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Induction of defense mechanisms in seedlings of oilseed winter rape inoculated with *Phoma lingam* (*Leptosphaeria maculans*)

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Abstract The aim of this study was to find the biochemical markers of blackleg, evoked by the fungus *Leptosphaeria maculans* in seedlings of oilseed winter rape. Analyses of antioxidant enzymes activity and reactive oxygen species detection were performed 24, 48 and 72 h after inoculation. Moreover, measurements of the total pool of phenolic compounds, *trans*-cinnamic acid and ferulic acid were carried out. The results proved the virulence of Ph L5 isolate towards the rape seedlings. An increase in total phenolic content, including *trans*-cinnamic acid, was observed 24 h after inoculation. High activity of phenylalanine ammonia-lyase is probably another symptom of the defense mechanisms, despite the fact that it was not accompanied by elevated phenolics level, which were probably rapidly exhausted during *Phoma lingam* inoculation. A drop in phenolics content was balanced by higher activity of SOD, CAT and non-specific peroxidases. Enhanced activity of these enzymes was a response to the reactive oxygen species overproduction. ROS accumulation in indirectly inoculated first true leaves after inoculation may suggest a systemic defense response.

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Introduction

Oilseed winter rape is exposed to diseases caused by fungal infections during its entire growth period. Nowadays, when the rape crops acreage is constantly being enlarged to grow plants used for biofuel production, this problem is becoming even more important. Moreover, oilseed rape cultivation tends toward limitation of pesticide usage, with the added benefits of increased environmental protection and quality of food based on rape-seed oil. Therefore, in many countries, improving the resistance of oilseed winter rape against pathogenic fungi becomes a vital issue.

Stem canker (blackleg) caused by the fungus *Leptosphaeria maculans*, anamorph: *Phoma lingam* (Tode ex Fr.) Desm., is one of the most damaging diseases suffered by oilseed winter rape and it causes large yield losses (Jędryczka 2007). *L. maculans* is a hemibiotrophic pathogen with an initial necrotrophic stage characterized by toxin (sirodesmins) production and necrotic spot formation (Fitt *et al.* 2006; Jindřichová *et al.* 2011; Kachlicki & Jędryczka 1994; Rouxel & Balesdent 2005). The first symptoms of blackleg can be observed at the seedling stage during autumn, but the disease develops throughout all the growth stages of the plant (Gladders & Musa 1980).

Plants attacked by the pathogenic fungus can activate many defense mechanisms to restrict the pathogens'

growth and/or to destroy them. Hypersensitive response (HR) under fungus infection is one of the most important defense mechanisms, and it results in both necrotization of infected tissues and protection of non-infected ones (Roussel *et al.* 1999). Cellular necrosis is probably a consequence of reactive oxygen species (ROS) accumulation, mainly hydrogen peroxide (H_2O_2) and superoxide radical ($O_2^{\bullet-}$) (Jindřichová *et al.* 2011). However, it should be stressed that ROS produced in the oxidative burst could reduce pathogen infection and also destroy the non-infected cells. Boosted production of ROS triggers increased activity of antioxidant enzymes, such as catalase (CAT), superoxide dismutase (SOD) and peroxidase (POX) (Foyer *et al.* 1994). All the above mentioned enzymes are involved in the defense mechanisms that control the concentration of $O_2^{\bullet-}$ and H_2O_2 in the infected plants (Bowler *et al.* 1992).

Induction of phenolics production, constituting another defense mechanism, may be of crucial importance for the restriction of pathogenic fungus expansion. Phenylalanine ammonia-lyase (PAL) catalyzes the conversion of *L*-phenylalanine to *trans*-cinnamic acid that is a precursor of other phenolic compounds. It was shown that the increase in PAL activity is associated with the defense responses under pathogen attack (Kavitha & Umesha 2008; Umesha 2006). Plant phenolics were reported to be low-molecular weight ROS scavengers. Some of them can be utilized during lignin biosynthesis, and as phytoalexins they inhibit pathogen development in infected and non-infected cells (Matern & Kneusel 1988; Płażek *et al.* 2005).

The biochemical response of oilseed winter rape to blackleg at the cellular level has not been well recognized so far. To find a reliable biochemical indicator of oilseed rape resistance to blackleg, we need to find out which defense mechanisms are activated in response to *L. maculans* attack. In this study we present biochemical responses induced by spores of *P. lingam* in seedlings of oilseed winter rape, determined 24, 48 and 72 h after inoculation. Analyses of the antioxidant enzymes activity (SOD, CAT, POX) and ROS level ($O_2^{\bullet-}$ and H_2O_2) were performed. Additionally, measurements of total pool of phenolic compounds, *trans*-cinnamic acid and ferulic acid were carried out. *Trans*-cinnamic acid was chosen as the precursor of other phenolics in the phenylpropanoid pathway, while ferulic acid was selected as a compound that could affect cell wall properties *via* its lignification.

