Pergamon

PII: S0031-9422(98)00146-0

Phytochemistry, Vol. 48, No. 4, pp. 601–606, 1998

Printed in Great Britain
0031-9422/98 $19.00+0.00

CONIFERYL ALCOHOL OXIDASE OPERATES THROUGH A BOUND FREE-RADICAL INTERMEDIATE

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(*Received* 19 *January* 1998)

Key Word Index—*Picea sitchensis*; Pinaceae; Sitka spruce; X *Cupressocyparis leylandii*; Cupressaceae; Leyland cypress; *Chamaecyparis lawsoniana*; Cupressaceae; Lawson cypress; *Ulmus glabra*; Ulmaceae; Wych elm; xylem; lignification; oxidase; coniferyl alcohol; electron paramagnetic resonance; free radical.

Abstract—Oxidase activity was enriched in extracts of the developing, lignifying xylem of Sitka spruce, Leyland cypress, Lawson cypress and Wych elm obtained by a procedure that selects cell-wall-associated glycoproteins. All of the xylem extracts were able to oxidise the monolignol, coniferyl alcohol, and had a greater affinity for coniferyl alcohol than ABTS. The oxidases were strongly inhibited by Cu-chelators and had inhibition profiles broadly similar to catechol oxidase-type polyphenol oxidases. Analysis of the oxidation products of coniferyl alcohol generated in the presence of the spin trap POBN, by electron paramagnetic resonance spectroscopy, showed that a spin adduct of parameters $a_N = 15.65$–15.74 G and $a_H = 2.73$–2.78 G had been generated which can be most readily assigned to a POBN adduct of the β-carbon-centred free radical of coniferyl alcohol. Electron paramagnetic resonance spectra of the extracts from the four species contained signals representative of Cu^{2+} and a free radical species of g value ~2.0036. The intensity of the free radical signal was diminished by addition of coniferyl alcohol but was restored upon aeration. The Cu^{2+} signals were similarly altered by this treatment, but diminution was less marked. Cycles of depletion then recovery of the free radical signal could be obtained by addition of aliquots of coniferyl alcohol followed by aeration which strongly suggests that the free radical is directly involved in the oxidation mechanism. We propose that coniferyl alcohol oxidase operates via a bound free radical which is re-oxidised via a charge relay mechanism involving bound Cu^{2+} ions and molecular oxygen.

INTRODUCTION

We have previously presented evidence that coniferyl alcohol oxidases participate in the oxidation and polymerisation of monolignols during the initial stages of lignification of developing xylem of Sitka spruce [1, 2] and Leyland cypress [3]. These enzymes appear to have an affinity for coniferyl alcohol and can oxidise and polymerise this monolignol to form insoluble lignin-like products [2, 3]. In this paper, we used an adapted extraction procedure that selects cell-wall-associated glycoproteins [4] to provide evidence for the presence of coniferyl alcohol oxidases in the lignifying, developing xylem of three conifers, *Picea sitchensis* (Sitka spruce), X *Cupressocyparis leylandii* (Leyland cypress) and *Chamaecyparis lawsoniana* (Lawson cypress) and an angiosperm, *Ulmus glabra* (Wych elm). We present electron paramagnetic resonance (EPR) evidence that coniferyl alcohol oxidases utilise a bound free radical in their oxidation mechanism.

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RESULTS AND DISCUSSION

Oxidase activity was enriched in the ConA bound extracts of xylem tissue from Sitka spruce, Lawson cypress, Leyland cypress and Wych elm (Table 1). The extracts readily oxidised coniferyl alcohol (CA) and the specific activity using coniferyl alcohol was 2–3 fold greater than with ABTS in all cases suggesting that the oxidases have an affinity for the oxidation of this monolignol. The extract from Wych elm had the lowest CA/ABTS oxidation ratio and this may reflect a different specificity of angiosperm oxidases towards monolignols as Wych elm wood lignin, in common with most angiosperms, contains greater levels of syringyl groups than lignin from conifers [5]. However,

Table 1. Oxidase activity in ConA bound extracts of developing xylem

	OXIDASE ACTIVITY		
	Total activity (nmol ABTS min^{-1})	Specific activity (nmol min^{-1} mg^{-1})	
SAMPLE		ABTS	CA
SITKA	791 ± 13	302 ± 7 [3.32][a]	1002 ± 42
WYCH ELM	146 ± 4	249 ± 8 [2.22]	553 ± 21
LAWSON	308 ± 8	89 ± 2 [2.89]	258 ± 17
LEYLAND	100 ± 1	73 ± 4 [3.10]	226 ± 13

a — figures in square parentheses represent the ratio of the specific activity against CA over that of ABTS.

a more detailed study of the specificity of monolignol oxidation is required to substantiate this suggestion. The discovery of oxidase activity capable of oxidising CA in the developing xylem of an angiosperm suggests that oxidases may participate in the lignification of xylem in a broad taxonomic range of trees.

