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CHANGES IN PEROXIDASE ACTIVITY IN CELL STRUCTURES OF LUPIN ROOTS INFECTED BY *FUSARIUM CULMORUM***

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Infection of lupin seedlings with *Fusarium culmorum* results in the increase in peroxidase activity with syringaldazine as a substrate, mostly in microsomes and cell walls, in the latter fraction somewhat later than in microsomes and cytosol. Less evident were the changes upon infection in peroxidase activity with guaiacol as a substrate.

Both qualitative and quantitative differences in the peroxidase forms were evidenced on electrofocusing of the enzyme in the examined subcellular fractions.

Numerous investigations carried out for many years have not fully elucidated the role of peroxidase in the defence reaction of plants to pathogens. The results on the response of this activity to infection are controversial [1, 2, 3]. To get an unequivocal answer it seems necessary to follow, in the process of infection, the peroxidase activity towards a specified substrate such as syringaldazine. It has been reported that the peroxidase of high affinity to syringaldazine participates in lignin biosynthesis, which is probably related to resistance mechanisms [4]. Besides, information on subcellular localization of the particulate enzyme form induced by infection may add to our understanding of the problem. Chibbar Huystee [5] are of the opinion that most of the newly synthesized peroxidase forms come from microsomes.

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The aim of the present work was to determine the peroxidase activity with guaiacol and syringaldazine as substrates in the microsomal, cytosol and cell wall fractions of lupin roots after inoculation with *Fusarium culmorum*.

**MATERIALS AND METHODS**

*Materials.* Lupin seeds (*Lupinus luteus* L. cv. Pałucki) were sterilized with 0.1% HgCl₂ for 1 min and with 50% ethanol for 2 min; next they were rinsed several times with distilled water and inoculated with a suspension of *Fusarium* spores (2 × 10⁶/ml).

The fungus was cultured on agar slants containing potato extract supplemented with glucose (20 g/l) at 26°C in the dark for 7 days. Then the culture was kept at 22°C for 5 days in the light in order to elicit sporulation. Spores were rinsed with distilled water and diluted to appropriate concentration.

Seedlings were inoculated 2 days after germination in sterile conditions. The control plants remained uninfected. Seedlings were grown till the 7th day in perlite at 100% humidity, 16 h photoperiod, and light intensity of 10 µE m⁻²s⁻¹, at 25°C. Roots after appropriate periods of time were homogenized in the mortar at 4°C in two alternative buffers: 1 M NaCl in 0.05 M acetate buffer, pH 5.6, or 250 mM sucrose, 3 mM EDTA, 70 mM Tris/HCl, pH 7.5. The extract obtained with 1 M NaCl was centrifuged for 20 min at 12,000 g and the supernatant was used to determine enzymatic activity. The sucrose extract was separated into three fractions by successive centrifugation for: (I) 10 min at 1000 g (cell wall fraction), (II) 20 min at 10,000 g and (III) 30 min at 100,000 g; after final centrifugation the obtained sediment and supernatant were used as microsome and cytosol fractions, respectively.

**Assay of peroxidase activity.** This was assayed colorimetrically with two substrates, guaiacol and syringaldazine. To assay the activity with guaiacol, 0.5 ml of the diluted enzyme, 0.5 ml of 0.05 M acetate buffer, pH 5.6, 0.02 M solution of guaiacol and 0.06 M H₂O₂ were used. The linear increase of absorbance at 480 nm was monitored for 1-4 min at 30°C. The activity with syringaldazine as a substrate was determined in the reaction mixture composed of 3.5 ml of Tris/HCl buffer, pH 7.5, 0.5 ml of 0.5 M H₂O₂, 20 µl of syringaldazine solution (3.1 mg/ml methanol per 2 ml dioxane) and 0.1 ml of the enzyme. The linear increase of absorbance was followed for 5 min at 30°C. No H₂O₂ was added to either of the controls. Such amounts of enzyme were added to the reaction mixture with guaiacol and syringaldazine to obtain the increase in absorbance 0.100-0.800 and 0.200-0.500, respectively.

**Isoelectric focusing in polyacrylamide gel.** The enzyme extracts purified by precipitation at 85% ammonium sulphate saturation and dialysis against 0.001 M acetate buffer, pH 5.6. were separated by isoelectric focusing in 4%
polyacrylamide gel with 0.8 M urea and 2.4% of Servalyt 3-10, as previously described [6]. The enzyme from the cell wall and the microsomal fractions was obtained by extraction with 1 M NaCl in 0.05 M acetate buffer, pH 5.6, for 20 min at 4°C, followed by salting out at 85% ammonium sulphate saturation. Isoelectric focusing was carried out in glass tubes (7 cm × 3 mm). The sample contained 40 μg of the enzymatic protein, as determined according to Lowry et al. [7]. As electrode solutions were used 0.01 M H₃PO₄ (anode) and 0.02 M NaOH (cathode). After 1 h prefocusing at 200 V, every 1 h the voltage was raised by 50 V to reach 400 V. Isoelectrofocusing was performed at 4°C during 24 h. The gels were immersed for 10 min in the mixture of 0.05 M benzidine, 0.05 M acetate buffer, pH 5.6, and 0.06 M H₂O₂ (1:1:1, by vol.). Densitometer readings were taken at 560 nm.