Materials and methods

Plant growth conditions The experiment was carried out on seedlings of oilseed winter rape (*Brassica napus* L.) cv. Lisek. The seedlings were grown in plastic pots (five seedlings per pot) 15 cm in diameter and 18 cm in height. Each pot was filled with a mixture of soil, peat and sand (2:1:1 v/v/v). The plants were grown in an air-conditioned greenhouse chamber, at 16/16°C (±2°C) day/night, and ~60% r.h. The plants were additionally illuminated (12h L : 12h D), so that PPFD (photosynthetic photon flux density) at the leaf level was ~200 $\mu mol\ m^{-2}\ s^{-1}$.

Inoculation of cotyledons with P. lingam Sixteen day-old seedlings (stadium of two cotyledons) were inoculated with the *P. lingam* spores according to the method described by Jędryczka *et al.* (1991). To allow the spore suspension infiltrate into the intercellular space, the seedlings were punctured with a needle in the middle of each half of the cotyledon. Then each puncture site was centrally injected with 10 μl of inoculum with a density of 1.5 x 10^7 spores ml^{-1}. As a consequence each seedling had four inoculation points in which the spore suspension was administered. Control plants were injected with 10 μl of sterile distilled water. Spore suspension and water were applied using a syringe with a needle. After that the seedlings were incubated in a shady place in the chamber at 18°C and the relative humidity was adjusted to 80%. The isolate (Ph L5) (Kachlicki & Jędryczka 1994) from the fungus *P. lingam*, used for this experiment, was obtained from the Institute of Plant Genetics of the Polish Academy of Sciences in Poznań.

Biochemical measurements and sample collection The measurements were performed 24 h, 48 h and 72 h after inoculation. Due to time-consuming procedures the histochemical analyses of H_2O_2 and $O_2^{\bullet-}$ were completed 48 h, 120 h, and 168 h after inoculation for H_2O_2 and after 48 h, 96 h, and 144 h for $O_2^{\bullet-}$. Another reason for this delay was our intention to estimate the level of defense mechanisms induction associated with ROS generation in the indirectly inoculated first true leaf. CAT, non-specific peroxidase and PAL activities were recalculated for protein content, estimated according to the procedure described by Bradford (1976).

Spectrofluorescence analysis of H_2O_2 Hydrogen peroxide content was calculated in the extracts from fresh leaves according to the procedure described by Ishikawa *et al.* (1993). Samples (150–200 mg of fresh weight) were homogenized in 1.5 ml of extraction buffer (50 mM potassium phosphate buffer pH 7.5, 5% trichloroacetic acid (TCA), 1 mM EDTA, 1% w/v polyvinylpyrrolidone) at 4°C. The homogenate was centrifuged at 14,000 rpm for 10 min. The reaction mixture contained 2.5 ml of 1.25 mM homovanillic acid, 2.5 μl of horseradish peroxidase (1380 U mg^{-1}) and 20 μl of the buffered extract. Hydrogen peroxide was detected with a spectrofluorometer (Perkin-Elmer LS 50B, Norwalk, CT, USA). The samples were excited at 315 nm and the detection was carried out between 400 and 450 nm. The slit width for both excitation and emission monochromators was adjusted to 10 nm. Hydrogen peroxide content was expressed in μl and recalculated in relation to fresh weight. The measurements were taken in three replicates.

Activity of catalase (CAT) (E.C. 1.11.1.6) Catalase activity was estimated according to Aebi (1984). Plant material (350–500 mg of fresh weight) was homogenized in the extraction buffer (50 mM potassium phosphate buffer pH 7.5, 1 mM EDTA) at 4°C. Plant extract (100 μl) was added to the reaction mixture consisting of 0.3 ml of 30% H_2O_2 in 50 mM potassium phosphate buffer pH 7.8 and 1 mM EDTA. Absorbance was analyzed at 240 nm. Enzyme activity was recalculated assuming that absorbance drop of ~0.0145 corresponds to degradation of 1 μmol H_2O_2 within 1 min. The measurements were taken in three replicates.