Oxidase activity was influenced by a number of effectors (Table 2). Although results are quoted for Sitka oxidase extracts, the main findings apply to all extracts, and the oxidation of coniferyl alcohol was similarly affected. The Cu-specific metal chelator, diethyl dithiocarbamate was a potent inhibitor, causing complete inhibition at 1 mM in the presence of

Table 2. Response of Sitka xylem oxidase activity to effectors

Effector	Concentration (mM)	% Control[a] Oxidase activity
Diethyl dithiocarbamate	1.0	1.6 ± 0.4
	0.1	18.3 ± 2.6
	0.01	70.2 ± 3.1
Dihydroxynaphthalene	0.01	0.8 ± 0.2
Phenylhydrazine	1.0	15.7 ± 2.1
p-Nitrophenyl hydrazine	1.0	30.1 ± 3.1
Tropolone	1.0	20.9 ± 1.2
Propyl gallate	0.01	3.3 ± 1.1
Salicyl hydroxamic acid	1.0	15.8 ± 3.1
Ferulic acid	0.1	12.3 ± 1.2
trans-Cinnamic acid	1.0	123.8 ± 1.3
Coniferyl alcohol	0.1	2.9 ± 1.1
NaCN	1.0	4.7 ± 1.4
NaN_3	1.0	0.9 ± 0.2
Tiron	1.0	17.1 ± 1.0
SOD	1000 u ml^{-1}	107.8 ± 4.6
Catalase	1000 u ml^{-1}	107.4 ± 3.3
SOD/catalase	1000 u ml^{-1}	98.2 ± 3.1

a — Oxidase activity was measured against ABTS.

200 mM Ca^{2+}, whereas EDTA or EGTA, which caused inhibition in desalted extracts [2], had no effect under these conditions. Oxidase activity was sensitive to a range of known polyphenol oxidase inhibitors [6] such as tropolone, propyl gallate, salicyl hydroxamic acid, phenylhydrazine and 2, 3-dihydroxynaphthalene. Oxidase activity was effectively abolished by azide or cyanide at 1 mM. Inhibition by coniferyl alcohol and ferulic acid appears to be due to preferential oxidation of these hydroxycinnamate derivatives [2]. Consistent activation by *trans*-cinnamic acid and inhibition by 2, 3-dihydroxynaphthalene (an *o*-diphenol mimic) and tropolone suggests that the enzyme is a catechol oxidase-like polyphenol oxidase [6, 7]. In fact, the inhibitor profile is similar to that reported for a coniferyl alcohol oxidase from the developing xylem of *Pinus strobus* [8] which the authors also classified as a catechol oxidase-like enzyme.

Oxidase activity was unaffected by catalase or superoxide dismutase. However, catalase solutions as supplied caused inhibition of oxidase activity (20–30% inhibition at 1000 units ml^{-1}) that was not dependent on enzymatic activity, as boiled inactive preparations caused equal inhibition (results not shown), but desalted active preparations caused no inhibition. This may be due to phenolic compounds present in the supplied preparation.

Oxidation of CA by extracts in the presence of the spin trap α-(4-pyridyl-1-oxide)-*N*-*t*-butylnitrone (POBN) produced a spin adduct that yielded an EPR spectrum with hyperfine coupling constants, $a_N = 15.65$–15.74 G and $a_H = 2.73$–2.78 G indicating the formation and trapping of a soluble free radical (Fig. 1). These coupling constants are similar to those reported for the POBN adducts of $\cdot CH_2CH_3$, $\cdot CH_2CH_2OH$ and $\cdot CH_2CH_2(C_6H_5)$ [9]. A plausible mechanism for the formation of the parent radical is the abstraction of the phenolic hydrogen atom to form the phenoxy radical (structure I) which has a resonance form which bears the unpaired electron at the β-carbon (structure II). It is postulated that structure II is the radical trapped to form the stable nitroxyl. Although spin traps can themselves demonstrate some preferences for the radicals they capture [9], the apparent predominance of the β-carbon radical would influence the dimeric products formed upon radical coupling and may explain the differences noted between the dimeric products produced from CA by the *Rhus* oxidase and peroxidases [10].

