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Elicitor-mediated induction of enzymes of lignin biosynthesis and formation of lignin-like material in a cell suspension culture of spruce (*Picea abies*)

Burkhard Messner & Meinrad Boll
Abt.Zellchemie, G.S.F., National Center for Environment and Health, München, P.O. Box 1129, D-85758 München-Oberschleissheim, Germany

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Abstract

Incubations of photomixotrophic suspension culture cells of spruce (*Picea abies*) (L.) (Karst) with an autoclaved cell wall preparation of *Rhizosphaera kalkhoffii* as elicitor led to a rapid increase of the activity of a number of enzymes involved in lignin biosynthesis. L-phenylalanine ammonia-lyase (EC 4.3.1.5) was induced about 10-fold, feruloyl-Coenzyme A reductase (EC 1.2.1.44) 4-fold, cinnamyl alcohol dehydrogenase ($NADP^+$) (EC 1.1.1.195) 2-fold and peroxidase (EC 1.11.1.7) about 1.5-fold. The induction of the enzymes, with the exception of the peroxidase, was transient, showing maximal activity within 3 days after elicitation. Extracellular peroxidase activity, determined in the culture medium, rapidly decreased on initiation of elicitation.

Concomitant with the increase of activity of the enzymes of lignin synthesis was a rapid clouding of the culture medium. Phloroglucinol-HCl staining revealed the presence of lignin-like material in the medium and also in the cells. The IR-spectrum of this material was identical with the IR-spectrum of authentic spruce lignin.

Abbreviations: PAL – L-phenylalanine ammonia-lyase, FCR – feruloyl-Coenzyme A reductase, CAD – cinnamyl alcohol dehydrogenase, POD – peroxidase

Introduction

Higher plants posses a number of defense mechanisms against infection. Disease resistance involves induction of host-synthesized phytoalexins, formation of lignin-like material, accumulation of glycoproteins and proteinase inhibitors and an increase of the activity of certain hydrolytic enzymes (Darvill & Albersheim 1984; Ebel 1986; Collinge & Shurarenko 1987; Bowles 1990). Such responses can be induced by infection and also by elicitor preparations from fungal cell walls or culture filtrates (Ebel 1986; Dixon 1986).

Some plants respond to infections with an increased formation of lignins (see below). This lignin is aimed to render advance of the pathogen within the tissue more difficult or to make it impossible (Friend 1981; Asada & Matsumoto 1987; Matern & Kneusel 1988). It would be of interest to know whether or not the constitutive lignin and the infection-induced lignin are similar or maybe identical. Lignin has a three-dimensional phenolic structure and it is

formed by polymerization of 4-coumaryl, coniferyl and sinapyl alcohols within the plant cell. The monomeric alcohols are derived from phenylalanine via the phenylpropanoid pathway, which also gives rise to flavonoid pigments, isoflavonoid phytoalexins and hydroxycinnamic esters. The specificity of the O-methyl-transferase reaction accounts for the specificity of gymnosperm and angiosperm lignins.

The infection-induced biosynthesis of phytoalexins has been studied in a great number of plant systems (for a review see Hahlbrock & Scheel 1989). Examples for an infection-induced lignification include wheat leaf and stem rust (whole plant) (Moerschbacher et al. 1986; Moerschbacher 1989; Ride et al. 1989; Southerton & Devervall 1990; Kogel et al. 1991), rice tissue culture and pathogenic fungi (Uchiyama et al. 1983), *Petunia* cell cultures (Hagendoorn et al. 1990), castor bean cell cultures (Bruce & West 1989) and cell cultures of *Pinus eliottii* and fungal elicitor (Lesney 1989).

We have established a green, photomixotrophic cell suspension culture of *Picea abies* (Messner & Berndt 1990). In the present communication we report on a system of an elicitor-mediated induction of enzymes correlated with lignin synthesis in this culture. Part of the work has been published in preliminary form (Glatzel et al. 1991; Messner & Boll 1991).