Activity of non-specific peroxidase The samples (350–500 mg of fresh weight) were homogenized in the extraction buffer (50 mM potassium phosphate buffer pH 7.0, 1 mM EDTA) at 4°C. The reaction mixture included 2 ml of 50 mM potassium phosphate buffer pH 7.8 with 1 mM EDTA, 12 μl of p-phenylenediamine, 12 μl of 30% H_2O_2 and 12 μl of plant extract. Activity of non-specific peroxidase was measured spectrophotometrically at 460 nm. Increased absorbance positively correlates with the accumulation of oxidized products of *p*-phenyldiamine in the presence of H_2O_2 (Bergmeyer 1965). The enzyme activity was recalculated per 1 mg of protein and expressed as the absorbance drop within 1 min. The measurements were taken in three replicates.

Activity of superoxide dismutase (SOD) (E.C. 1.15.1.1) SOD activity was quantified according to McCord & Fridovich (1969). The samples were homogenized in the extraction buffer (50 mM potassium phosphate buffer pH 7.8, 10 mM EDTA) at 4°C. The reaction mixture consisted of 50 mM potassium phosphate buffer pH 7.8 with 1 mM EDTA, buffered solution of 1 mM cytochrome *c*, 1 mM xanthine and the plant extract. One unit was defined as the amount of enzyme necessary for 50% inhibition of cytochrome *c* in a coupled system with xanthine and xanthine oxidase. The absorbance was measured at 550 nm and the enzyme activity was recalculated per 1 mg of protein and expressed in cytochrome *c* units. The measurements were taken in five replicates.

Detection of SOD isoforms (Mn-SOD, Cu/Zn-SOD, Fe-SOD) Electrophoresis was performed at 4°C, 180 V in 13% polyacrylamide gel (Laemmli 1970). For each lane 15 μg of protein extract was applied. Identification of individual SOD isoforms was carried out according to the procedure described by Beauchamp & Fridovich (1971). After electrophoresis the gels were incubated for 30 min in the dark at room temperature in the staining buffer consisting of 50 mM potassium phosphate buffer pH 7.8, 1 mM EDTA, 28 mM TEMED, 0.03 mM riboflavin and 2.45 mM NBT. After that the gels were exposed to daylight until the SOD activity bands became visible. To inhibit Cu/Zn-SOD and Fe-SOD isoforms the gels were stained in the buffer containing 5 mM H_2O_2. Selective inhibition of Cu/Zn-SOD was executed by the gel incubation in the buffer containing 3 mM KCN. Mn-SOD isoform was not inhibited by either H_2O_2 or KCN.

Histochemical detection of H_2O_2 accumulation Hydrogen peroxide in leaves and cotyledons was detected according to Thordal-Christensen *et al.* (1997) with our modification. The leaves and cotyledons were infiltrated in darkness for 15 min under pressure of 0.8 MPa with 3,3'-diaminobenzidine (DAB) solution. DAB aqueous solution was prepared using 1 mg DAB per 1ml H_2O, and pH was adjusted with 0.1 M HCl to 3.8. To remove chlorophyll from tissues, the leaves and cotyledons were rinsed several times with hot (60°C) ethanol (96%) at low light conditions. The colored spots of H_2O_2 accumulation, which appeared on the leaves and cotyledons, were scanned.

Histochemical identification of superoxide radical ($O_2^{\bullet-}$) Dye-based identification of superoxide radical ($O_2^{\bullet-}$) in the leaves and cotyledons was performed employing the method described by Doke & Ohashi (1988). Plant material was infiltrated in darkness for 15 min under pressure of 0.8 MPa with a solution containing 0.5% (w/v) nitroblue tetrazolium (NBT), 10 mM potassium phosphate buffer pH 7.0 and 0.005% (w/v) Triton X-100. After that the plant material was exposed to light for 15 min. The leaves and cotyledons were rinsed several times with hot (60°C) ethanol (96%) to remove chlorophyll from plant tissue. The colored spots (dark blue) of superoxide radical accumulation in the leaves and cotyledons were scanned.

Activity of L-phenylalanine ammonia-lyase (PAL) (E.C. 4.3.1.5.) Measurement of PAL activity was carried out according to the method described by Peltonen & Karjalainen (1995). Extraction procedure was performed at +4°C. The samples (100–200 mg of fresh weight) were homogenized in the extraction buffer (50 mM Tris-HCl pH 8.5, 14.4 mM 2-mercaptoethanol, 5% w/v polyvinylpyrrolidone). The plant extract (500 μl) was added to the reaction mixture of 2.5 ml of 0.2 % *L*-phenylalanine in 50 mM Tris-HCl (pH 8.5). The reaction mixture was then incubated for 24 h at 38°C. After that the absorbance at 290 nm was measured. The enzyme activity was expressed as μmol of cinnamic acid produced within 1 min per 1 mg of protein. The measurements were taken in five replicates.

