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The possible involvement of peroxidase in defense of yellow lupine embryo axes against *Fusarium oxysporum*

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Summary

Peroxidase activity (EC 1.11.1.7) towards phenolic substrates, i.e. pyrogallol, syringaldazine and guaiacol, and ascorbate peroxidase activity (EC 1.11.1.11) were analyzed in embryo axes of *Lupinus luteus* L. cv. *Polo* cultured on Heller medium for 96 h after inoculation with the necrotrophic fungus *Fusarium oxysporum* f.sp. Schlecht *lupini*. Four variants were compared: inoculated embryo axes cultured with 60 mM sucrose (+Si) or without it (—Si), and non-inoculated embryo axes cultured with 60 mM sucrose (+Sn) or without it (—Sn). Between 0 and 96 h of culture, peroxidase activity towards the phenolic substrates increased in all variants except —Si, where a decrease was noted in peroxidase activity towards syringaldazine and guaiacol, but not towards pyrogallol. In +Si tissues, a considerable increase in enzyme activity towards these substrates was recorded starting from 72 h of culture. Lignin content of +Si tissues increased already at the first stage of infection, i.e. 24 h after inoculation. Additionally, in +Sn tissues, high ascorbate peroxidase activity was observed during the culture. Its activity increased in +Si tissues, beginning at 72 h after inoculation. However, this was lower than in +Sn tissues. At 72 h after inoculation, a considerably stronger development of the infection was observed in —Si than in +Si tissues during our earlier research [Morkunas, I. et al., 2005. Sucrose-stimulated accumulation of isoflavonoids as a defense response of lupine to *Fusarium oxysporum*. Plant Physiol Biochem 2005; 43: 363—73]. Both peroxidases assayed towards phenolic substrates and ascorbate peroxidase was less active in —Si tissues than in —Sn tissues. Hydrogen peroxide concentration was much higher in —Si than in

Abbreviations: cAPX, cytosolic ascorbate peroxidase; GPX, guaiacol peroxidase; H_2O_2, hydrogen peroxide; PPX, pyrogallol peroxidase; +Si, inoculated cultured with sucrose; —Si, inoculated cultured without sucrose; +Sn, non-inoculated cultured with sucrose; —Sn, non-inoculated cultured without sucrose; SPX, syringaldazine peroxidase

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+Si tissues. These results indicate that peroxidases may be some of the elements of the defense system that are stimulated by sucrose in yellow lupine embryo axes in response to infection caused by *F. oxysporum*.

Introduction

Changes in peroxidase activity occurring under stress conditions in response to the action of both abiotic and biotic conditions have been widely investigated. However, little is known about the involvement of soluble carbohydrates, and especially sucrose, in the defense response connected with peroxidase activity during the pathogen–plant interaction. Numerous studies indicate that sugars in plants function as a metabolic resource, but they are also important regulators of many processes associated with growth, maturation and senescence (Jang and Sheen, 1997; Koch, 1996). A few studies have revealed that they may also be involved in the regulation of defense responses initiated by a pathogen attack (Engström and Strömberg, 1996; Herbers et al., 1996; Morkunas et al., 2005). Their regulatory activities include both the repression and activation of many genes, and several distinct sensing and transduction mechanisms are likely to be involved (Chiou and Bush, 1988; Jang and Sheen, 1997). There are some studies indicating that sugar signaling in plants also occurs by a hexose-dependent, but hexokinase-independent pathway (Ehness et al., 1997), as well as by a sucrose-dependent pathway (Chiou and Bush, 1988). Studies by Herbers et al. (1996) showed that hexoses could induce the expression of many genes, also including plant resistance genes that determine the production of peroxidase and pathogenesis-related PR proteins. Moreover, Ehness et al. (1997) revealed the existence of correlations between independent transduction pathways of a signal triggered by glucose as well as a signal generated as a result of the action of a fungal elicitor. The existence of multiple pathways indicates that sugar-signal transduction processes are relatively complex in plants (Halford et al., 1999).