Electron paramagnetic resonance spectra of the extracts from the four species contained signals representative of Cu^{2+} and a free radical species of g value ~2.0036 (Fig. 2a–d). In all samples the presence of type II Cu (typified by $A_{//} \sim 180$G) was confirmed, although there is some evidence for type I Cu. For example, in the EPR spectrum of the Lawson cypress extract, the second parallel feature shows a broader absorption than would be expected for type II Cu in isolation [11]. Prolonged accumulation in this region

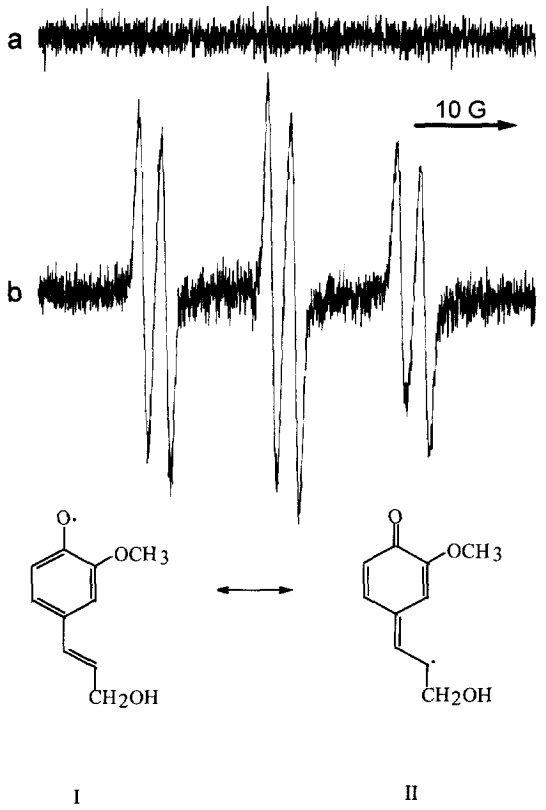

Fig. 1. EPR spectra of xylem extracts in the presence of spin trap, POBN. a = extract + spin trap; b = extract + spin trap + coniferyl alcohol. I = phenoxyl radical formed by abstraction of the phenolic hydrogen atom, II = β-carbon centred tautomer. Spectrum b is postulated to be that of the POBN adduct of II.

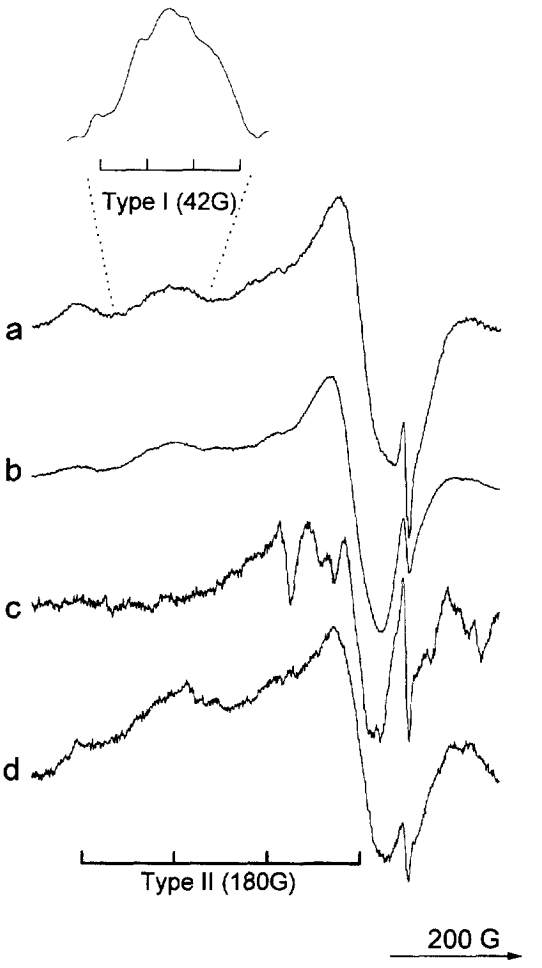

Fig. 2. EPR spectra of oxidase extracts from: a, Sitka spruce; b, Lawson cypress; c, Leyland cypress and d, Wych elm. The inset above Fig. 2a illustrates the fine structure of features associated with the second parallel feature of type II Cu^{2+}, and was obtained by prolonged accumulation across this region.