Total phenolics analysis The plant material for the total phenolics and *trans*-cinnamic acid and ferulic acid measurements was homogenized in 80% ethanol. The homogenate was centrifuged at 13,000 rpm for 15 min at +4°C. Total concentration of phenolics was determined using the Folin-Ciocalteu method of Singleton & Rossi (1965). Supernatant (0.1 ml) was added to the measurement solution consisting of 0.9 ml of distilled water, 0.5 ml of 25% Na_2CO_3 and 0.125 ml of Folin-Ciocalteu reagent which was previously diluted two times with distilled water. The absorbance was measured at 760 nm. Chlorogenic acid was used as a standard. The measurements for each genotype were taken in five replicates.

Trans-cinnamic and ferulic acid content Before analysis the chlorophyll present in the supernatant was removed by several extractions with hexane, until no green color was visible. The *trans*-cinnamic and ferulic acid were separated by silica gel thin-layer chromatography (TLC, silica gel 60 F_{254} 0.25 mm thick, Merck & Co., Inc., Rockville, MD, USA). The mixture of benzene, acetic acid and MeOH (45:8:4 v/v/v) was used as a developing solvent. Phenolics were located on the TLC plates as spots under UV radiation. They were scraped off the silica gel and eluted with 2 ml of 80% ethanol.

The concentration of phenolics was determined with a spectrofluorometer (Perkin-Elmer LS 50B). To determine ferulic acid content the samples were excited at 300 nm and the fluorescence emission was detected at 434 nm. The slit widths for both excitation and emission monochromators were adjusted to 10 nm.

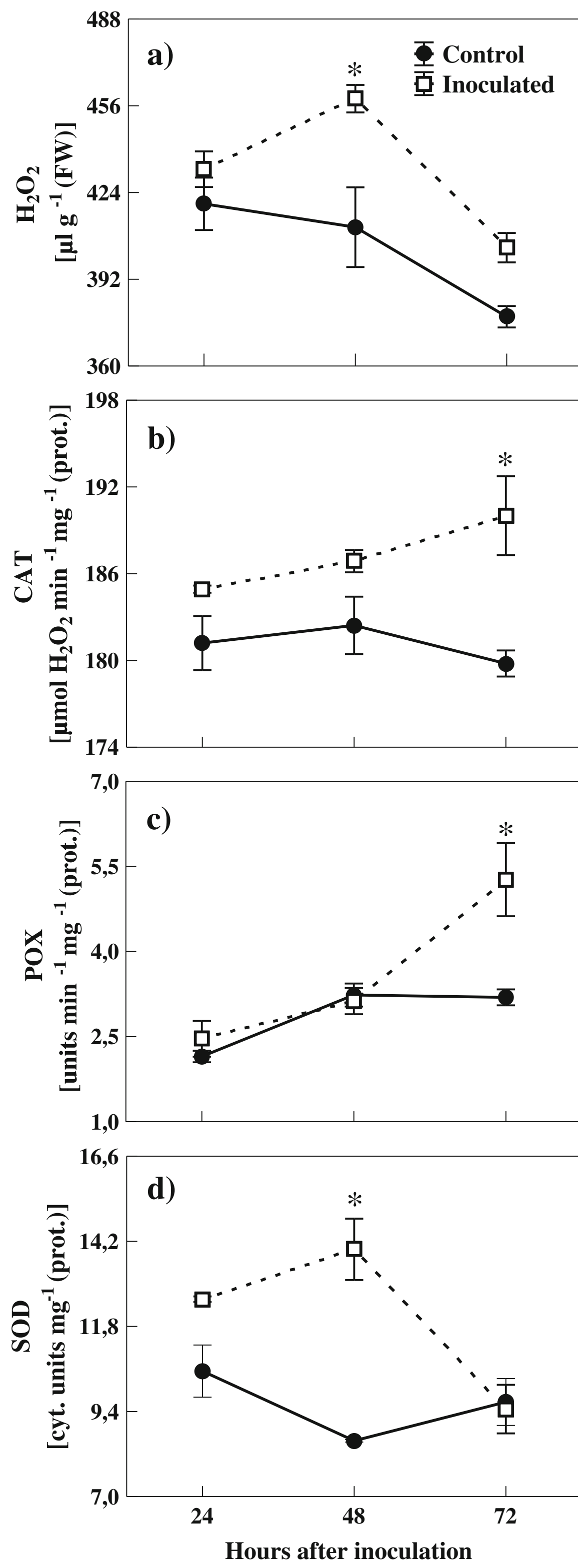
In order to determine *trans*-cinnamic acid concentration the samples were excited at 290 nm and the fluorescence emission was detected at 320–350 nm. The slit widths for both excitation and emission monochromators were adjusted to 15 nm. The measurements of phenolics and both acids were taken in five replicates.