Materials and methods

Plant material

Cell suspension cultures

Seven day old photomixotrophic cell suspension cultures of *Picea abies*, which were obtained from green callus cells, were used for the experiments. They were cultivated in Murashige and Skoog basal medium (MS), supplemented with the growth regulators benzylaminopurine (4.4 μM) and napthylacetic acid (16.1 μM) plus three percent sucrose. The preparation of the callus cells from surface-sterilized seeds via seedlings and the establishment and propagation of the cell suspension cultures have been described in detail (Messner et al. 1991).

Fungal material and elicitor preparation

Rhizosphaera kalkhoffii was a gift from W.F Oswald, München. It was maintained on malt agar ($30\,g\,l^{-1}$ malt extract, $10\,g\,l^{-1}$ agar). The preparation of the fungal cell wall fraction has also been described in detail (Messner et al. 1991).

Incubations

Cell suspension cultures (20 mg fresh weight ml^{-1} medium) were incubated with fungal cell wall preparation ($50\,\mu g\,ml^{-1}$ medium) in 300 ml Erlenmeyer flasks (30 ml incubation volume) under rotary shaking (50 rpm) at 27°C in continuous white light (Osram L 40W/77, 1000 Lux) (Messner & Berndt 1990).

Preparation of crude extracts for enzyme assays

These were prepared for the different enzymes as follows:

- PAL and CAD: 200 mg suspension culture cells were homogenized in 2.0 ml 0.1M borate buffer, pH 8.8, containing 10 mM β-mercaptoethanol, in a Potter Elvehjem homogenizer for 1 min. The homogenate was then sonified for 30 sec (Branson sonifier B-12). These procedures were carried out at 4°C. Cell debris was removed by centrifugation for 5 min in a microlitre centrifuge ($15\,000 \times g$). One millilitre of the supernatant was passed through a 5 ml column of Sephadex G-25 to remove compounds interfering with the optical assay. The protein-containing fraction (2 ml) of the eluate was used for enzyme assay.
- FCR: The suspension culture cells were homogenized and sonicated as above but in 2.0 ml 0.2 M potassium phosphate buffer, pH 7.5, containing 10 percent (v/v) polyethyleneglycol and 10 mM β-mercaptoethanol. The crude extract (supernatant of the $15\,000 \times g$ centrifugation) was subjected to fractionation with ammonium sulphate. Activity precipitating between 35 and 75 percent saturation with ammonium sulphate was dissolved in a small volume of the above buffer and used for enzyme assay. The ammonium

sulphate cut (35–75 percent) precipitated all FCR activity present (cf. Lüderitz & Grisebach 1981).

— POD: Suspension culture cells were homogenized and sonicated as above but in 2.0 ml 0.02 M potassium phosphate buffer, pH 7.0, containing 0.1 percent (w/v) polyvinylpyrrolidone 40. The supernatant of the 15 000 × *g* centrifugation was used for enyzme assay. Extracellular POD was determined directly in the cell-deprived medium.

Enzyme assays

Enzyme activity was measured spectrophotometrically: PAL was assayed according to Hahlbrock et al. (1981), FCR and CAD as described by Lüderitz & Grisebach (1981). POD was measured with p-phenylenediamine as substrate according to Schulz (1985). Specific activity is expressed as $\mu Kat \cdot kg\ protein^{-1}$. POD activity is expressed as $\Delta E\ 485\ nm \cdot min^{-1} \cdot mg\ protein^{-1}$ (Schulz 1985; Ludewig et al. 1989). Protein was determined by the method of Bradford (1976), using bovine serum albumin as standard.

Lignin-like material

The lignin-like material was determined with phloroglucinol/HCl as follows:

Medium (quantitative determination): one ml of medium was centrifuged (15 000 × *g*, 5 min), the pellet was dissolved in DMSO under sonification (1 min). Then 0.1 ml of 4 percent phloroglucinol/ethanol (w/v) were added and mixed. The mixture was quantitatively transferred into a cuvette, 0.7 ml of 37% HCl were added and mixed. Extinction at 555 nm was determined after 30 sec.