(Fig. 2a; inset) identifies an $A_{//}$ component of ~42 G which is indicative of type I Cu. The additional isotropic features from the Leyland cypress sample (Fig. 2c) result from Mn^{2+}, probably carried through from the Concanavalin-A Sepharose column, but its absence in all other samples suggest it to be adventitious.

The intensity of the free radical signal seems unrelated to the specific oxidase activity of the extract. For example the Wych elm extract has a low free radical signal but has a high specific activity. However, the total activity present in the sample subjected to EPR (which is essentially proportional to the total activity figures in Table 1) appears to be more relevant as the spectra from Sitka and the Lawson samples (which had the greatest total activity) were the most intense. The link between total oxidase activity and free radical and Cu^{2+} signal intensity may be relevant as Udagama-Randenyia and Savidge [8] reported that a highly purified sample of coniferyl alcohol oxidase from developing xylem of *Pinus strobus* gave an EPR spectrum with no discernible Cu^{2+} or free radical signals. However, no spectra were shown.

The intensity of the free radical signal was diminished by addition of coniferyl alcohol (Fig. 3) but was restored upon aeration. Although the data in Fig. 3 was obtained using the Lawson xylem extract, similar effects were noted using the other extracts. The total Cu signal (as measured by peak height at g⊥) was less obviously affected by this treatment but similar cycles of depletion and recovery could be discerned especially upon addition of greater amounts of CA. These cycles of depletion of the free radical/Cu signals caused by addition of CA and their recovery upon aeration indicates the involvement of the free radical in the oxidation of CA, which is then re-oxidised by a redox couple with Cu and oxygen (see Fig. 4). The catalytic role of copper is supported by inhibition studies (Table 2) and the involvement of a free-radical intermediate in the catalytic mechanism would explain the susceptibility of the oxidase to reducing agents such as tropolone, propyl gallate, salicyl hydroxamic acid (Table 2) and ascorbate and dithiothreitol [2].

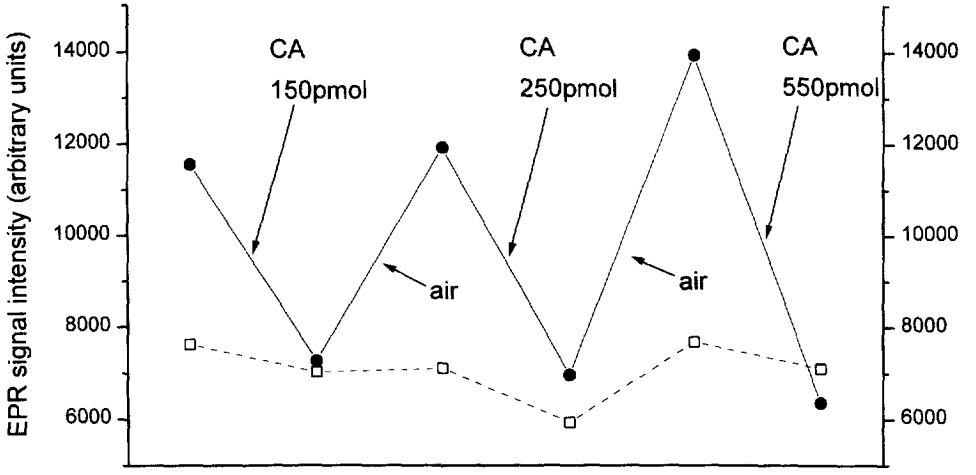

Fig. 3. EPR signal intensity for Cu^{2+} (□) and free radical (●) during the step-wise addition of coniferyl alcohol/aeration.

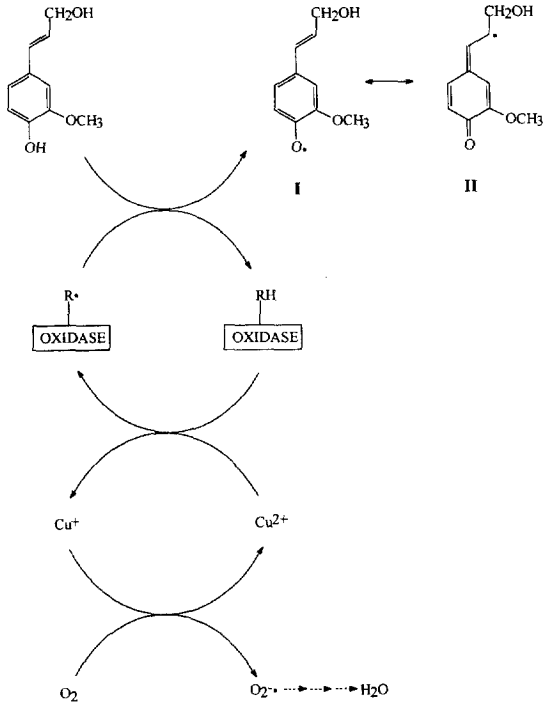

Fig. 4. Schematic of the proposed mechanism of oxidase action. Arrows pointing right indicate oxidation reactions, arrows pointing left indicate reductions. I and II denote the same radicals as in Figure 1.