Statistical analysis The results were statistically evaluated using the Statistica software for Windows, version 10. The data were analyzed using Student's t-test at P=0.05. Statistically significant differences are marked on the Figures with asterisks.

Results

Twenty-four hours after inoculation, hydrogen peroxide content was very close to the control. Maximum concentration of H_2O_2 was detected 48 h after inoculation and was significantly higher than in the control seedlings. A decrease in H_2O_2 level was found 72 h after inoculation and was slightly higher than that observed in the control plants (Fig. 1a). Hydrogen peroxide level in the control seedlings was relatively constant, and the lowest value was perceived 72 h after water inoculation.

Figure 1b shows the changes in CAT activity following the inoculation in relation to the control seedlings. In general, pathogen-inoculated seedlings exhibited higher CAT activity than the control ones. However, statistically significant differences were



◀ **Fig. 1** Change in hydrogen peroxide level (a) and the activity of antioxidant enzymes CAT (b), POX (c), and SOD (d) observed 24, 48 and 72 h after inoculation. Each point represents the mean ± standard error. *P < 0.05 *vs* control, Student's t-test

observed only 72 h after inoculation. Similarly, significant increase in the activity of non-specific peroxidise (POX) was observed after this time (Fig. 1c). Twenty-four and 48 h after inoculation POX activity in pathogen-inoculated seedlings was the same as in the controls, whereas the activity of superoxide dismutase was significantly higher 48 h after inoculation (Fig. 1d). SOD activity 24 and 72 h after inoculation was the same as or very similar to the control seedlings.

SOD isoforms were identified using different inhibitors (Fig. 2). Native polyacrylamide gel electrophoresis and staining revealed the presence of the manganese isoform Mn-SOD (not inhibited by either H_2O_2 or KCN), two isoforms of Zn/Cu-SODI and Zn/Cu-SODII (activity of both isoforms inhibited by 5 mM H_2O_2 or 3 mM KCN). The iron isoform Fe-SOD (activity inhibited by 5 mM H_2O_2) was detected. In the case of zinc-copper isoform (Zn/Cu-SODI), two or three additional sub-isoforms were detected (C48, I72). Seventy-two hours after inoculation two sub-isoforms of Zn/Cu-SODI were identified in the study plants (the third band was barely visible), and only one band was visible in the control plants. Forty-eight hours after inoculation one Zn/Cu-SODI isoform was visible in the inoculated plants and three in the control seedlings. In the course of pathogenesis SOD isoforms did not exhibit significant changes in activity (similar band intensity) in the control and inoculated seedlings (Fig. 2). However, Zn/Cu-SODII bands evaluated 24 h after inoculation exhibited slightly higher intensity than those observed for control plants. Similarly, 48 h after inoculation Zn/Cu-SODI bands exhibited higher intensity than those observed for control plants (three isoforms). Inversely, after 72 h the intensity of Zn/Cu-SODI bands was lower in the inoculated samples.

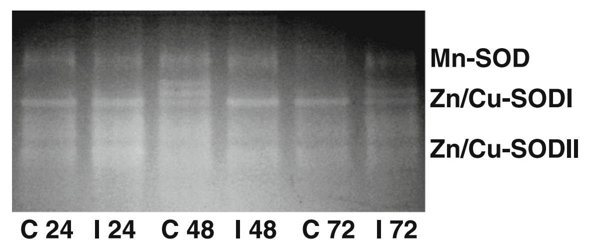


Fig. 2 Superoxide dismutase (SOD) isoforms of control (C) and inoculated (I) plants observed 24, 48 and 72 h after inoculation

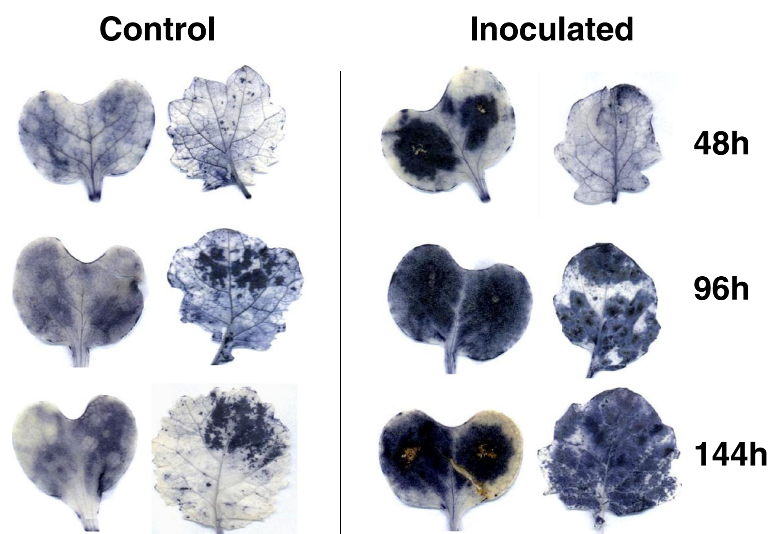


Fig. 3 Histochemical identification of superoxide radicals ($O_2^{\bullet-}$) in the cotyledons and leaves carried out 48, 96 and 144 h after inoculation

