfiltrated, fungal cultures represent complex mixtures of substances derived from the pathogen isolate. Fungal culture filtrates may contain a spectrum of secondary metabolites like polysaccharides, oligosaccharides, proteins, glycoproteins, unsaturated fatty acids, growth regulators as auxin, cytokinins and gibberellic acid, along with toxins that may play a role as co-determinants of pathogenicity during disease development (Svábová and Lebeda, 2005). The application of filtrates to plant cultures *in vitro* can trigger the elicitation of various defence responses, including the induction of enzymes such as peroxidases, β1,3-glucanase, and chitinase, and the synthesis and accumulation of phytoalexins, phenols and phenolic acids (Lebeda and Svábová, 2010).

A phenotypic response of plants to filtrates, similar to that elicited by fungal inoculation, is one of the basic pre-requisites for the possibility to substitute fungus treatment with filtrates for *in vitro* experiments. Based on the phenotypic responses of pea cultivars in our experiments, it can be concluded that fungal filtrates are able to mimic symptoms induced by *F. solani* inoculation. Our results show that the microfiltrated variant of *F. solani* filtrate was more efficient and have a higher inhibitive effect on *in vitro* pea explants compared with the autoclaved filtrate (Fig. 3). The process of autoclaving is responsible for inactivation or decomposition of some compounds as thermo-labile proteins which are involved in the pathogenicity and the expression of symptoms. The responses of pea explants showed significant genotypic differences in parameters such as percentage of surviving explants, length and weight of roots. Cv. Adept expressed a significantly higher tolerance to the negative effect of filtrate, whereas other cultivars were more sensitive and did not differ in tolerance. This relationship is in agreement with the expression of resistance degree *in planta* (Fig. 2). From the viewpoint of selectivity, the microfiltrated variant (20F) was the most efficient. A comparable phenomenon, inhibition of callus growth, was reported for chickpea treated with *F. oxysporum* filtrates (Singh *et al.*, 2003) and in cell culture of soybean treated with *F. solani* f. sp. *glycines*, where a decrease of viability of mesophyll cells was observed and correlated with foliar symptom severity (Li *et al.*, 1999).

Oxidative stress, including ROS production, was reported in different plant species after inoculation or natural fungal attack by *Fusarium* spp. or other pathogens, e.g. in the pathosystems bean-*Botrytis cinerea* (Unger *et al.*, 2005), carnation-*F. oxysporum* (Van Pelt-Heerschap and Smit-Bakker, 1999), chickpea-*F. oxysporum* (Singh *et al.*, 2003), potato-*F. sambucinum* (Ray and Hammerschmidt, 1998), and yellow lupine-*F. oxysporum* (Morkunas and Gmerek, 2007).

Plants are protected against ROS damage by an antioxidant system which includes enzymes of ROS catabolism such as superoxide dismutase and peroxidases (Lebeda *et al.*, 2001). Rapid production of ROS in the apoplast in response to pathogen attack has been proposed to orchestrate different defensive barriers against

the pathogens (Torres *et al.*, 2006). This trend was observed in resistant lines of *Medicago truncatula* which revealed rapid increase of iPOX after inoculation with *Phoma medicaginis* (Djebali *et al.*, 2007), and in mungbean (*Vigna radiata*) after treatment with an elicitor from *Macrophomina phaseolina* (Vidhyasekaran *et al.*, 2002).

Cell wall-bound peroxidases also belong to potential sources of ROS in plants (Able, 2003; Polkowska-Kowalczyk and Maciejewska, 2001). Generally, the response of resistant genotypes is characterised by an increase of activities of extracellular POXs, i.e. membrane-bound and ionic, during the first 24 h and then a stable increased level or gradual decrease. The *in vitro* response of POX in cv. Komet is more similar to the trend recorded by the more resistant cv. Adept. There are two possible explanations for this reaction: cv. Komet is either more resistant than previously reported, or it may have a very good adaptability to *in vitro* conditions which may also cause the increased activity of POX. In our experiments, susceptible cv. Menhir showed delayed increase of mPOX and iPOX as a result of late pathogen recognition, possibly following a weak strengthening of the cell wall as well as a late start of cell signalling (Fig. 7, 8). Low values of cPOX activities may be a result of the lack of resistance gene expression by sensitive cv. Menhir. Similar results were recorded in *Solanum* cell culture of a sensitive genotype after treatment with culture filtrate of *Phytophthora infestans,* where the activity of intracellular peroxidases did not change, whereas extracellular POX activity increased by 30% (Polkowska-Kowalczyk and Maciejewska, 2001).

Several papers dealing with the study of POX activities do not differentiate the forms of POX and record only total POX activity (Djebali *et al.*, 2007; Singh *et al.*, 2003; Vidhyasekaran, 2002), whilst other studies differentiate POX types (Cachinero *et al.*, 2002; Morkunas and Gmerek, 2007; Polkowska-Kowalczyk and Maciejewska, 2001; Vance *et al.*, 1976). POX influences not only plant-pathogen interactions, but also the growth and ageing of cells (Passardi *et al.*, 2004), and morphogenesis, i.e. somatic embryogenesis (Vranová *et al.*, 2002). The differences in the amount of POX types are probably also strongly influenced by specific *in vitro* conditions, such as hermetic sealing, limited space, limited exchange of gases and metabolic waste products and the content of nutritive substances and phytohormones in the media.

Our results represent one of the first studies focused on the *P. sativum-F. solani* interaction *in vivo* and *in vitro*, from the viewpoint of morphological changes as well as changes in the activity of peroxidases in plants and explant cultures exposed to the fungus and fungal filtrates. Results suggest that responses to *F. solani* filtrates mimic the physiological response *in planta* as well as *in vitro*. The activity of three POX types, cytosolic, membrane-bound and ionic, is suitable for the assessment of the adaptability to stress, including biotic stress caused by *F. solani* and its filtrates. The resistant response is characterised by the immediate increase of all three types of POX. After an immediate increase of POX activity, a successive decrease comes as a result of plant adaptation to the infection. Conversely, the sensitive response is specific in later start of POX increase, as a result of late pathogen recognition, and the decrease of POX activity is parallel to plant cell death.

Recent studies have shown that there is some positive relationships between the pathogenicity of *F. solani* and pathogenesis processes on pea plants grown *in planta* and *in vitro*. It is evident that culture filtrates of *F. solani* could mimic the activity of inoculation by the pathogen, i.e. they could be used for *in vitro* selection. However, a detailed understanding of the physiological and biochemical processes responsible for this interaction remains unclear.

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