Involvement of peroxidases in the formation of the brown coloration of heartwood in *Juglans nigra*

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Received 9 April 2001; Accepted 28 September 2001

Abstract

Oxidase activities were investigated within the cross-section of walnut trunk in relation to the brown staining of heartwood, especially in the transition zone where the colour change occurs. The distribution of peroxidase activity was investigated using 3,3'-diaminobenzidine (DAB) or guaiacol as a substrate. Generally, the highest activity was found in the cambial zone and in the middle sapwood. This activity was mainly vacuolar. However, during autumn a peak of activity was observed in the transition zone with DAB, but not with guaiacol. Immunohistolocalization of the peroxidase revealed that the protein was present in the transition zone even if the enzymatic activity was not detectable. Flavan-3-ols were abundantly localized in the transition zone and it is hypothesized that they are physiological substrates of peroxidases. By contrast, polyphenoloxidases do not seem to be implicated in heartwood formation.

Key words: Heartwood, peroxidase, walnut.

Introduction

Wood colour is one of the most important factors of wood quality. Cross-sections of the trunk of mature trees usually present two different zones, a pale-coloured outer one: the sapwood, and a dark-coloured inner zone, the heartwood. The pale-coloured sapwood contains storage material (starch and lipids) while the dark colour of the heartwood relies on the presence of various organic substances named extractives. The nature of the extractives gives the specific colour of the heartwood. The heartwood is of greater value than the sapwood for many purposes owing to its greater durability or richer colour. In black walnut which is a very valuable wood most of the extractives are phenolics (Label et al., 2000).

At the boundary between sapwood and heartwood, storage material disappears and newly formed phenolics are accumulated (Burtin et al., 1998).

The brown colour of heartwood is primarily related to the oxidation of phenolic compounds. Browning reactions are generally catalysed by polyphenoloxidases (PPO) (Lee and Whitaker, 1995) or peroxidases (PO) (Higuchi, 1997) and result in the formation of quinones which subsequently polymerize to varying degrees, leading to brown pigments (Rouet-Mayer et al., 1990). Oxidation proceeds via H₂O₂ or other peroxides for PO whereas PPO use molecular oxygen. Nevertheless, this difference is not completely clear cut, as some PO may oxidize phenolics or other substrates with O₂ (Gaspar et al., 1982). In addition, non-enzymatic oxidation has also been shown to occur in several species (Croux, 1989).

The activity of phenol oxidative enzymes has been studied previously, either biochemically or cytologically, on different woody species, i.e. *Juglans* (Nelson, 1977); *Acacia* (Shah et al., 1981); Cryptomeria (Nobuchi et al., 1982), and Quercus (Eberman and Stich, 1985). Nevertheless, a more precise study of the involvement of these enzymes in wood browning is essential for a better understanding of heartwood formation. Within the framework of an important European project on walnut wood quality, the aim of this work was to characterize the radial distribution and seasonal variations of phenol oxidases in a *Juglans nigra* trunk and to localize the corresponding proteins in situ by immunohistochemistry, and to correlate them with the presence of their potential phenolic substrates, especially in the transition zone between sapwood and heartwood.

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Materials and methods

Seventeen-year-old trees of *Juglans nigra* were selected in an INRA station in the south of France (Bormes les Mimosas). Every 2 months, samples of a wood core were collected with a borer (15 mm diameter) at 1.5 m above ground level from trees as similar as possible. Fresh tissues were immediately used for histochemistry. Wood fragments were cut into 25 μm sections on a sliding microtome. Photographs have been chosen, which were taken during two characteristic seasons, summer and autumn.

Polyphenoloxidase histolocalization

Polyphenoloxidase (PPO) activity was revealed using 25 mM DL-DOPA (DL-3,4-dihydroxyphenylalanine) as a substrate, in phosphate–citrate 0.1 M buffer pH 4.6. PPO activity, when present, was visualized as brown–dark staining. Negative controls were previously incubated in 10 mM tropolone, a specific inhibitor for PPO activity.

Results

Definition of the transition zone

Transverse sections had 9–10 growth rings of sapwood and about 7 rings of heartwood detected visually by its darker colour due to a greater amount of extractives (Fig. 1A). A visible transition zone, between these two major zones, is not a constant feature in walnut (Nelson, 1977). Nevertheless, a narrow zone (about 2 rings) was generally localized under UV light by its autofluorescence (EA Magel, personal communication) although this fluorescence was not visible under UV microscopy on 25 μm sections. The heartwood/sapwood boundary was visualized under the light microscope by brown granulations present in the cells of the first ring of heartwood (Fig. 1B). Therefore, on a cross-section of wood, growth rings were visualized from the sapwood/heartwood boundary (LC0). (Fig. 4)

Histology and histochemistry of walnut wood

Both sapwood and heartwood presented different types of cells, i.e. vessels, fibres and living parenchyma cells (uniseriate or multiseriate rays and axial cells).