In this study, we were interested in determining whether disturbed carbohydrate metabolism, found in sink tissues during the seed germination stage, may modulate the response of these tissues connected with the activity of intracellular, cytoplasmic peroxidases to the infection caused by the pathogenic fungus. In the course of the pathogen–plant interaction, peroxidases are key enzymes in the detoxification systems, involved in scavenging reactive oxygen forms, whose increased generation is closely associated with the induction of plant defense reactions (Gay and Tuzun, 2000). They are also involved in the oxidation of isophenols to forms that are more toxic towards the pathogens (Takahama and Hirota, 2000) and in the cross-linking of cell wall proteins and lignification of cell walls (Gay and Tuzun, 2000; Lagrimini and Rothstein, 1987). This may limit the penetration of pathogen toxins into the cells and thus the spread of the infection (Lurie et al., 1997). Thus, due to their multifunctional nature, these enzymes are sensitive markers of stress conditions. Moreover, under the influence of pathogenic microorganisms or other stress factors, peroxidases are the first enzymes to alter their activity and/or the composition of their isoenzymes (Lamb and Dixon, 1997; Ye et al., 1990).

Perturbations in carbohydrate metabolism occurring during seed germination (which is a heterotrophic phase in plant life, caused by a delay in mobilization of storage materials in cotyledons) may lead to the weakening of their defense mechanisms and to the increased susceptibility of seeds to infections caused by pathogenic fungi (Morkunas et al., 2004, 2005). The experimental protocol used in this study is based on a model system: embryo axes from germinating yellow lupine seeds were cultured in vitro on a medium with or without sucrose after inoculation with a spore suspension of *Fusarium oxysporum* f.sp. *lupini*. This pathogen causes *Fusarium* wilt and pre-emergent root rot and post-emergent seedling rot. Application of the above model system aimed to compare two situations during seed germination: (1) when the embryo axis receives a pool of soluble sugars thanks to proper mobilization of storage materials; and (2) when mobilization of storage materials is delayed due to unfavorable environmental conditions, which can lead to sugar starvation of the embryo axis. We used yellow lupine because seeds of this species were used also in our earlier experiments in this field (Morkunas et al., 2002, 2004, 2005).

In the present study, we analyzed participation of sucrose in defense responses of germinating yellow lupine to inoculation with a pathogenic fungus.

More precisely, we analyzed the effect of the presence or absence of this sugar on peroxidase activity during the pathogen–plant interaction. Peroxidases were characterized by their ability to oxidize hydrogen donors, such as pyrogallol, syringaldazine, guaiacol, and additionally ascorbate. At the same time, changes in hydrogen peroxide (H_2O_2) concentration were investigated in embryo axes non-inoculated and inoculated with *Fusarium oxysporum*, cultured on a medium with sucrose as well as a medium without this sugar. The infection and the development of the necrotrophic pathogen in embryo axes of yellow lupine are controlled by higher sucrose levels, as has been previously reported (Morkunas et al., 2005). Therefore, in this study, we also attempted to determine whether this might be a consequence of a relatively fast lignification of embryo axes under the influence of sucrose.

Materials and methods

Plant material

Yellow lupine (*Lupinus luteus* L. cv. *Polo*) seeds of the S-elite class were used in the experiments. The seeds were surface-sterilized, immersed in sterile water, and left in a phytotron at 25 °C. After 6 h of imbibition, the seeds were transferred onto filter paper (in Petri dishes) and immersed in a small amount of water in order to support further imbibition. After subsequent 18 h, seed coats were removed from the imbibed seeds and cotyledons were cut off to isolate embryo axes. Half of embryo axes were immediately inoculated (the procedure is described below) and within 20 min (i.e. at 0 h) the inoculated and non-inoculated embryo axes were inserted in groups of 4 in Whatman filter paper and transferred to sterile glass test tubes (diameter 3 cm, height 13.5 cm) containing 14 mL of mineral Heller medium (Heller, 1954). The embryo axes were suspended so that the radicle was partly immersed in the medium, but some empty space was left between the paper and the medium in order to allow better aeration. After removal of cotyledons, embryo axes were dependent on the carbon source provided by the medium. Four variants were compared: inoculated embryo axes cultured with 60 mM sucrose (+Si) or without it (−Si), and non-inoculated embryo axes cultured with 60 mM sucrose (+Sn) or without it (−Sn). The embryo axes were incubated in darkness at 25 °C. Samples were collected for analyses at 0 h and after 24, 48, 72, and 96 h of culture, when they were frozen in liquid nitrogen for the analysis of peroxidase activity. The activity of both phenolic peroxidase and ascorbate peroxidase was measured at the same time for all culture variants.

