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New Phytol, 101(487-494)

Authors

Ronald, P C
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1985

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Author(s): Pamela Ronald and Kenneth Soderhall
Source: *New Phytologist*, Vol. 101, No. 3 (Nov., 1985), pp. 487-494
Published by: Blackwell Publishing on behalf of the New Phytologist Trust
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New Phytol. (1985) **101**, 487–494

PHENYLALANINE AMMONIA LYASE AND PEROXIDASE ACTIVITY IN MYCORRHIZAL AND NONMYCORRHIZAL SHORT ROOTS OF SCOTS PINE, *PINUS SYLVESTRIS* L.

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(*Accepted 17 July 1985*)

SUMMARY

Phenylalanine ammonia lyase was characterized in roots of *Pinus sylvestris* L. The K_m for the pine root enzyme with phenylalanine as a substrate was $1{\cdot}2 \pm 0{\cdot}4 \times 10^{-4}$ M. The enzyme had a pH activity optimum of 9 and the subunit molecular weight was 70 to 72 kD as determined by Western blotting. Enzyme activity could be inhibited by D,L-2-aminooxy 3 phenylpropionic acid at 1 μM. Treatments with zymosan, pectinase, light or kinetin and naphthylacetic acid did not induce higher phenylalanine ammonia lyase or peroxidase activity in pine roots. No significant differences were observed in phenylalanine ammonia lyase or peroxidase activity in mycorrhizal and nonmycorrhizal short roots in the *P. sylvestris–L. laccata* symbiosis 15 weeks after cultivation.

Key words: *Laccaria laccata*, mycorrhiza, peroxidase, phenylalanine ammonia lyase, *Pinus sylvestris* L.

INTRODUCTION

The enzyme phenylalanine ammonia lyase (PAL) catalyzes the elimination of ammonia from L-phenylalanine to form trans-cinnamic acid (Koukol & Conn, 1961). This is the first reaction in the biosynthesis of phenylpropanoid compounds in higher plants. The activity of PAL undergoes dramatic changes in several plant tissues as a response to parasitic attack, exposure to light, or wounding (for reviews see Hanson & Havir, 1981; Jones, 1984). PAL is involved in the biosynthesis of antimicrobial compounds (phytoalexins) as well as providing the cell with precursors for lignin synthesis. Several workers have reported that polysaccharides from fungal cell walls elicit higher PAL activity in plant tissues. Recently, Albersheim and coworkers (Ossowski *et al.*, 1984; Sharp, McNeil & Albersheim, 1984a; Sharp, Valent & Albersheim, 1984b; Sharp *et al.*, 1984c) have shown that the process of phytoalexin induction may only be specific for carbohydrates containing β-1,3;β-1,6 linked glucose units (for a review see Darvill & Albersheim, 1984).

The possible involvement of PAL for the maintenance or rejection of fungi in a symbiotic system such as mycorrhizas is, however, not known. To investigate whether PAL is involved in establishment of mycorrhizas, we partially characterized the enzyme from roots of *P. sylvestris*. The inducibility of PAL activity by known elicitors was tested and the level of PAL activity in the *P. sylvestris – L. laccata* interaction was assayed in mycorrhizal and nonmycorrhizal short roots.

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0028-646X/85/110487+08 $03.00/0

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MATERIALS AND METHODS

Growth of fungi

Laccaria laccata (stock S-238 from Randy Molina, Corvallis, Oregon) was cultivated as described previously (Ramstedt & Söderhäll, 1983). After four weeks of growth, mycelia of *L. laccata* were rinsed with 100 mM Tris buffer, pH 7·5, lyophilized and frozen until use. Growth of *L. laccata* for cultivation of mycorrhiza was modified as described below.

Mycorrhizas

P. sylvestris seeds were obtained from Påarp Plantors Lugnets Plantskola in Bålsta, Sweden. Seeds were surface sterilized for 10 min in 10% sodium hypochlorite and germinated on 1% agar. *L. laccata* was grown in a mixture of expanded clay (Leca) and modified Melin Norkrans' medium (MMN) (Marx, 1969) and the pH adjusted to 4. Fe-citrate was substituted for Fe-sulphate in the MMN medium. After 1 month of growth, the fungal-clay mixture was used for inoculation of one half of the seedlings and the rest were kept free of fungi as the nonmycorrhizal control. All plants were grown in a greenhouse at 18 °C with 18 h light per day for the first 4 weeks. Then they were transferred to incubation chambers and grown at 20 °C with 12 h light per day. All plants were watered three times a week with a nutrient solution composed according to Ingestad (1979).