Cells (qualitative determination): Cells were washed free of lignin-like material-containing incubation medium by using fresh medium. Then the cells were stained with 4 percent phloroglucinol/ethanol and 37% HCl (1:1).

Biochemicals

Coniferyl alcohol was a product of Fluka Chemie, Buchs, Switzerland, feruloyl Coenzyme A was prepared by W. Heller according to the method of Stöckigt & Zenk (1975). All other biochemicals were obtained from Sigma Chemical Co., St. Louis, USA, (Deisenhofen, Germany).

Results

Elicitor stimulation of the activity of the enzymes of lignin biosynthesis

Incubation of *Picea abies* suspension culture cells with a cell wall preparation of *Rhizosphaera kalkhoffii* led to an increase of the activity of the enzymes correlated with the biosynthesis of lignin. This increase was accompanied by a rapid clouding of the culture medium, due to the accumulation of lignin-like material. Induction of the investigated enzymes, PAL, FCR, CAD and POD, is summarized in Fig. 1 A-C and Fig. 2.

Seven day old suspension culture cells were used for the elicitation experiments without further change of the medium, as PAL exhibited a pronounced transient increase of its activity whenever the cell cultures were transferred into fresh medium (Messner et al. 1991). Such increase would interfere with the elicitor-mediated induction of PAL. The transient increase, termed 'dilution effect' could be up to 10-fold. It had a maximum after 1–2 days of incubation and the activity subsequently declined to the original level within 3-5 days (Messner et al. 1991).

The induction of the enzymes of lignin synthesis by elicitor required the presence of a carbohydrate. Sucrose concentrations of 0.3–0.5 percent, as found in the culture medium after 7 days of growth (see above), were sufficient for a maximal induction of the enzymes. With 0.25 percent sucrose in the medium, maximal induction was still possible.

PAL: The induction of PAL by fungal elicitor is shown in Fig. 1 A. Activity rapidly increased about 10-fold within 24 h of incubation with elicitor and then declined. Activity of PAL also increased slightly in the control cells without elicitor (Fig. 1 A, curve 2). Such small increase was seen when the cell suspension culture, after having been cultivated in the same medium for 7 days (see above) solely was transferred into new