Histochemical assay of the leaves and cotyledons revealed higher content of $O_2^{\bullet-}$ in the seedlings inoculated with the pathogen (Fig. 3). In cotyledons, 48 h following inoculation, local colored spots (dark blue) encircled the areas of superoxide radical accumulation. After 96 h and 144 h the whole inoculated cotyledon exhibited a high level of $O_2^{\bullet-}$. Similarly, 96 h and 144 h after inoculation, elevated accumulation of $O_2^{\bullet-}$, manifested as dark blue spots, was discernible in indirectly inoculated true leaves. It should be stressed here that histochemical estimation of $O_2^{\bullet-}$ is very sensitive to mechanical injuries. In our experiment, the leaves were punctured with a needle used for inoculation; hence the accumulation of superoxide radical was observed even in control plants which were water inoculated (Fig. 3).

The analysis revealed plant tissue accumulation of H_2O_2 after inoculation (Fig. 4). However, intensity of H_2O_2 generation was higher in the cotyledons than in indirectly inoculated true leaves. Forty-eight hours after inoculation, H_2O_2 content was detected as local dark brown spots on the cotyledons and after 120 h its concentration rose in the cotyledons and partly in the first true leaf. Then, 168 h later, a higher content of H_2O_2 was detected in both whole cotyledons and in the first true leaf in comparison to the control plants. The control plants exhibited low H_2O_2 accumulation 48 and 120 h after water inoculation. Hydrogen peroxide content rose in the cotyledons of the control plants 168 h after water inoculation, probably as a consequence of oxidative stress induced by leaf aging and mechanical injury during the analysis.

Figure 5a shows significant growth in PAL activity 48 h (maximum increase) and 72 h after inoculation in comparison with the control plants. Twenty-four hours after inoculation *L*-phenylalanine ammonia-lyase activity was the same as for control seedlings, in which it was relatively constant. The highest content of *trans*-cinnamic acid was detected 24 h following the pathogen inoculation (Fig. 5b). Cinnamic acid level 48 and 72 h after inoculation was similar to the control. In contrast, a statistically significant increase in ferulic acid concentration was observed 48 h after pathogen inoculation (Fig. 5c). Figure 5d presents changes in the total content of soluble phenolics. A significant boost in the phenolics content was noticed 24 h after inoculation. The subsequent hours of pathogenesis showed a reduction in the total content of phenolic compounds to the level observed in the control seedlings (48 h, 72 h).

Discussion

Plants activate certain defense mechanisms in response to a pathogen attack, including oxygen burst connected with the generation of reactive oxygen species, mainly superoxide radicals ($O_2^{\bullet-}$) and/or hydrogen peroxide (H_2O_2). However, ROS are able to destroy both pathogen cells and host cells. We have proved the virulence of Ph L5 isolate in relation to cv. Lisek. Increased $O_2^{\bullet-}$ accumulation around the inoculated areas was found in the cotyledons of this oilseed winter rape variety 48 h after inoculation with *P. lingam* spores (Fig. 3). Moreover, 96 h later, a high level of $O_2^{\bullet-}$ spread over

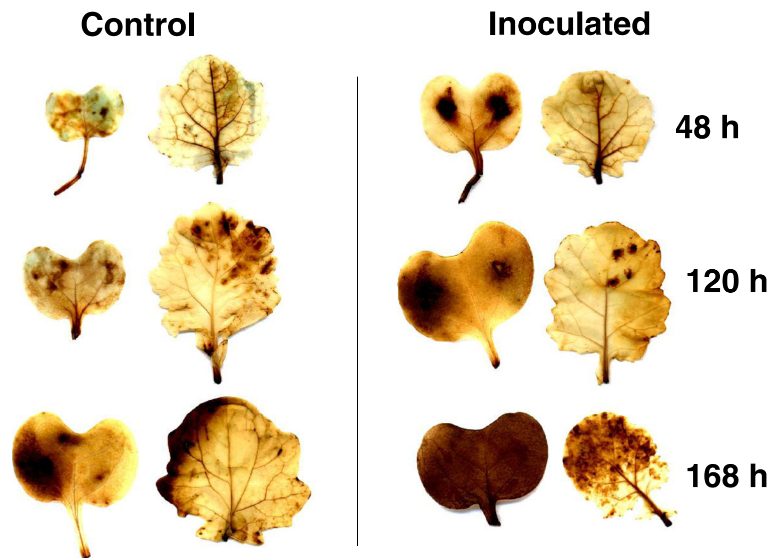


Fig. 4 Dye-based detection of H_2O_2 accumulation in the cotyledons and leaves carried out 48, 120 and 168 h after inoculation