Determination of extent of mycorrhizal colonization

Short roots of 10 randomly selected mycorrhizal and control plants were examined under the light microscope 8 weeks after planting and again before harvest to determine the extent of mycorrhizal colonization.

Homogenization of plant and fungal material

L. laccata: 120 mg lyophilized tissue was ground in an ice cold mortar with 4% (w:v) polyvinylpyrrolidone (PVP) in 100 mM Tris–HCl buffer, pH 7·5 containing 1 mM EDTA, 2 mM $MgCl_2$, 0·5 mM phenylmethylsulphonidefluoride and 1 mM β-mercaptoethanol, and then further homogenized in a glass piston homogenizer. The homogenate was centrifuged at 10000 ***g*** for 20 min and the resulting supernatant was desalted on a Sephadex G-25 column (PD-10, Pharmacia, Sweden).

P. sylvestris mycorrhizal and nonmycorrhizal short roots: 15 week old mycorrhizal or nonmycorrhizal short roots of 12 plants were carefully cut from the main root and homogenized in an ice cold glass piston homogenizer in 500 μl sodium phosphate buffer (100 mM) pH 7·7 containing 1 mM EDTA, 1 mM β-mercaptoethanol and 4% (w:v) PVP. The homogenates were centrifuged at 10000 ***g*** for 20 min and the crude supernatants were used for the assays.

Enzyme assays

All assays were recorded in a Hitachi 100-80 spectrophotometer. One μkatal of enzyme activity is defined as the amount of enzyme catalyzing the formation of 1 μmol product per s under standard assay conditions. For PAL, the change in cinnamic acid concentration was estimated every 30 min for 3 h by measuring the absorbance at 290 nm against a control reaction containing no L-phenylalanine. The reaction mixture contained: 0·033 M sodium borate buffer, pH 8·8, 10 mM

L-phenylalanine, and 100 μl enzyme extract in a total volume of 600 μl and was incubated at 30 °C (Zucker, 1965). Two measurements were made for each extract. Peroxidase was assayed using 2,2′-Azino-di (3-ethyl benzthiazoline-6-sulphonic acid) (ABTS), (Boehringer Mannheim, F.R.G.) as chromogen (Childs & Bardsley, 1975). The reaction mixture contained 100 mM sodium citrate phosphate buffer pH 3·7, 3 mM ABTS, and 3 mM hydrogen peroxide. The amount of extract that gave a change in absorbance of 0·05 to 0·7 per min (1–5 μl) when measured at 414 nm was used in each reaction. The control reaction contained no enzyme extract. Three replicate determinations were made with each extract. Protein concentrations were determined in triplicate according to Bradford (1976) using bovine serum albumin as a standard.

Inhibition experiments

The inhibitor, D,L-2 aminooxy 3 phenyl propionic acid (D,L-AOPP), was synthesized according to Testa *et al.* (1963) and Undheim, Bamberg & Sjöberg (1965). *Rhodoturula glutinus* PAL was purchased from Sigma Chemical Company, St Louis, MO (No. P-9519). *P. sylvestris* extracts were prepared as follows: Plants were grown for 3 weeks in a greenhouse with continuous light at 22 °C and the roots were homogenized as described for short roots. The extract was then fractionated by $(NH_4)_2SO_4$ precipitation (60% saturation) for 2 h, centrifuged 15 min at 36 000 ***g*** and dialyzed overnight against 50 mM Tris–HCl buffer, pH 8·8. The assays were carried out as described in the legend to Figure 1.