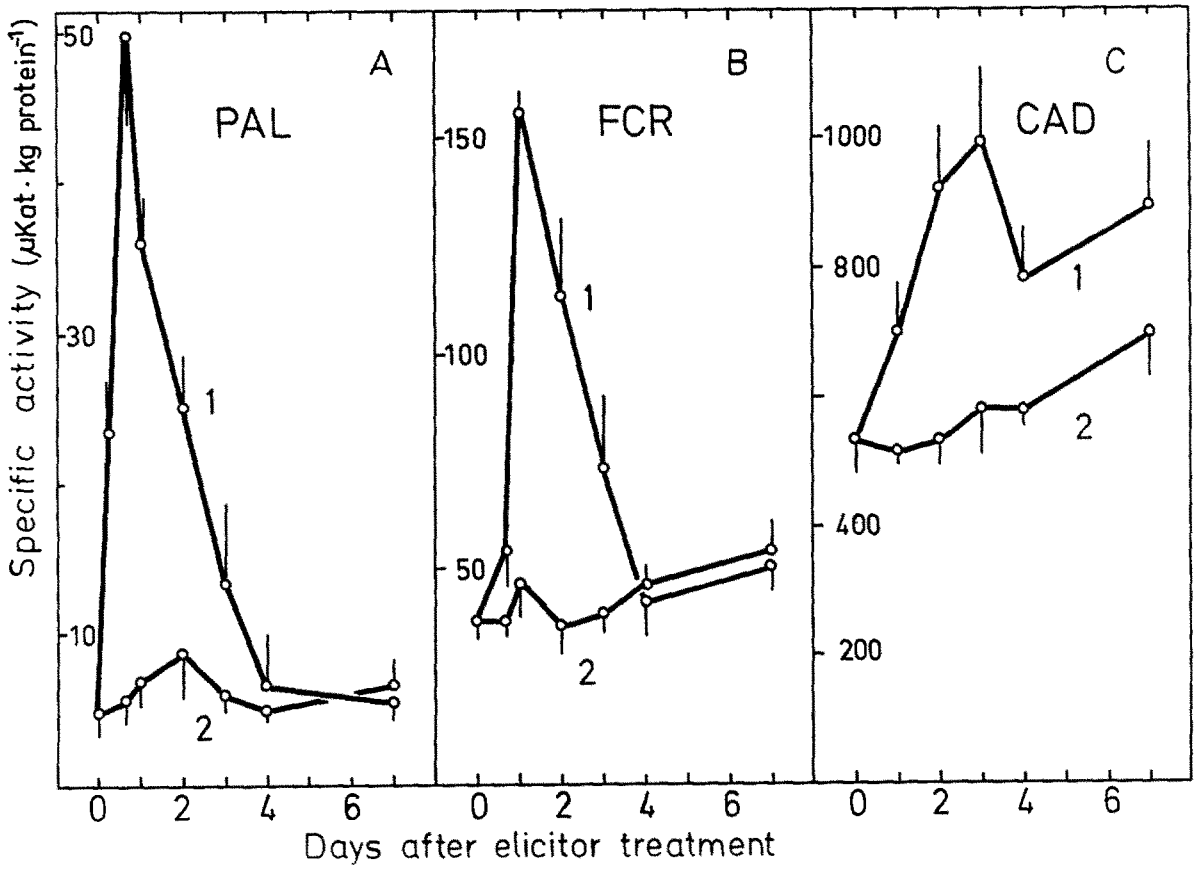

Fig. 1. Induction of enzymes of lignin biosynthesis in cell suspension cultures of *Picea abies* by a cell wall elicitor from *Rhizosphaera kalkhoffii*. (*A*) L-phenylalanine ammonia-lyase (PAL), (*B*) feruloyl-Coenzyme A reductase (FCR), (*C*) cinnamyl alcohol dehydrogenase (CAD). Cell suspension cultures (20 mg fresh weight ml^{-1} medium) were precultivated for 7 days (transient increase of PAL, see text). Specific activity after 7 days was 4.8 ± 0.25 (PAL), 38 ± 0.34 (FCR) and 550 ± 60 (CAD) (Time zero of the figure). The cultures were then incubated (no change of the medium) with (1) or without (2) fungal elicitor (50 μg ml^{-1} medium). Each value is the mean ± S.E. of 4–6 replicate experiments.

flasks and it was called 'transfer stress' (Messner et al. 1991). The transient increase of activity, caused by manipulations of transferring cells (new medium or new flasks) was found only with PAL. Activity of the other enzymes investigated was not affected.

FCR: Fig. 1 B shows the elicitor-mediated induction of FCR. The time course of the changes of the activity was similar to that of PAL. Induction of the activity was 4-fold. As can be seen there was no increase of FCR activity in the control incubations caused by the transfer of the culture into new flasks.

CAD: Activity of CAD was induced after treatment of the cell culture with fungal elicitor (Fig. 1 C). The increase of activity was about 2-fold. Activity of CAD gradually increased in the non-elicited control incubations (Fig. 1 C, curve 2). This might be the reason why the elicitor-induced activity did not return to its original level during incubation and the induction by elicitor actually also was transient. Increase of CAD activity during growth (2 weeks) of suspension culture cells of spruce has often been observed (Galliano et al. 1993).