Preparation of spore suspension and inoculation

Fusarium oxysporum f.sp. *lupini* strain K-1018 (further referred to as *F. oxysporum*) was obtained from the Collection of Plant Pathogenic Fungi, Institute of Plant Protection, Poznań. The pathogen was incubated at 25 °C in Petri dishes (diameter 9 cm) on a potato dextrose agar (PDA) medium (Difco, pH 5.5) in the dark. After 3 weeks of growth, *F. oxysporum* spore suspension was prepared. The spore suspension was obtained by washing the mycelium with sterile water and shaking with glass pearls. The number of spores was then determined using a Bürker hemocytometer chamber. The embryo axes were inoculated with the spore suspension at a concentration of 5×10^6 of spores per 1 mL. Inoculation was performed by injecting 10 μl of spore suspension into the upper part of the embryo axis shoot, and additionally by also spraying the upper part of the embryo axis shoot with the inoculum.

Extraction and assay of peroxidase activity towards phenolic substrates

Frozen embryos (300 mg) were homogenized at 4 °C with a mortar and pestle in 1.2 mL of 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA and 10% (w/v) polyvinylopyrrolidone (PVP). The slurry was centrifuged at 15 000*g* for 30 min at 4 °C, and the supernatants were used for enzyme assays. The protein concentration in the samples was estimated according to Bradford (1976) by using bovine serum albumin as a standard.

Peroxidase (EC 1.11.1.7) activity was measured spectrophotometrically using the following phenolic substrates: pyrogallol, syringaldazine and guaiacol.

Peroxidase activity towards pyrogallol was measured according to Nakano and Asada (1981). This method includes the measurement of the content of purpurogallin – a product of pyrogallol oxidation. The assay mixture contained 50 mM sodium phosphate buffer (pH 7.0), 40 μL enzyme extract, 180 mM pyrogallol and 2 mM H_2O_2. Absorbance was recorded at 430 nm.

Activity of peroxidase towards syringaldazine was assayed according to the modified method of

Imberty et al. (1985) consisting in the measurement of the content of the colored product of syringaldazine oxidation. The assay mixture contained 50 mM potassium phosphate buffer (pH 7.0), 60 μL enzyme extract, 41.6 μM syringaldazine and 4 mM H_2O_2. Absorbance was recorded at 530 nm.

Peroxidase activity towards guaiacol was assayed according to the modified method of Maehly and Chance (1954). This method consists of the assay of tetraguaiacol – a colored product of guaiacol oxidation in the investigated sample. The assay mixture contained 50 mM potassium phosphate (pH 7.0), 100 μL enzyme extract, 20 μM guaiacol and 6 mM H_2O_2. Absorbance was recorded at 470 nm. The activity of the enzyme was expressed as U per 1 mg of protein.

Extraction and assay of cytosolic ascorbate peroxidase (cAPX) activity

The enzyme (EC 1.11.1.11) was extracted at 4 °C by using 50 mM potassium phosphate buffer at pH 7.0 (1.2 mL per 300 mg of frozen tissue), containing 1 mM EDTA, 1 mM sodium ascorbate, and 1% PVP. Samples were ground in a mortar and centrifuged at 15 000*g* for 30 min. The activity of cAPX was determined with a modified method of Nakano and Asada (1981). The assay mixture contained 50 mM potassium phosphate (pH 7.0), 60 μL enzyme extract, 10 mM ascorbic acid and 2 mM H_2O_2. Absorbance was recorded at 265 nm. The activity of the enzyme was expressed as U per 1 mg of protein.

Assay of hydrogen peroxide

The embryo axes were ground in 100 mM potassium phosphate buffer at pH 7.0 with Polyclar AT, in a chilled mortar on ice. The homogenates were centrifuged at 15 000*g* for 20 min. The concentration of H_2O_2 was determined according to Bergmeyer and Bernt (1974). The reaction mixture contained: 400 μL of plant extract, 2 mL of KH_2PO_4/K_2HPO_4 pH 7.0 buffer (100 mM), 50 μL of horseradish peroxidase (147 U mL^{-1}), and 50 μL of ABTS solution (50 mM). The concentration of H_2O_2 was estimated by measuring absorbance at 415 nm against a calibration curve and was expressed as μmol H_2O_2 g^{-1} fresh weight (FW).