all the cotyledons and leaves. Gawlik & Wojcik (1997) reported $O_2^{\bullet-}$ overproduction in bean leaves 3 h after inoculation with the spores of *B. cinerea* and *B. fabae.* In the same experiment they discovered that the superoxide radical level was barely enhanced in places distant from the inoculation site. Significant participation of reactive oxygen species in plant adaptation to biotic stresses evoked by pathogens was also discussed by others (Kondo *et al.* 1993; Libik-Konieczny *et al.* 2011; Torres 2010). Our experiment confirmed intensified accumulation of H_2O_2 in rape tissues in the response to inoculation with *P. lingam* (Fig. 4). Hydrogen peroxide can be formed from superoxide radical as a result of the disproportional reaction catalyzed by superoxide dismutase. It has already been proved that high H_2O_2 content increases resistance to a pathogen (Barna *et al.* 2012). Elevated H_2O_2 level is frequently observed during pathogen infection (Bowler & Fluhr 2000; Lee *et al.* 1999; Neill *et al.* 2002). Additionally, in the course of pathogenesis, H_2O_2 molecules participate in the signal transduction and in this way can initiate phytoalexin production, which in turn inhibits the pathogen development (Kuźniak & Urbanek 2000). Heightened concentration of H_2O_2 and superoxide radical in indirectly inoculated first true leaves may suggest rather a systemic defense response.

It is well known that $O_2^{\bullet-}$ and H_2O_2 production accompany the hypersensitive response (HR) that improves resistance to the blackleg. It was shown that the HR in the cotyledons of *B. napus* can be induced by inoculation with an avirulent isolate of *L. maculans* or an elicitin (cryptogein) which can mimic the effect of an avirulent isolate of *L. maculans* (Roussel *et al.* 1999). Plant interaction with the avirulent isolate can lead to a more rapid response to infection and can provide improved protection during subsequent interactions with virulent pathogens (Kuc 1987). Jędryczka *et al.* (1991) reported HR in oilseed winter rape treated with *P. lingam* toxins. However, it should be mentioned that while HR limits biotrophs, the necrotrophs can profit from HR-related necroses. Since *L. maculans* can be considered a necrotroph during early stages of infection, the necrosis caused by ROS overproduction can accelerate cell death. Therefore, the necroses can spread as a result of HR, which is a form of programmed cell death (PCD), or following phytotoxicity of fungal toxins.

In the present study, the spectrophotometric measurements, in contrast to PAGE analysis, revealed enhanced SOD activity 48 h after inoculation, which may be considered an early defense mechanism (Fig. 1d). Jindřichová *et al.* (2011) showed elevated activity of peroxidases (guaiacol and ascorbate) and superoxide dismutase in both *L. maculans*-infected and elicitor-treated cotyledons of *B. napus*. Enhanced activities of antioxidant enzymes, such as catalase, non-specific peroxidase and superoxide dismutase, are frequently observed as response to ROS generation (Barna *et al.* 2012; Libik-Konieczny *et al.* 2011). In this experiment reduced SOD activity 72 h after inoculation was balanced by higher activity of other enzymes, *i.e.,* catalase (Fig. 1b) and non-specific peroxidase (Fig. 1c).