POD: Peroxidases (total peroxidase activities) of the suspension cells were also affected by incubation with fungal elicitor. Cell suspension cultures of spruce contained cellular and extracellular POD activities, the latter being secreted into the medium. Extracellular POD had a 6–8 fold higher specific activity than the cellular enzyme (Fig. 2), and this relation remained unchanged during cultivation of the cells. Cel-

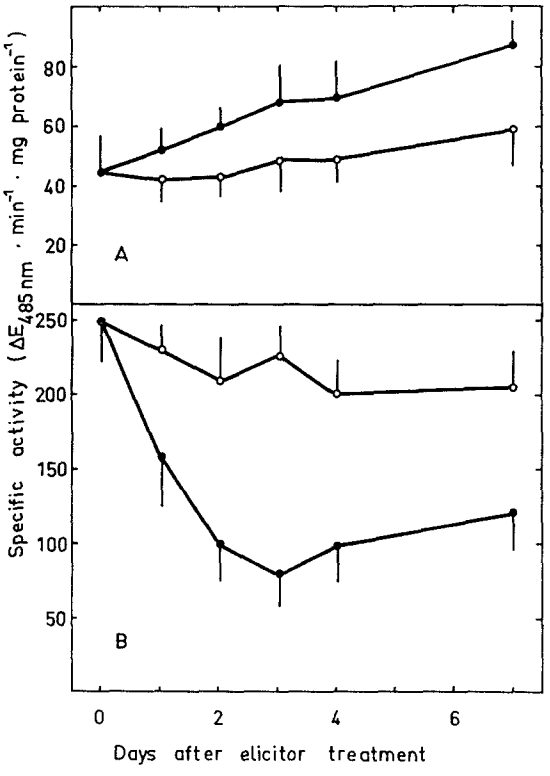

Fig. 2. Changes of cellular (*A*) and extracellular (*B*) peroxidase activities in cell suspension cultures of *Picea abies* on elicitation with a cell wall preparation of *Rhizosphaera kalkhoffii*. Cell suspension cultures (20 mg fresh weight ml^{-1} medium) were precultivated for 7 days (transient increase of PAL, see text). Specific activity of POD after 7 days was 45 ± 4.1 (cellular activity) and 250 ± 27.2 (extracellular activity) (time zero of the figure). The cultures were then incubated (no change of the medium) with (closed symbols) or without (open symbols) fungal elicitor (50 µg ml^{-1} medium). At the indicated times POD activity was determined in the cells and in the medium. Values are specific activity (mean ± S.E.) of 3 identical experiments.

lular POD activity increased about 50 percent over the control during 7 days of elicitation (Fig. 2 A). The induction here was not transient and activity did not return to its original level on continuation of elicitation. While the cellular activity of POD gradually increased after elicitation, extracellular POD activity rapidly declined to a significantly lower level within 1–2 days and subsequently began to slowly increase again (Fig. 2 B). The decline which was about 50 percent here (Fig. 2 B) could also be more than 75 percent in other experiments (Messner & Boll 1993).

Elicitor-induced formation of lignin-like material

Concomitant with the increase of the enzymes of lignin biosynthesis was a rapid accumulation of lignin-like material in the medium. This material which can be considered a native, low-molecular lignin is soluble in DMSO under sonification (Dimmel et al. 1985). It is also soluble in dioxan (Obst & Kirk 1988) and this is a characteristic feature for low-molecular lignins. However, DMSO, being a more polar solvent, was given preference here.

Turbidity in the medium strongly increased after elicitation (Fig. 3 A). The difference seen at the beginning of the experiment was due to the addition of the fungal elicitor material to the experimental flask. The increase in turbidity of Fig. 3 A, curve 2, is the result of growth of the cell suspension cultures. Thus, the values of