Determination of lignin content

Lignins were assayed according to the procedure of Doster and Bostock (1988). Lupine embryo axes were treated for 48 h with twice-changed methanol, using 5 mL of methanol per 1 g of tissue. Next the embryo axes were dried in a desiccator and ground in a mortar. Then, 20 mg of dry matter placed in Teflon vials were mixed with 5 mL of 2 N HCl and 0.5 mL of thioglycolic acid. The vials were tightly closed and heated at 95 °C for 4 h. The samples were centrifuged at 1000*g* for 20 min. The precipitate was washed with deionized water and extracted with 5 mL of 0.5 N NaOH for 18 h at room temperature. The NaOH extract was separated by centrifugation. The precipitate was washed with 4 mL of deionized water and centrifuged, and the obtained supernatant was added to the NaOH extract. The extract was then acidified with 1 mL of concentrated HCl, placed at the temperature of 5 °C for 4 h and centrifuged. The obtained precipitate was dissolved in 10 mL of 0.5 N NaOH. Absorbance was measured at 280 nm by using a Perkin Elmer Lambda 11 spectrophotometer. Lignin content was expressed in absorbance units obtained from 10 mg of dry matter.

Statistical analysis

All analyses were performed in at least three replicates in three independent experiments. Statistical analysis performed using the Student's *t*-test, with the level of significance $\alpha = 0.05$; the standard deviation was calculated and its range is shown in the figures.

Results

Changes in peroxidase activity assayed towards phenolic substrates

The enzyme's activity generally increased between 0 and 96 h of in vitro culture, except for the −Si variant, i.e. inoculated axes cultured without sucrose (Fig. 1). Moreover, the infection resulted in a considerable increase in peroxidase activity assayed towards the above-mentioned hydrogen donors in +Si embryo axes, primarily beginning 72 h after inoculation. Thus, after 72 and 96 h, the activity of pyrogallol peroxidase (PPX) was 44% and 46% higher, that of syringaldazine peroxidase (SPX) was 59% and 66% higher, while that of guaiacol peroxidase (GPX) was 24% and 33% higher, respectively, as compared to +Sn embryo axes (Figs. 1A–C). As early as 24 h after inoculation a higher SPX activity was found in +Si embryo axes than in +Sn embryo axes (Fig. 1B). Moreover, PPX and SPX activities were higher than GPX activity 72 h after inoculation in +Si tissues.