Induction experiments

Seeds of *P. sylvestris* L. were obtained from the Institute for Forest Improvement, Ekebo, Svalöv, Sweden and the same seed lot was used in all the induction experiments. Zymosan, a crude source of β-1,3;β-1,6 linked glucose units from the cell walls of *Saccharomyces cerevisiae* was purchased from Sigma Chemical Company. Seed sterilization and growth of the plants were performed according to Grönroos & von Arnold (1985) with the following modifications: Hoagland's medium (Schropp & Scharrer, 1933) was used for the nutrient solution, and plants were grown in 160 μE m^{-2} s^{-1} light. After 2 weeks, 1 cm root sections were cut and soaked in the following solutions: sodium phosphate buffer pH 5·9, 0·25 M mannitol, 1 mg ml^{-1} pectinase, zymosan (1, 3, 10, 100 μg ml^{-1}) or 1 mg l^{-1} naphthylacetic acid + 0·2 mg l^{-1} kinetin. All solutions were made up in sodium phosphate buffer, pH 5·9. The segments were incubated in darkness at 30 °C. After 0, 5, 9, 13, 22 and 48 h the root segments were rinsed three times in 100 mM sodium phosphate buffer, pH 7·7 and homogenized and assayed as described for short roots. To test the effect of light, the plants were grown for 11 d as described above and then moved to the dark for 17 h. The plants were separately treated in three ways. One group was exposed to u.v. light for 2 to 4 h and another to white light for 15 to 24 h. Plants in these two groups were harvested, homogenized and assayed for enzyme activity as described above. Control plants, the third group, were harvested under green light immediately after the dark treatment.

Protein blotting

The parsley PAL and the parsley PAL antiserum used in the blotting experiments were the kind gift of Dr Elmon Schmelzer (Max-Planck Institute, Köln, FRG). The PAL was prepared as follows: Extract from 8 g of frozen light induced parsley cells (Zimmerman & Hahlbrock, 1975) was precipitated at 40%

$(NH_4)_2SO_4$ saturation, the supernatant was then subjected to 70% $(NH_4)_2SO_4$ precipitation, the pellet was solubilized in 10 mM Tris–HCl, pH 7·5, saturated to 100% $(NH_4)_2SO_4$ then centrifuged at 10000 *g* for 15 min, resuspended in 50 mM Tris pH 8·8 and dialyzed for 5 h against the same buffer. The pine extract was prepared as described for the inhibition experiments. Thirty micrograms of partially purified PAL from parsley or pine was first subjected to polyacrylamide gel electrophoresis in a 10–15% gradient gel containing sodium dodecyl sulphate according to Blobel & Dobberstein (1975). After electrophoresis, the proteins were transferred to nitrocellulose sheets (Towbin, Staehelin & Gordon, 1979) and then incubated with the parsley-PAL antiserum diluted 1 : 1000 times (Schröder, Betz & Hahlbrock, 1976). Subsequently, the filters were incubated with horseradish peroxidase (Sigma Chemicals) conjugated to *Staphylococcus aureus* protein A (Pharmacia). The conjugation was prepared according to Engvall (1978) to an initial concentration of 350 μg ml^{-1} and then diluted 1 : 1000 times. The filters were then stained with diaminobenzidine (Towbin *et al.*, 1979). The stained blots were scanned with a densitometer at an absorbance of 550 nm. Molecular weight standards contained: lysozyme (14·3 kD), carbonic anhydrase (30 kD), ovalbumin (46 kD) and bovine serum albumin (69 kD) (Sigma Chemical Company).

RESULTS

PAL extracted from roots of pine shared several properties in common with PAL from parsley, *Petroselinum hortense*, and the yeast *R. glutinus* as shown in Table 1. D,L-AOPP caused a 50% reduction in PAL activity of pine PAL at a concentration of 1 μM. L-AOPP is known to be a specific inhibitor of PAL activity in *R. glutinis* (Amrhein & Gödeke, 1977). *R. glutinus* PAL was inhibited to 50% at a concentration of 0·6 μM D,L-AOPP (Fig. 1). The parsley derived PAL antiserum crossreacted prominently with a pine protein of molecular weight of about 70–72 kD and to a lesser extent with several low molecular weight proteins (Fig. 2). The antiserum reacted most prominently with a 55–58 and a 70–72 kD protein in the parsley extract (data not shown).

In order to test the effect of known elicitors on PAL activity in roots, we first incubated roots of intact pine plants in a solution containing zymosan, a crude source of β-1,3;β-1,6 linked glucose units or exposed the plants to u.v. or white light for various times. No increase in activity was observed. We then incubated

Table 1. *Properties of phenylalanine ammonia lyase*

	*Pinus sylvestris**	*Petroselinum hortense*†	*Rhodotorula glutinus*‡
Molecular weight of subunits (kD)	72	75–78§	83
K_m (M)¶	$1{\cdot}2 \times 10^{-4}$	$2{\cdot}4 \times 10^{-4}$ (H) $3{\cdot}2 \times 10^{-5}$ (L)	$2{\cdot}5 \times 10^{-4}$
pH optimum	9	8–9	8–9
L-AOPP sensitivity	Inhibition		Inhibition‖

Data from: * The present paper; † Zimmerman & Hahlbrock (1975); ‡ Hodgins (1971); Havir & Hanson (1973); § Dr E. Schmelzer (1985). Max-Planck Institute, Köln, FRG (personal communication); ‖ Amrhein & Gödeke (1979).