The living cells of the xylem contained numerous starch grains stored equally in ray and axial parenchyma cells (Fig. 1C, arrows), that stained intensely with iodine (Sakar and Howarth, 1976). Vanillin reacts strongly with both flavan-3-ol monomers and polymers.

Peroxidase immunolocalization

Fresh trunkwood cross-sections (25 μm) of trees harvested in summer were incubated for 3 h in a TBS buffer (25 mM TRIS-HCl pH 7.4; 150 mM NaCl; 3 mM KCl) supplemented with 0.2% (w/v) Tween 20 and 5% (w/v) milk proteins. Wood sections undergo four washings of 10 min each in TBS buffer before immunostaining.

The immunoreaction occurred overnight at 4 °C in TBS buffer supplemented with 5% (w/v) milk proteins and with 1:500 diluted anti-peroxidase antibody (anti-rabbit IgG antibody developed in goat; Sigma Aldrich) labelled with fluorescein isothiocyanate (FITC). Washing in TBS was followed by mounting on microscope slides with glycerol–gelatin and observation under fluorescent microscope using an excitation wavelength of 495 nm.

The first control was performed by following the whole process, but omitting the first antibody. A second type of control followed the normal process using an anti-peroxidase antibody saturated with its specific antigen.

Fresh trunkwood cross-sections (25 μm) of trees harvested in summer were incubated for 2 h at room temperature in TBS containing an 1:200 diluted anti-rabbit antibody (anti-rabbit IgG antibody developed in goat; Sigma Aldrich) labelled with fluorescein isothiocyanate (FITC). Washing in TBS was followed by mounting on microscope slides with glycerol–gelatin and observation under fluorescent microscope using an excitation wavelength of 495 nm.

This antibody towards wood peroxidases was confirmed by immunoblotting.

After washing in TBS buffer, sections were incubated for 2 h at room temperature in TBS containing an 1:200 diluted anti-rabbit antibody (anti-rabbit IgG antibody developed in goat; Sigma Aldrich) labelled with fluorescein isothiocyanate (FITC). Washing in TBS was followed by mounting on microscope slides with glycerol–gelatin and observation under fluorescent microscope using an excitation wavelength of 495 nm.

The first control was performed by following the whole process, but omitting the first antibody. A second type of control followed the normal process using an anti-peroxidase antibody saturated with its specific antigen.
Flavans, shown red with HCl–vanillin reagent, were always observed in ray cells where peroxidase activity and starch grains had almost completely disappeared (Figs 2B, 1E, 4). Note that flavans or starch grains or peroxidase activity appeared to be present in the same cells, but at different development stages. In the innermost cells of sapwood, the red coloration was slight and homogeneous, revealing vacuoles filled with flavans, whereas there was only a heterogeneous, but intense, pink-brown coloration of granulations in the first cells of the heartwood, easily observable in radial sections (Fig. 2C).

Detection of oxidase activities
Polyphenoloxidase activity was revealed in differentiating xylem close to the cambium, but it was never detected in the inner zone of the trunk.
Peroxidase activity was revealed, as for PPO activity, in differentiating xylem, but also along the cross-section of the trunk to the inner sapwood (Fig. 4). This activity was revealed routinely either with guaiacol or DAB, but the two substrates did not reveal the enzyme activity equally. Figures 2D and E show cross-sections of the outer sapwood stained with guaiacol (D) and DAB (E). Staining with DAB was generally more intense than with guaiacol. The coloration was predominantly localized inside the ray parenchyma cells, but DAB also revealed peroxidase activity in axial parenchyma cells (arrows), although it was not so intense. Generally, a higher activity was observed in the latewood rather than in the earlywood of each annual growth ring. Multiseriate rays were more often coloured than uniseriate rays. Furthermore, in multiseriate rays, PO activity was also very unequally distributed: some cells were strongly coloured whereas others did not show any activity. This
unequal distribution is better shown on radial sections (Fig. 2F). Staining with DAB was localized both inside the vacuole and more intensely at the periphery of cells, i.e. in cytoplasm and pits (Fig. 3A, arrow). Both KCN (an inhibitor of haem-containing enzymes) and heat-treated samples showed complete loss of PO activity (Fig. 3B). Aminotriazole (a potent inhibitor of catalase) did not affect staining intensity.