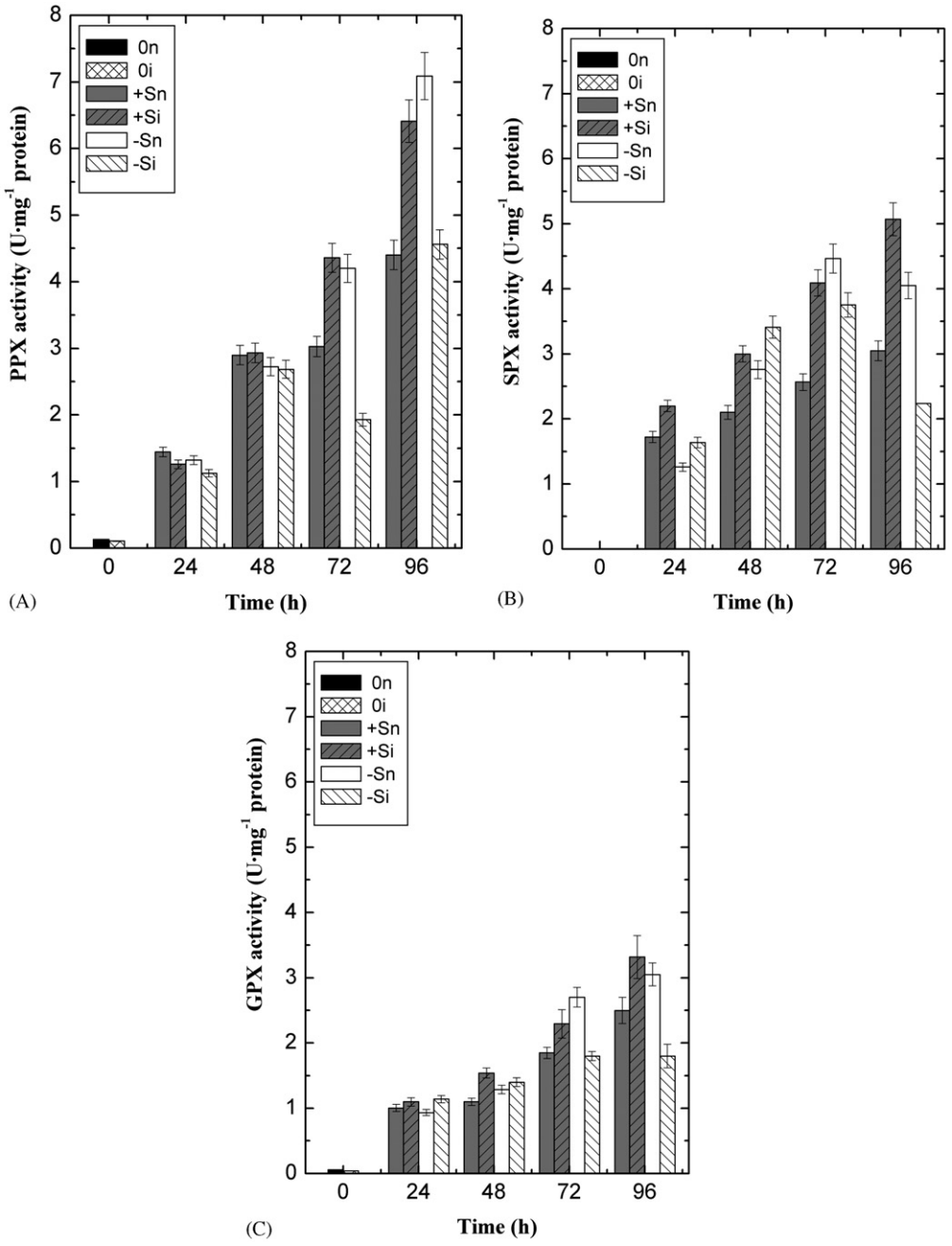

Figure 1. Changes in peroxidase activity towards pyrogallol (PPX), syringaldazine (SPX) and guaiacol (GPX) in yellow lupine embryo axes inoculated with *F. oxysporum* and cultured in vitro on Heller medium with or without sucrose 0n, non-inoculated, at 0 h (i.e. immediately before transfer onto medium); 0i, inoculated, at 0 h; +Sn, non-inoculated cultured with 60 mM sucrose; +Si, inoculated cultured with 60 mM sucrose; −Sn, non-inoculated cultured without sucrose; −Si, inoculated cultured without sucrose.

Additionally, carbohydrate starvation itself caused an increase in GPX, PPX, and SPX activities, which was conspicuous starting from 72 h of culture. In −Si embryo axes until 48 h after inoculation, higher SPX and GPX activity was observed compared to −Sn embryo axes. Moreover, it should be emphasized that, beginning at 72 h after inoculation, in −Si tissues lower GPX, PPX, and SPX activities were observed relative to −Sn tissues. Thus, in −Si embryo axes after 72 and 96 h, PPX activity was 54% and 35% lower, SPX activity was 16% and 45% lower, while GPX activity was 33% and 41% lower, respectively, than in −Sn embryo axes.

Changes in ascorbate peroxidase (cAPX) activity

Changes in cAPX activity (Fig. 2) were distinctly different from those observed for peroxidase assayed towards phenolic substrates. Between 0 and 24 h of in vitro culture, a considerable increase was observed in cAPX activity in all variants of the experiment. Moreover, during the culturing a high cAPX activity was found in +Sn tissue. An increase in cAPX was also found in infected axes. In +Si embryo axes it increased between 0 and 24 h of culture, and between 48 and 72 h after inoculation. However, the activity of this enzyme in +Si tissues throughout the culture time was lower than in +Sn tissues. Moreover, cAPX activity in +Si embryo axes from 24 to 72 h after inoculation (0.6–1.8 U mg^{-1} protein) was higher than in −Si embryo axes (0.45–0.75 U mg^{-1} protein). Relatively high fluctuations in cAPX activity during the culturing of embryo axes on a medium without sucrose were found, not only as a result of the infection with a necrotrophic fungus *F. oxysporum*, but also as a result of carbohydrate starvation alone. Moreover, it must be stressed that in −Si embryo axes 96 h after inoculation, i.e. when the development of fusariosis was strong, an increase was observed in cAPX activity as compared to 72 h after inoculation.

Hydrogen peroxide

Already at 0 h, i.e. immediately after the removal of the seed coat and cutting of the cotyledons, a high concentration of H_2O_2 was found (Fig. 3). Moreover, after tissue inoculation, higher H_2O_2 content was observed both immediately after tissue injection at 0 h, (i.e. up to 20 min after inoculation) and during culturing, i.e. from 24 to 96 h, in comparison to non-inoculated tissues. In +Sn and in +Si embryo axes starting from 24 h after culture, a decrease in H_2O_2 content was observed. In −Si tissues, H_2O_2 level remained within the range from 5.8 to 7.9 μmol g^{-1} FW during culturing and was considerably higher than in −Sn tissues. Moreover, starting from 72 h after inoculation, when the development of infection in −Si tissues was distinctly stronger than in +Si tissues, the level of H_2O_2 generation was also higher.