Although the extracts subjected to EPR are greatly enriched in oxidase activity, they are by no means pure samples of oxidase (e.g. the Sitka extract contains at least 8 major proteins bands on SDS-PAGE [2]). Therefore, it is possible that the free radical and the Cu ions are not resident on one enzyme and a number of coupled redox reactions may be involved. Nevertheless, the EPR spectra of purified, oxidase-enriched samples contain similar, if less intense, free radical and Cu signals (results not shown). Therefore, the simplest working hypothesis is that the free radical and Cu operate as part of the catalytic cycle of a single oxidase enzyme.

Free radicals are being increasingly recognised as intermediates in the enzymatic conversion of substrate to product [12] and as integral components of enzyme structure. Catalytically-important tyrosyl radicals have been identified in ribonucleotide reductase [13] and prostaglandin H synthase [14], glycyl radicals within pyruvate formate lyase [15], and trihydroxyphenylalanine (TOPA) quinone derived radicals in amine oxidases [16]. The free radical observed in the present study is clearly not a tyrosyl radical as it lacks the characteristic 18 G splitting associated with this species [13]. It is also unlikely to be a glycyl radical as the glycyl radical of pyruvate formate lyase is unstable to molecular oxygen [15] and, in the present case, the free radical is formed in the presence of oxygen. Of the known free radical enzymes, the free radical in xylem extracts most closely resembles that of *E.coli* amine oxidase [16] which utilises a radical derived from TOPA quinone. Enzymes with TOPA quinone derived radicals are inactivated by phenylhydrazine derivatives via the formation of an irreversible TOPA-Schiff base intermediate [17]. In contrast, the oxidase activity of xylem extracts is only partially inhibited by phenylhydrazine or *p*-nitrophenylhydrazine (Table 2) and oxidase activity against CA and ABTS is completely restored if these reagents are removed by desalting (results not shown).

Diamine oxidase (DAO), which utilises a TOPA quinone derived radical, has been identified as a cell wall associated glycoprotein in lentil and pea [18] and has been mooted as a possible source of hydrogen peroxide in cell walls [19]. If DAO were present in the xylem extracts, one can construct a scheme whereby DAO generates hydrogen peroxide (by oxidation of a suitable substrate) which is then utilised by peroxidases to oxidise CA or ABTS. However, the pres-

ence of a suitable substrate for DAO in our extracts is unlikely and one would expect phenylhydrazine derivatives to inactivate DAO. In addition, we have found that the pre-incubation of xylem extracts with 1–5 mM putrescine, cadaverine or spermidine does not enhance or reduce the rate of oxidation of ABTS (results not shown) which, in combination with the lack of inhibition by catalase, suggests that DAO generation of hydrogen peroxide is not involved. In summary, the nature of the free radical associated with the coniferyl alcohol oxidase activity of developing xylem extracts remains to be defined.

METHODS AND MATERIALS

Plant material

Branches of Sitka spruce [*Picea sitchensis* (Bong) Carr] were obtained from the Forest Research clone bank at Ledmore, Perth and Kinross, UK. Individual healthy specimens of Leyland cypress [X *Cupressocyparis leylandii* (A.B. Jacks & Dallim.)] and Lawson cypress [*Chamaecyparis lawsoniana* (A. Murr.) Parl.] were obtained from local gardens and were sampled in early to mid May 1997 when new xylem development was in progress. Branches of Wych elm [*Ulmus glabra* (Huds.)] were obtained from a specimen in the grounds of our Institute in late May 1997. The branches and trunks were cut into manageable lengths and removed to the laboratory where the bark was peeled from the wood. New developing xylem was then scraped from the old wood using razor blades and stored frozen at −20°.