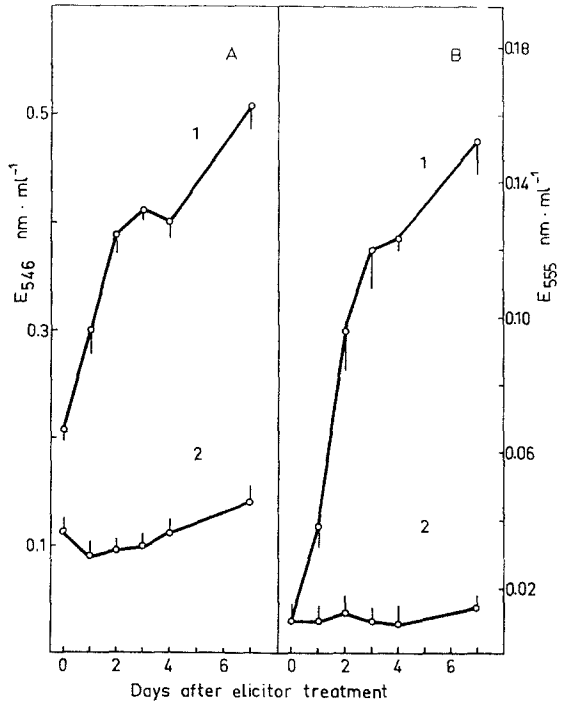

Fig. 3. Release into the medium of lignin-like material in cell suspension cultures of *Picea abies* on elicitation with a cell wall preparation of *Rhizosphaera kalkhoffii*. (*A*) turbidity, (determined at 546 nm), (*B*) phloroglucinol/HCl stainable material. Cell suspension cultures (20 mg ml^{-1} medium) were precultivated for 7 days (transient increase of PAL, see text) (time zero in the figure). The cultures were then incubated (no change of the medium) with (1) or without (2) fungal elicitor (50 µg ml^{-1} medium). Values are mean ± S.E. of 4 replicate experiments.

Fig. 3 A, curve 1 are the sum of the accumulation of lignin-like material in the medium plus growth of the cells.

Figure 3 B depicts the formation of lignin-like material as measured with the phloroglucinol/HCl staining method. Accumulation continued during the entire period investigated and levels which were 15-fold higher than the initial extinction measured were found after 7 days of elicitation. Cell cultures and also the fungal material added to the culture did not contain any lignin (Fig. 3 B, curve 2). Lignin-like material was also determined as the thioglycolic acid derivative (Bruce & West 1989). Estimates of this determination correlated well with those of the phloroglucinol/HCl method (Glatzel et al. 1991). The IR-spectrum of the present lignin-like material was found to be identical with the IR-spectrum of authentic spruce lignin (Wegener et al. 1983) (Heller W, pers. comm.).

Lignin-like material was detected not only in the medium but also in the cells of the elicited suspension culture (see Experimental section for estimation). The concentration of this material in the cells increased during elicitation as seen by an increasingly intense colour of phloroglucinol/HCl staining (not shown).

Discussion

While the numerous investigations on the infection-induced lignification in cell cultures cited in the Introduction contained only limited information on the enzymes involved in this process, the present study presents information on four of the enzymes of the lignin pathway. In a very recent study some details on the enzymes of fungal elicitor-mediated induction of lignin synthesis in *Pinus banksia* were reported (Campbell & Ellis 1992). The authors also find a transient increase of the activity of both PAL and CAD, while induction of cellular POD was not transient. Induction of the enzymes reported in the present study proceeds in a chronological sequence, in which PAL becomes induced first of all, showing the most extensive increase of activity (Fig. 1 A), followed by FCR with a less pronounced induction of activity (Fig. 1 B). Increase of CAD and of cellular POD (Fig. 1 C and Fig. 2 A) is later, it is still slower and significantly less intense. A similar chronological sequence of induction of enzyme activity was found in the UV-A light-induced induction of the enzymes of anthocyanin synthesis in cell cultures of *Daucus carota* (Gläßgen WE & Seitz HU, pers. comm.). Kinetics of the changes of CAD in response to ozone stress are very similar to those reported here for the elicitor-mediated induction of lignin enzymes (Galliano et al. 1993).

Activity of CAD was very high in the non-elicited cells (compare basal activities of the enzymes in Fig. 1 A–C) and it is only slightly induced on elicitation. This poses the question of CAD being a true regulatory activity in lignin biosynthesis. An explanation for the small increase would be that, also in spruce, a number of isoenzymes of CAD exist, as it was shown for other plant systems (Wyrambik & Grisebach 1975; Mansell et al. 1976; Pillonel et al. 1992; O'Malley et al. 1992). It could be suggested that of these CAD isoenzymes, a lignin-specific activity becomes strongly induced, while the other isoenzymes remain unaffected. Thus, the sum of all CAD activities, which is measured, is only slightly higher on elicitation.