Changes in lignin content

A consequence of inoculation was an increase in lignin content of both +Si and −Si embryo axes (Fig. 4). Lignin content of +Si tissues in the first stage after infection, i.e. at the time point of 24 h was nearly twice as high as in +Sn tissues, which needs to be especially stressed. High lignin levels in +Si tissues were also observed in the successive

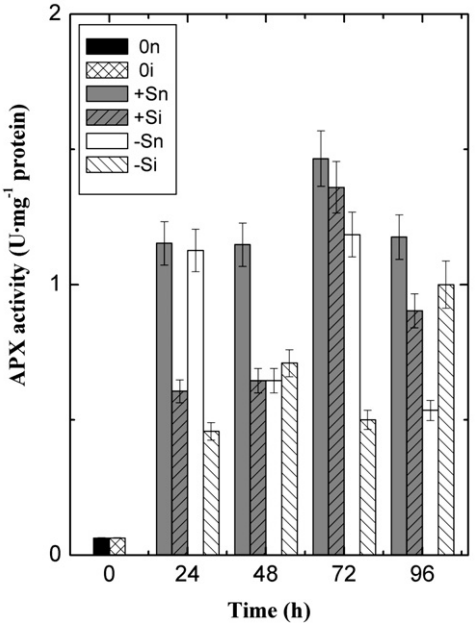

Figure 2. Changes in cytosolic ascorbate peroxidase (cAPX) activity in yellow lupine embryo axes inoculated with *F. oxysporum* and cultured in vitro on Heller medium with or without sucrose.

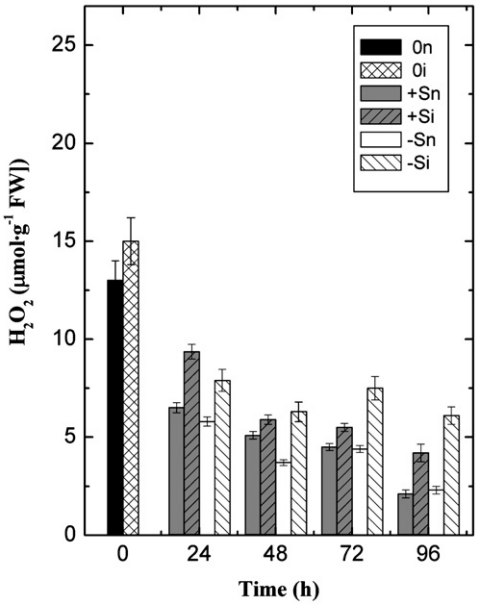

Figure 3. Concentration of H_2O_2 (μmol H_2O_2 g^{-1} FW) in yellow lupine embryo axes inoculated with *F. oxysporum* and cultured in vitro on Heller medium with or without sucrose.

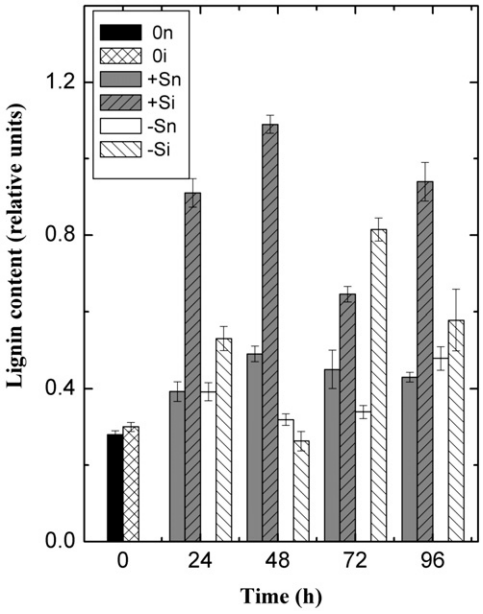

Figure 4. Changes in lignin content of yellow lupine embryo axes inoculated with *F. oxysporum* and cultured in vitro on Heller medium with or without sucrose, expressed in absorbance units obtained from 10 mg of dry matter extracted with methanol and dissolved in 0.5 N NaOH.

time points after inoculation. In −Si axes, a considerable increase in lignin content was observed as late as 72 h after inoculation.