¶ K_m was determined from both Lineweaver–Burk and Eadie–Hofstee plots. H, high; L, low substrate concentrations.

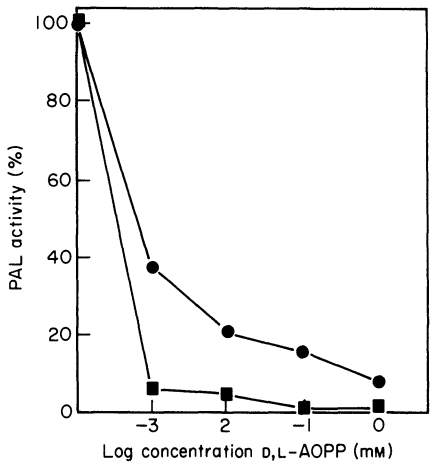

Fig. 1. Inhibition of PAL activity by D,L-2 aminooxy 3 phenyl propionic acid (D,L-AOPP). Varying concentrations of the inhibitor or a water control were added to the enzyme which was incubated for 5 min at room temperature. 100% PAL activity corresponds to the control reaction incubated with water. ●, *Pinus sylvestris*; ■, *Rhodoturula glutinis*.

root segments of pine in solutions known to enhance PAL activity in other plants (Hanson & Havir, 1981) as described in detail in Materials and Methods above. None of the treatments enhanced PAL activity in pine root segments. The PAL activity remained at approximately 2×10^{-5} μkatal mg protein^{-1}.

No fungal colonization could be observed on any of the nonmycorrhizal control plants. After 8 weeks, 85% of the short roots of plants inoculated with *L. laccata* had formed mycorrhizas. Clamp connections of *L. laccata* could easily be seen at 40 × magnification. After 15 weeks, still no mycorrhizas had formed on the control

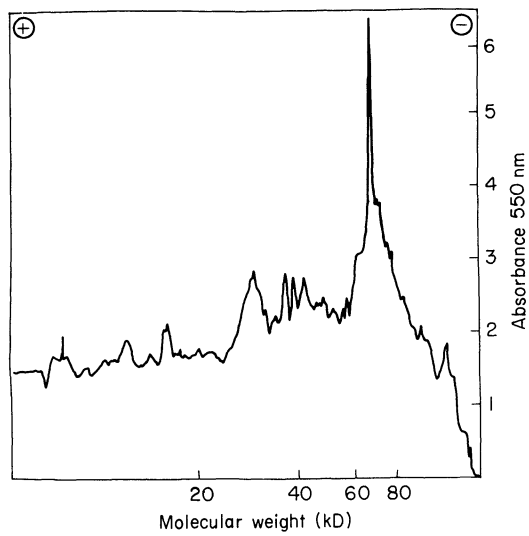

Fig. 2. Molecular weight determination of the PAL subunits of *Pinus sylvestris*. The graph represents the scanning of a protein blot probed with a parsley derived PAL antiserum (arbitrary units).

Table 2. *PAL and peroxidase activity in mycorrhizal and nonmycorrhizal short roots of* Pinus sylvestris *and* Laccaria laccata

	PAL activity (μkatal mg protein$^{-1} \times 10^{-5}$)	Peroxidase (μkatal mg protein^{-1})
Mycorrhizal short roots	8·3 ± 0·7	1·8 ± 0·2
Nonmycorrhizal short roots	3·4 ± 0·1	1·7 ± 0·3
Laccaria laccata	1·9 ± 0·5	0·003 ± 0·003

The values for short roots are means ± SD of three experiments. For *Laccaria laccata*, the values are means of two experiments. Each experiment is a mean of two replicates (PAL) or three replicates (peroxidase).

plants and the mycorrhizal plants had greater than 85 % colonization. Enzyme activity measurements of extracts from mycorrhizal and nonmycorrhizal plants showed that peroxidase was the same. No significant peroxidase activity was detected in *L. laccata*. In contrast, PAL activity was approximately two times higher in the mycorrhizal short roots compared to the nonmycorrhizal short roots, but most of the higher activity is probably due to the PAL activity present in *L. laccata* (Table 2).