Activity of FCR is also induced in response to elicitation, showing transient kinetics (Fig. 1 B). In contrast, the activity of this enzyme in parsley leaves did not increase after treatment with elicitor (Matern & Kneusel 1988).

The activity of PAL in cell suspension cultures changes during longer periods of the cells in culture. When spruce cells were propagated in the liquid medium for long periods of time (including periodical 7 day replacements of the culture medium) the activity of PAL decreased. After 12 months in culture, basal activity of PAL decreased from 13 ± 1.8 (Messner et al. 1991) to about 5.0 ± 0.8 (Fig. 1 A). However, extent of the induction of activity of PAL in response to elicitor treatment remained the same throughout the entire period. It is not known whether the activity of other enzymes also changed after long periods of time of the cells in culture. The reasons for the change in activity and the consequences for the cells are not known.

Activity of PAL strongly increased in response to elicitation or on transfer of the cells to new medium (dilution effect) (Messner et al. 1991).

However, the increase of PAL caused by the dilution effect does not induce formation of lignin. Rather, extent of the increase of activity in response to elicitor treatment plus that caused by the dilution effect (determined in the same experiment) was the sum of the two treatments. The possibility exists that by elicitation and by the dilution effect two different species of PAL become induced.

Increase of the cellular activity of POD after elicitation is in accordance with the observations made in *Pinus banksia* (Campbell & Ellis 1992). In the present investigation, activity of the extracellular POD was also studied. Extracellular POD which includes activities located in the intercellular space as well as activities ionically bound to the cell walls are known to be involved in the polymerization of lignin percursors and other molecules with cell wall material (Castillo 1986). Recent studies (Polle & Seifert 1992) revealed that in young needles of spruce lignin accumulated during the lignification period (3–4 weeks) and this was accompanied by an increase in extracellular POD activity, showing 3 new isoenzymes. These isoenzymes disappeared when lignin accumulation was accomplished. While these results unequivocally demonstrated a participation of extracellular POD in lignin synthesis under conditions of cell growth, studied here was the reaction of extracellular POD in response to the action of an elicitor, and the elicitor caused a decrease (inactivation) of the extracellular POD (Messner & Boll 1992). As synthesis of lignin nevertheless increased, this would imply that the lower level of the activity of POD is still sufficient to catalyze an increased polymerization of the lignin precursors as these are supplied by the preceeding steps of the biosynthetic sequence in which the activity of enzymes is induced (see Fig. 1).

The decrease of extracellular POD activity on elicitor treatment revealed that the inactivation probably was caused by an effector, arising from the cells after contact with the fungal cell wall material (Messner & Boll 1992). Appearance of the effector was very rapid. It was most active immediately after initiation of elicitation and it subsequently became increasingly ineffective, probably due to inactivation. As the cells continued to secrete POD into the medium, the level of extracellular POD slowly increased again as the effector disappeared (Fig. 2 B). This probably is the reason why inactivation of POD by the effector cannot become complete.

The elicitor-induced inactivation of extracellular POD might rather be a target of still another defense reaction mechanism operating in the plant cell as recent investigations have shown that other extracellular enzymes (β-glucosidase, acid phosphatase) also become inactivated in response to fungal elicitation (Messner & Boll 1992, 1993).

Campbell & Ellis (1992) have determined the elicitor-induced production of lignin only in the cells of their suspension culture while here large quantities of lignin-like material were found in the medium of the cell culture (Fig. 3). A similar release of lignin into the medium has been described (Brunow et al. 1990; Simola et al. 1992), also with suspension cells of *Picea abies*. It is interesting, that this lignin was released during standard growth conditions of the cells in culture while in the present study the lignin-like material was produced only in response to the action of an elicitor and without elicitor the medium did not contain any lignin at all. This would offer an unique possibility to differentiate between a pathogen-induced lignin and a lignin which is produced under ordinary growth conditions.

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