Discussion

The regulation of carbon partitioning between source (cotyledon) and sink (embryo axis) tissues in germinating seeds is not only important for their growth and development, but also offers insight into the underlying regulatory mechanism. The availability or lack of sugars triggers many metabolic responses (Koch, 1996; Roitsch, 1999). Therefore, the sugar-sensing system controls metabolic pathways and developmental decisions in plants, also including the signaling pathway for the signal generated in the pathogen–plant interaction (Ehness et al., 1997). This fact may be essential for the resistance of the embryo axis in the germinating seed to infection caused by pathogenic fungi. In our previous studies, high levels of soluble sugars were shown to inhibit both the infection and development of the disease caused by a necrotrophic fungus, *Fusarium oxysporum*, in the tissues of yellow lupine (Morkunas et al., 2002, 2005). Peroxidases may be involved in the mechanism of this resistance, as seems to be indicated by the results of a considerable variation in their post-infection activity in embryo axes cultured on a medium with sucrose and a medium without this sugar. In contrast to −Si embryo axes, starting from 72 h after inoculation in +Si embryo axes, a considerable increase was found in peroxidase activity assayed towards all studied phenolic substrates (Fig. 1). At the same time after tissue inoculation, a higher H_2O_2 content was observed both in +Si and −Si axes than in non-inoculated tissues, i.e. +Sn and −Sn (Fig. 3). The rate of peroxidation and of polymerization in lignin biosynthesis are highly dependent on the amount of H_2O_2 present.

The role of peroxidase assayed towards phenolic substrates in the resistance of embryo axes with higher carbohydrate levels may consist of the oxidation of isophenols or their derivatives to metabolites that are more toxic to pathogens, and in the strengthening of the cell wall by early initiation of the lignification process, which would limit the penetration of pathogen toxins into the cells and the spread of the infection. This is be suggested by the high increase in lignin content in +Si tissues already in the first stage after the onset of infection, i.e. 24 h after inoculation (Fig. 4). Since that moment an increase was observed in peroxidase activity assayed towards syringaldazine (Fig. 1B), likely because peroxidase involved in lignification is characterized by a high affinity to this substrate (Imberty et al., 1985), especially its anion forms located in the cell wall (Boudet, 2000; Maranon and van Huystee, 1994).

According to Hammerschmidt (1984), an effective inhibition of the development of infections caused by necrotrophs is possible only when the induction of lignin synthesis occurs within a short time after inoculation.

In view of the above, a special role may be played by a very strong lignification of +Si tissues as early as 24 h after inoculation, much higher than in the case of −Si tissues (by about 60%). This fact may be one of the causes for the higher resistance to infections in +Si axes than in −Si axes (Morkunas et al., 2005). This might constitute one of the elements of the complex protective action of sucrose on the lupine resistance to *Fusarium* infections.

As was shown in our previous study (Morkunas et al., 2005) on the same experimental material, increased lignification is even more effective in +Si tissues because already from 48 h after inoculation especially in +Si embryo axes a considerable increase was observed in the level of free aglycones of isoflavonoids, constituting an important element of their defense system. Thus, a high concentration of these compounds in tissues is one of the factors

facilitating the defense of +Si axes against *F. oxysporum*.

Studies by Gomez-Vasquez et al. (2004), conducted on suspension cells of *Manihot esculenta*, showed that 48 h after elicitation an increase in phenolic levels was observed in cells, together with a fourfold increase in symplasmic peroxidase activity. However, their levels were too low in the cells to inhibit the development of the infection. Phenolic levels in combination, and when oxidized, may contribute to the defense, because oxidation of esculetin and scopoletin by peroxidase, and of esculetin by tyrosinase enhanced their fugitoxicity up to 20 times.

In addition, El Hassni et al. (2004) revealed that in plants pre-treated with a hypoaggressive *Fusarium* isolate (ADH), accumulation of phenolics (mainly of non-constitutive hydroxycinnamic acid derivatives) and peroxidase activity were high. Those researchers also found that those two parameters may be involved in the date palm resistance to *Fusarium oxysporum* f.sp. *albedinis* (Foa).

Moreover, the considerable increase in peroxidase activity assayed towards phenolic substrates and ascorbate peroxidase 72 h after inoculation in +Si embryo axes may also indicate a high efficiency of the antioxidant system in these tissues in contrast to −Si embryo axes. The observed decrease in the activity of these enzymes in −Si tissues may be connected with a high concentration of H_2O_2 observed at that time, so it could be regarded as evidence for overwhelming of this antioxidant enzyme. The concentration of H_2O_2 in −Si tissues between 24 and 72 h after inoculation, remained at the level of 7–8 $\mu mol\, g^{-1}$ FW and was much higher than in non-inoculated axes, where in +Si tissues a decrease was observed in H_2O_2 concentration during culture.