Generally, there was a steady decrease in PO activity from the cambium to the heartwood boundary where it completely disappeared (Fig. 4). As shown in Fig. 3C and D, respectively stained with guaiacol (C) and with DAB (D), peroxidase activity in the transition zone was only observed in some isolated ray cells. A very clear relationship was observed between the decrease of PO activity in living cells of ray parenchyma and their starch content (Fig. 4).

However, during autumn (September and October) a peak of PO activity (visualized with DAB but not with guaiacol) was observed in the transition zone (Fig. 3E). PO activity was mainly localized in the tyloses of the transition zone which appeared dark with DAB (Fig. 3E) whereas they were not coloured with guaiacol (data not shown). In the middle sapwood where tyloses were not present, contact parenchyma cells presented a positive coloration with DAB, but not with guaiacol (Fig. 3F).

Furthermore, in addition to the PO activity located inside the cells, an activity was also revealed in the xylem cell walls of the youngest growth ring, near the cambium.

**Immunohistolocalization**

Immunolocalization showed the abundance of PO protein all over the section, mainly in tyloses of the xylem vessels of the transition zone (Fig. 3G), in ray parenchyma cells of sapwood and transition zone, in parenchyma cells associated with the vessels, and in axial parenchyma. Controls (1–2) did not show any fluorescence (Fig. 3H).

**Discussion**

The aim of this work was to study heartwood coloration in relation to histolocalization of enzyme activities known to be implicated in browning processes. *Juglans nigra* belongs to semi-ring porous tree species and heartwood is normally initiated after 10–15 years of tree’s growth. PPO activity was not detected in the innermost sapwood, whereas it was present in the outer periphery of sapwood which may suggest its implication in lignification as it has been shown for different laccases (Ranocha *et al.*, 1998).

PO activity was easily detected with two substrates, i.e. guaiacol and DAB. Using guaiacol, the activity was very high in the youngest annual rings in the trunk, then it regularly decreased right up to the internal rings of sapwood. Ota *et al.* previously showed that PO activity (using guaiacol as a substrate) was the highest in the outermost xylem of kiri wood and negligible in other parts (Ota *et al.*, 1991). The cytochemical results obtained with KCN and aminotriazole as inhibitors indicated that peroxidase and not catalase contributed to the oxidation of the substrate.

As for PPO, the high PO activity found in the cell walls of the cambial zone probably indicates the importance of these enzymes in the lignification process. This localization pattern may also explain the peroxidase-mediated cross-linking of different polymers of the wall (Gaspar *et al.*, 1991).

Latewood cells always presented a higher PO activity than earlywood cells. For other plants, in Robinia for example, two key enzymes of flavonoid biosynthesis show a higher activity in earlywood and the authors concluded that heartwood formation begins in earlywood (Magel *et al.*, 1991). In *Juglans nigra* it must be postulated that heartwood formation begins in latewood.

In sapwood parenchyma, PO activity was revealed inside the cells where its localization is likely vacuolar as is well known in many cases (Welinder, 1992; Mäder, 1992). However, some activity may also be associated with the secretory pathway (endoplasmic reticulum, Golgi apparatus) or on the plasmalemma, as suggested by the strong activity found around and near the pits. Previously Patel and Bhat noticed that ‘pits showed higher activity as compared to the other parts of the cell’ (Patel and Bhat, 1981). However, this localization needs to be confirmed on the ultrastructural level.

An increase in PO activity as indicated by DAB coloration was observed in the transition zone during September and October. A high PO activity during the dormant period, generally based on biochemical assays, has previously been noticed in the transition zone of several woody species: *Juglans* (Nelson, 1977), *Picea* (Estebauer *et al.*, 1978), *Quercus* (Eberman and Stich, 1982), *Paulownia* (Ota *et al.*, 1991), and *Larix* (Korori *et al.*, 1998). The higher peroxidase activity observed in the transition zone supports the view of several authors that this zone is in general characterized by an enhanced physiological activity. For instance, Shah *et al.* presented results on various enzymatic histochemical localization: activities of adenosine triphosphatase, lipase, acid phosphatase, succinate dehydrogenase, and peroxidase were shown to increase in the transition zone compared with the outer and medium sapwood (Shah *et al.*, 1981). An increase in the activity of specific enzymes of phenolic metabolism (phenylammonialyase and chalcone synthase) has been observed in the transition zone compared with the outer sapwood (Magel *et al.*, 1991; Magel and Hübner, 1997).
An important PO activity was also observed in the tyloses of the transition zone, this activity probably contributed to the browning of the heartwood tissues. In xylem vessels which had no tylosis, xylem parenchyma cells around them showed an intense colouring with DAB. This proved to be intense peroxidase activity in cells which will give further tylosis.