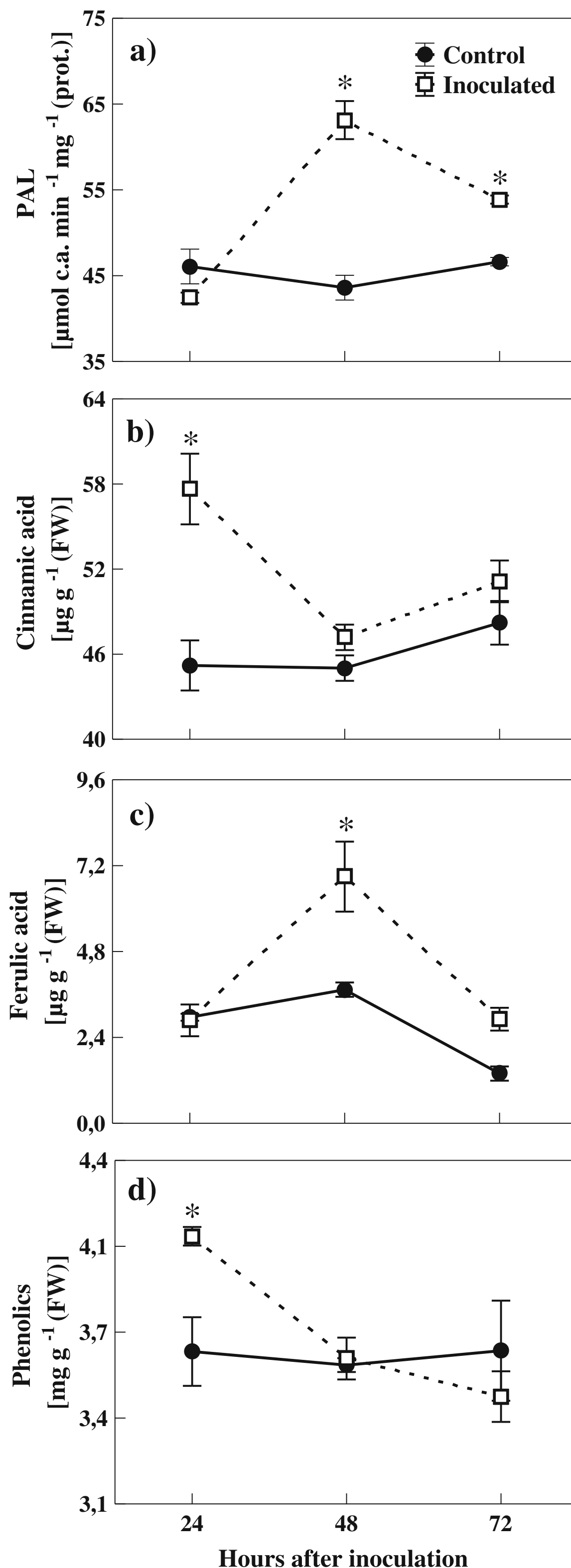


Fig. 5 Change in PAL activity (a), cinnamic acid (b), ferulic acid (c), and total phenolics content (d) observed 24, 48 and 72 h after inoculation. Each point represents the mean ± standard error. *P < 0.05 *vs* control, Student's t-test

Increased activity of phenylalanine ammonia-lyase, a crucial enzyme in the synthesis of phenolic compounds (Sanchez-Ballesta *et al.* 2000), was observed in our study 48 and 72 h after inoculation. Similar responses to a pathogen attack were found in other experiments (Ishihara *et al.* 1999; Shadle *et al.* 2003). It can be suggested that low PAL activity 24 h after inoculation is due to phenolic compounds accumulation (high phenolics content including *trans*-cinnamic acid), exceeding their utilization possibilities. In the course of pathogenesis, low phenolics content may reflect their utilization in the defense mechanisms, *e.g.* cell wall lignification. Elevated content of ferulic acid, observed 48 h after inoculation, could be the result of its possible function in cell growth regulation, through ester-linked to cell wall polysaccharides (Fry 1979, 1982). This fact suggests that triggering the production of ferulic acid may contribute to making the cell walls be less extensible and to form a barrier to pathogen penetration. PAL is considered a key enzyme in synthesis of other plant phenolics, *e.g.* salicylic acid, an important signaling molecule in plant defense response to pathogens (Rivas-San Vicente & Plasencia 2011). However, it was shown that resistance in the *A. thaliana – L. maculans* pathosystem is independent of salicylic acid signaling (Bohman *et al.* 2004). El Hadrami *et al.* (2010) proved the importance of the phenolic compounds in the defense mechanism in canola cultivars challenged with *L. maculans* and *L. biglobosa*.

In summary: Our results have proved the virulence of the Ph L5 isolate towards cv. Lisek. A boost in total phenolic content including trans-cinnamic acid was observed 24 h after inoculation. High activity of PAL could be another marker of the defense mechanisms, despite the fact that it was not accompanied by the growth in phenolics content, which was probably rapidly exhausted during *P. lingam* inoculation. Reduced phenolics content was balanced by higher activity of SOD, CAT and non-specific peroxidases. Elevated activity of these enzymes was a response to ROS overproduction. ROS accumulation in indirectly inoculated first true leaves may constitute evidence of a systemic defense response. The results presented here are the first step towards finding reliable biochemical indicators of blackleg, caused by the *P. lingam* infection. In order to achieve this goal, similar studies should be

performed for other oilseed rape genotypes that differ in resistance to *P. lingam* phytotoxins.

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