DISCUSSION

The enzyme PAL in gymnosperms has received very little attention. Kutsuki & Higuchi (1981) compared activities of PAL in reaction wood and opposite wood in several gymnosperms but did not characterize the enzyme. No studies have been published on the possible importance of PAL in the interaction between the host plant and the fungal symbiont in symbiotic systems, such as ectomycorrhizas or endomycorrhizas.

We found that PAL of pine had several properties in common with PAL characterized in various angiosperms and fungi (Table 1 and Zimmerman & Hahlbrock, 1975). We obtained slightly lower molecular weight values, 70–72 kD, for both parsley and pine PAL subunits than the 75–78 kD that has been reported for parsley (Dr E. Schmelzer, Max-Planck Institute, Köln, West Germany). On our blots, the parsley antiserum also cross reacted with a 70–72 kD protein in the parsley extract but did not cross react with a control protein of approximately the same molecular weight (bovine serum albumin, 69 kD). It appears, therefore, that the reaction observed with pine is specific. The low molecular weight bands in pine may be due to an instability of the large enzyme subunits or to impurities in the antiserum (Schröder *et al.*, 1976). The K_m, the pH optimum and sensitivity to the inhibitor D,L-AOPP for pine root PAL were also similar to that reported for parsley and yeast (Table 1).

Pine root PAL and peroxidase activity could not be enhanced by treatments used to induce higher PAL activity in other plants. These results suggest that, under the conditions tested, pine roots do not respond to elicitors or to stress conditions by increasing synthesis of the enzyme PAL as has been found in other systems (Jones, 1984). In contrast, an increase of PAL and peroxidase activity in wounded hypocotyls was observed after 72 h of treatment (unpublished results). The relatively long time period required for an enhancement of PAL activity in pine hypocotyls may reflect an involvement of the enzymes in lignin synthesis rather than phytoalexin synthesis.

Another question we asked was whether mycorrhizal fungi could suppress PAL activity in the roots to avoid being exposed to phytoalexins and/or to decrease

lignin synthesis during penetration of host root cells. Table 2 shows that the mycorrhizal fungus *L. laccata* did not affect the PAL activity in pine short roots although our results must be interpreted cautiously since the exact amount of *L. laccata* hyphae in the short roots was not determined. These results are consistent with those obtained by Piché, Fortin & LaFontaine (1981), who found no differences in phenolic substances in mycorrhizal and nonmycorrhizal short roots of *Pinus strobus* infected with *Pisolithus tinctorius* and they concluded that the phenolics in the mycorrhizal short roots are inherent to the host plant and are not produced as a reaction to the ectomycorrhizal fungi. Coleman & Anderson (1985), however, have reported that Douglas fir callus, initiated from roots, responds non-specifically to culture filtrates of ectomycorrhizal fungi by production of brown oxidized phenolics. This reaction may be an activation of phenoloxidase/peroxidase which will yield melanin-like pigments, since we have recently shown that phenoloxidase is present as a proenzyme in plants which can be activated by trypsin or Ca^{2+}/Mn^{2+} ions (Söderhäll, Carlberg & Eriksson, 1985). Anderson (1985) found that root surfaces of several plant species had potent peroxidase activity and suggests that the oxidative capacity of the enzyme could have antimicrobial activity.

In conclusion, our results show that PAL in pine roots has similar properties to that of PAL isolated from other sources but, under the conditions tested, the enzyme cannot be enhanced in activity by treatments used to induce PAL activity in angiosperms. Nonmycorrhizal and mycorrhizal short roots had approximately the same amount of PAL and peroxidase activity indicating that *L. laccata* does not suppress or increase the activity of this enzyme 15 weeks after colonization. Probably several factors are regulating the symbiosis between mycorrhizal fungi and their host roots. The means by which mycorrhizal fungi overcome plant defences and establish a symbiosis remain to be elucidated.

Acknowledgements

We thank Dr Klaus Hahlbrock (Max-Planck Institute, Köln, W. Germany) for the gift of the antiserum to parsley PAL, Elmon Schmelzer (of the same Institute) and Roland Grönroos (Institute of Physiological Botany, Uppsala, Sweden) for valuable discussions, Joakim Rödin and Dr Lars Rask (Wallenberg Laboratory, Uppsala, Sweden) for help with protein blotting, and Eva Damm (Agricultural University, Uppsala, Sweden) for assistance in growth of the mycorrhizae.

This work was supported by the Swedish Agricultural Research Council (S93:1). Pamela Ronald was a recipient of a Fulbright Fellowship.

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