Extraction procedure

When required, xylem tissue (~ up to 400 g per extraction) was defrosted then extracted by an adaptation of the method outlined previously (see [2, 4]). In brief, the tissue was placed in a 5-fold excess of homogenisation buffer [25 mM MOPS pH 7 containing 0.5% v/v polyvinylpyrrolidine (PVP) and 0.5 mM phenylmethyl sulphonyl fluoride (PMSF)] and homogenised in a Waring blender, then using an Ultra-Turrax disintegrator as before [2]. After filtration through double-layered muslin, the insoluble residue was re-homogenised in fresh ice-cold buffer which lacked PVP or PMSF. This procedure was repeated 3 times. MOPS was preferred to Tris because MOPS (pKa = 7.2) has a better buffering capacity at pH 7.0 and Tris has been reported to sequester and complex copper from enzymes [20].

The insoluble residue (crude cell walls) was then extracted with 25 mM MOPS pH 7.0 containing 200 mM $CaCl_2$ (ConA buffer) for 1 hr on ice with stirring. The slurry was filtered through muslin and the ConA extract clarified by filtration though glass fibre filters (Whatman GF/A).

Procedure for chromatography on Concanavalin-A Sepharose

Small (~5–7 ml bed vol.) columns of Concanavalin-A Sepharose were pre-equilibrated in ice-cold ConA buffer containing 1 mM $MnCl_2$ to load the lectin with Mn^{2+} ions as directed by the supplier (Pharmacia) then washed in ConA buffer at 1 ml/min. The ConA extract was applied to the Concanavalin-A column at 1 ml/min and the eluate discarded. After a wash with a 5-fold column vol. of ConA buffer, the bound fraction, which is enriched in cell-wall-associated glycoproteins, was eluted using ConA buffer containing 100 mM α-methyl mannoside. No oxidase activity was present in the load eluate or the ConA washes, although considerable peroxidase activity was lost. This bound fraction was desalted into deionised H_2O by passage through PD-10 desalting columns (Pharmacia) and conc. as required using centrifugal membrane units with theoretical Mm cut-offs of 50 k (Centriplus-50 units, Amicon) which retained the oxidase activity.

Assays for oxidase activity, coniferyl alcohol oxidase activity, protein content and response to effectors

Oxidase activity was measured by monitoring the initial rate of increase in A_{420} due to the formation of the oxidised chromophore of 2, 2'-azinobis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) as described previously [2]. Coniferyl alcohol oxidase activity was measured by a continuous spectrophotometric assay following the initial rate of decline in A_{260} due to coniferyl alcohol (CA). CA was present at 0.1 mM in 100 mM NaOAc pH 5 and the basal rate of CA oxidation without enzyme was measured as control. The extinction coefficient for CA was estimated for each assay but was around $1.8 \times 10^4 M^{-1} cm^{-1}$. Protein content was measured by the dye-binding method [21].

Effector studies were carried out using the ConA bound fraction as enzyme source. Effectors were added to the assay mixture, the reaction started by the addition of enzyme and the increase in A_{420} over a fixed time. The assay contained a final conc. of 1.73 mM ABTS. Suitable controls were constructed. Catalase (Sigma product no C-100) and superoxide dismutase (Sigma product no. S-2515) were made up in H_2O then desalted into H_2O prior to use, by passage through PD-10 columns. Catalase activity was checked by monitoring O_2 evolution from H_2O_2 with an O_2 electrode.

Electron paramagnetic resonance spectroscopy

Spectra were obtained with a Bruker ESP300E spectrometer operating at X-band frequencies (~9.4 GHz). Samples (about 120 μl) were frozen in liquid N_2 and transferred to a quartz finger Dewar containing liquid N_2. Full scan spectra (1000 G sweep

width) were acquired with 2.5 mW microwave power, 10 G modulation amplitude and 100 kHz modulation frequency. Spectra were also obtained for all samples of the free radical alone, during EPR titrations, and were obtained at 100 μW microwave power. At 77 K, the onset of saturation of the free radical signal was approximately 70 μW. Liquid phase EPR spectra of the POBN adducts were obtained from a 180 μl sample in a quartz flat cell (path length $\sim$1 mm) at ambient temp. (20°) with microwave power 10 mW and modulation amplitude, 1 G.

Acknowledgements—We thank Dr Steve Lee of Forest Research for access to the Sitka spruce clonebank, Mr Ken Taylor for Lawson cypress trees and Mr Les Bisset, Curator of the Botanic Gardens, University of Dundee for help with classification of the tree species. We also thank Andrew Richardson and Julie Duncan for their hard work and Dr Ian Morrison and Dr Bernard Goodman for their advice. We acknowledge funding from The Scottish Office Agriculture, Environment and Fisheries Department.

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