An increase in generation of H_2O_2 (which is a relatively stable reactive oxygen form) as a consequence of inoculation of embryo axes may constitute one of their defense strategies aiming at destruction of the pathogen. However, this does not exclude the possibility of H_2O_2 originating from the necrotrophic fungus *F. oxysporum* itself. Recent investigations have shown that necrotrophic pathogens use oxidative processes during their attack and invasion of plant tissue (Mayer et al., 2001).

The inhibitory effect of high H_2O_2 concentration on peroxidase activity was also reported by Messner and Boll (1994), who studied extracellular peroxidase activity during oxidative burst initiated as a result of elicitation of spruce cells. Thus, the decrease in H_2O_2 amounts observed in +Si tissues starting from 48 h after inoculation may be connected with the beginning of a considerable induction of the activity of these peroxidases.

Additionally, the reported high H_2O_2 level in lupine embryo axes from 0 h is an additional confirmation of the results published in our previous paper (Morkunas et al., 2004) for germinating seeds grown on perlite, where we showed that the moment when the axis penetrates the seed coat was connected with a rise in reactive oxygen species levels. This fact had been reported earlier by Schopfer et al. (2001) for germinating radish seeds.

Moreover, in this study we found that −Sn embryo axes lacking sucrose reacted to the stress caused by sugar starvation. In the peroxidase activity assayed towards phenolic substrates generally an increase in the activity was observed starting from 72 h of culture. However, −Si embryo axes were subjected to the additional stress of inoculation with a pathogenic fungus, so a distinct decrease was found in the activity of peroxidases, particularly of PPX and cAPX, in comparison to −Sn embryo axes. After 96 h this effect was manifested mainly for GPX, PPX, and SPX.

It should be emphasized that phenolic peroxidases may play an important role in the control of H_2O_2 level. In the published literature, a principal role in scavenging this reactive oxygen form is attributed to cytosolic ascorbate peroxidase (cAPX), which shows slightly different trends in its activity after the infection of lupine axes than those of phenolic peroxidase. Although in +Si axes an increase was found in cAPX activity both between 0 and 24 h and between 48 and 72 h after inoculation, its activity throughout the time of culture was lower than in +Sn axes (Fig. 2). Moreover, we showed that sucrose itself caused a relatively high cAPX activity in +Sn tissues throughout the culture time. In infected tissues the activity of this peroxidase fluctuated during culturing.

The results of investigations conducted by de Gara et al. (1996) on wheat and pea embryos showed the existence of a close correlation between cAPX activity and ascorbate contents during seed germination. Along with an increase in ascorbate, an increase in cAPX activity was also observed. Thus, it cannot be excluded that its activity might be connected with the level of ascorbate, which is a substrate for the reaction catalyzed by it.

Studies by Muckenschnabel et al. (2002) conducted on leaves of *Arabidopsis thaliana* infected with a necrotrophic pathogen, *Botrytis cinerea* showed that tissue infection caused as much as a 30-fold decrease in ascorbate level.

Our experiments showed that, as a result of infection, cAPX activity was generally lower than in non-inoculated tissues, except for −Si axes after 96 h of culture.

A post-infection decrease in cAPX activity in comparison to a control was also previously reported by Gayoso et al. (2004) in roots of *Capsicum annuum* infected by *Phytophthora capsici*.

As was reported by Mayer et al. (2001), while analyzing the fluctuations in peroxidase activity in host tissues, it is necessary to consider that necrotrophs have their own system of antioxidative enzymes. As a result, they are capable of inactivating reactive oxygen forms. Moreover, a large number of peroxidases have been reported from fungi (Nakayama and Amachi, 1999) and elevated levels of peroxidase activity in host tissue have been repeatedly reported (Eshdat et al., 1997).

The results obtained in this study indicate that peroxidases, next to isoflavonoids with antimicrobial action reported in our previous paper (Morkunas et al., 2005), may be involved in defense mechanisms of yellow lupine embryo axes with a high endogenous sucrose level against *F. oxysporum*, aiming at the limitation of the infection and the development of the pathogen. Peroxidases may be involved in the early lignification of these tissues, but their participation in the oxidation of phenolic compounds cannot be excluded, which may enhance their fungitoxicity.

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