Considering this cluster of observations it must be said that the transition zone is a zone of enhanced enzymatic activity (of which peroxidase is an important compound due to its key role in the browning), which is subject to seasonal variations. These enhanced activities together with cytological modifications are considered by some authors (Frey Wyssling and Bossard, 1959) as proof of altered metabolism, and by others as an increase of metabolism (Shah et al., 1981). Immunolocalization results proved that PO proteins were present in the cells of the transition zone even in summer when activity was not detected by staining with DAB. They were active only during the dormant season, and this probably corresponded to a reactivation of latent forms of PO or to the presence of another isoenzyme with different substrate specificity. Furthermore, the absence of staining with guaiacol furnished evidence for specific activity or greater affinity towards DAB.

Not only are enzyme activities needed for the biosynthesis of heartwood substances, but also the substrates (storage materials). Storage materials, mainly starch in Juglans, could provide, following degradation, primary metabolites needed for extractive formation. Numerous biochemical studies revealed that, during sapwood/heartwood transformation, storage material is consumed (Shah et al., 1981; Nobuchi et al., 1982). The carbon skeletons obtained might be used by the cells to produce phenolics within the heartwood (Magel et al., 1991, 1994). In Juglans PO activity and starch were both located in the same ray parenchyma cells. The starch content decreased gradually from the outer to the inner sapwood. The disappearance of storage material was concomitant with the appearance of small vesicles of soluble phenolics in the same parenchyma cells. These polyphenols then polymerized in insoluble forms in heartwood. A similar phenomenon has been observed for a long time in wood and other plant materials where the disappearance of starch from plastids has been correlated with the vacuolar accumulation of phenolics (Beckman et al., 1972; Mueller and Beckman, 1976; Hillis, 1987; Gutmann, 1993). The results with Juglans nigra localize these observations.

Among walnut wood phenolics, special attention was given to condensed tannins which show red in the transition zone with the HCl–vanillin reagent. They derive from the condensation of flavanols (Gutmann and Feucht, 1991) and may form strong complexes with carbohydrates and proteins leading to irreversible protein damage (Everdeen et al., 1988). This might explain the peroxidase inactivity (without eliminating antibody binding) and, consequently, the contradiction between immunofluorescence and enzyme activity results. These complexes probably correspond to the brown-red granulations shown in the outer rings of heartwood using the vanillin reaction. In the inner part of the heartwood, complexation is more important and there was no longer any reaction with vanillin.

**Fig. 4.** Graphic representation showing the radial distribution patterns of starch (A), peroxidase activity (B), and flavans (C) in the trunk cross-section, from the bark to the pith. TZ, Transition zone; LC0, sapwood/heartwood boundary.
Whatever the substrate utilized, PO activity was not homogeneous in the different cells of a ray which proves that all the ray cells are not functionally identical. This had been previously pointed out by different authors: for instance Chattaway observed that ‘there is a functional heterogeneity not only between the different files of cells that compose a ray, but even within a single file of cells’ (Chattaway, 1951) and Crévecoeur et al. noted a heterogeneous localization of peroxidase from cell to cell in the shoot apical meristem of Spinacia (Crévecoeur et al., 1997). In the case of Juglans nigra, this heterogeneity was also found for flavans revealed by vanillin-HCl. According to the literature, phenolics are produced within the cytoplasm and cluster together forming droplets in the vacuoles, which later develop into a single large vacuole full of phenolics (Higuchi, 1997). During the process of duraminization leading to heartwood, cytoplasm and organelles of living ray cells degenerate and there is finally a release of vacuolar content associated with the death of cells (Iwanowska et al., 1994). It has been shown previously that peroxidases involved in heartwood formation, and their substrates, are located in two different types of ray parenchyma cells (Dehon et al., 2001). The browning process which characterizes heartwood formation implies a cell to cell decompartmentation or an intercellular transport to allow enzymatic oxidation of phenolic substrates in the transition zone. It is not inconceivable that endo/exocytotic events play an important role in the radial trafficking of these compounds. Sauter speculated on a mechanism for symplastic transfer by which solute vesicles are transferred from cell to cell in the tangential pit areas of the ray cells (the intense coloration of the pits in Juglans may strengthen this hypothesis) (Sauter, 1982).

The high PO activity observed in the autumn in the transition zone and the high concentration of flavans (and certainly other phenolics as juglone derivatives which are abundant in Juglans; Burtin et al., 1998) simultaneously with the disappearance of storage material, are the main parameters which explain the appearance of brown pigments during heartwood formation.

Acknowledgements

This work was developed within the framework: FAIR CT96-1887 Walnut-BRAINS research programme. L. Dehon is grateful to the European Community for a research fellowship. We gratefully acknowledge the expert technical assistance of C. Chalies.

References