RESULTS

Table 1 and figure 1 present the peroxidase activity in the cell fractions of lupin roots, both control and infected with *F. culmorum*. In microsomal fraction as early as 5 h after the infection the enzyme activity with guaiacol as a substrate was increased by about 30% comparing with control. The similar difference between the infected and non-infected roots persisted for 48 h. The corresponding difference in the peroxidase activity determined with syringaldazine was even higher, i.e. by about 50% after 5 h and about 72% after 48 h.

Although in cytosol no appreciable difference in the activity with guaiacol was noted 5 h after the infection, the differences between the infected and

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<thead>
<tr>
<th>Cell fraction</th>
<th>Time after infection h</th>
<th>Peroxidase activity, units/g.f.fr.w.</th>
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<tr>
<td></td>
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<td>with guaiacol</td>
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<td>noninfected</td>
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<tr>
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<tr>
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Table 1
Peroxidase activity in microsome, cytosol and cell wall fractions in lupin roots noninfected and infected with *Fusarium culmorum*
non-infected roots after 24 h and 48 h were similar to that in microsomes (Fig. 1B). The corresponding differences in the peroxidase activity with syringaldazine were insignificant after 5 h and 24 h; only slight differences occurred after 48 h.

In cell walls the peroxidase activity after the infection, measured with guaiacol, did not differ significantly from that of control (Fig. 1C). However, the activity towards syringaldazine increased markedly, although the response appeared later than in microsomes (Fig. 1C): 5 h after the infection the enzyme activity in the infected roots was similar to that of the control but after 24 h it was higher by about 20%, and after 48 h by about 40%.

In the isoelectric focusing profile of the peroxidase extracted from the infected roots with 1 M NaCl solution ten active bands were found, whereas only eight in controls. The two new bands (1' and 2') which appeared were of low isoelectric point. Moreover, quantitative differences were evident, namely the activity in the infected roots was significantly increased while the activities of bands 5 and 6 were lower. Likewise, on separation of the peroxidases from the microsomal fraction of infected roots by isoelectric focusing (Fig. 2B) a new band (1') appeared, the activity of band 2 was increased, and in bands 4 and 5 it was decreased. The increase of the band 2 activity after the infection was accompanied by a rise in band 3 activity.

**Fig. 1.** Peroxidase activity in (A), microsomes; (B), cytosol; and (C), cell walls; measured with guaiacol ○; and syringaldazine Δ; as a substrate. The activity is expressed as percentage of the control values.
Fig. 2. Densitometric scan of peroxidase extracted with 1 M NaCl from whole lupin roots (A), and from: microsomes (B), cytosol (C) and cell walls (D). Isoelectric focusing was performed as described in Methods. Upper parts of the diagrams refer to control, and lower parts to the roots infected with *Fusarium* culture.
The appearance of band 1' and an increase in the activity of band 3 were also observed on electrofocusing of the peroxidase extracted from the infected cell walls (Fig. 2D). Besides, within the low isoelectric point range the activity of band 1, and at higher pH values the activity of band 7 were enhanced. In cytosol no new bands appeared after the infection but some changes in the band intensity at lower values of the isoelectric point were observed. A marked increase in the intensity of band 1 was visible after inoculation (Fig 2D). In contrast, considerable quantitative differences occurred in the activities of bands 10, 11, 13 and 14 at higher pH values.

DISCUSSION

Inoculation of lupin seedlings with Fusarium culmorum stimulated peroxidase activity, noted as early as 5 h after the infection in cytosol and microsomes, and somewhat later in cell walls. It is of interest that the increase in peroxidase activity in microsomes as well as in cell walls was more significant when syringaldazine, instead of guaiacol, was used as a substrate. Some authors [8] suggest that the molecular peroxidase isoforms which participate in lignification, considered to be one of the plant defence mechanisms, demonstrate high activity with syringaldazine. Besides, it has been found that the forms which catalyse polymerization of lignin precursors are anionic peroxidases [9]. In the inoculated roots both quantitative and qualitative differences, especially at low isoelectric point values, were observed on isoelectric focusing.

To sum up, the results obtained indicate that the peroxidase forms involved in lignification are located in microsomes and cell walls. The delay in the increase in peroxidase activity, with syringaldazine as a substrate, in the cell walls of infected roots, could be explained by synthesis of the enzyme in microsomes followed by its transport to cell walls, where it affects the lignification involved in the plant defense mechanisms.

In cytosol, unlike in microsomes and cell walls changes in the isoelectric profile were more distinct in the bands appearing at higher values of the isoelectric point. The question whether a relationship exists between these changes and the defense reaction of the plant will be the aim of further study.

REFERENCES


