Cellular Localisation of Polyphenol Oxidase and Peroxidase Activity in *Litchi chinensis* Sonn. Pericarp

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Abstract. Cellular localisation of visual browning and oxidative activity studies were conducted to determine the relative significance of polyphenol oxidase (PPO) and peroxidase (POD) activities during pericarp browning. Pericarp browning was first observed on the protuberance apices and subsequently extended uniformly over the entire pericarp surface. Anatomically, browning was highly localised and restricted to the epicarp and the upper mesocarp. PPO and POD activities were highest in the epicarp, with progressively less activity in both the mesocarp and endocarp. *In situ* localisation of oxidative activity using tissue blots confirmed high epicarp PPO activity. POD activity, although primarily restricted to mesocarp vascular tissue, was also detected in the epicarp. We believe that litchi pericarp browning is due to highly localised oxidative activity in the epicarp and upper mesocarp. As PPO and POD activities were significantly higher in this tissue and browning was not observed when both enzymes were selectively inhibited, it is postulated that both PPO and POD activities are associated with litchi pericarp browning. The current theory that litchi pericarp browning is only caused by PPO activity needs to be re-appraised to determine the relative role of POD activity.

Introduction

Pericarp browning is the most important commercial problem associated with the marketing of fresh litchi fruit (Snowdon 1990). Where browning has been studied in other species, it has been attributed to polyphenol oxidase activity (PPO)*, also referred to as catechol oxidase, tyrosinase or *o*-diphenol oxygen oxidoreductase (Mayer and Harel 1979). Although there is widespread acceptance of the role of PPO in litchi pericarp browning (Tan and Li 1984; Joubert 1986), the evidence is not conclusive. Using immature fruitlets Joubert and Van Lelyveld (1975) demonstrated increased PPO activity in the necrotic pericarp tissue. In conjunction with earlier work by Akamine (1960), these results may have contributed to the acceptance of PPO's involvement in litchi pericarp browning. Where PPO activity has been determined during injury development, results are inconsistent. Lin *et al.* (1988a) demonstrated a rapid increase in PPO activity during the first 48 h of storage. Under similar conditions Zauberman *et al.* (1991) did not observe any significant change in PPO activity during the same period. However, they did demonstrate that inhibition of PPO activity using sulfite application could control litchi pericarp browning.

Although litchi browning has been widely attributed to PPO activity, other oxidative enzymes such as peroxidase (POD) have also been assayed from litchi pericarp (Joubert 1986). Lin *et al.* (1988b) and Huang *et al.* (1990) both demonstrated increased POD activity during fruit storage, concurrent with tissue browning. While the significance of POD activity has been well documented in other species such as peaches (Flurkey and Jen 1978), mangoes (Zauberman *et al.* 1988) and walnuts (Piffaut and Metche 1991), its relative importance in litchi pericarp browning has been overlooked. As PPO and POD activity cause similar tissue browning, it is possible that both enzymes are involved in pericarp browning.

Differentiation of PPO and POD activity during litchi pericarp browning has not been clearly established. PPO and POD oxidise similar phenolic substrates (Kahn 1985), and it is relatively difficult to clearly differentiate their functional roles. PPO causes hydroxylation of the monohydroxyphenols to *o*-dihydroxyphenols, and dehydrogenation of the *o*-dihydroxyphenols to *o*-quinones (Mayer and Harel 1979). Browning results from the non-enzymatic polymerisation of the *o*-quinones to melanins. POD activity in the presence of hydrogen peroxide will also act on *o*-dihydroxyphenols

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*Abbreviations used: PPO, polyphenol oxidase; POD, peroxidase; DIECA, diethyldithiocarbamate; PMSF, phenylmethylsulfonylfluoride; L-DOPA, L-3,4-dihydroxyphenylalanine; PVP-40, polyvinylpyrrolidone (soluble); tropolone, 2,4,6-cycloheptatrienone.

causing a similar discoloration (Kahn 1985). Where differentiation of POD activity has been determined in litchi pericarp, assays were performed in the presence of both hydrogen peroxide and guaiacol, and PPO inhibitors such as DIECA. Although this approach has been widely reported, it is far from ideal. Kahn (1985) indicated that DIECA will non-selectively inhibit POD, while the addition of hydrogen peroxide, considered necessary for POD activity, may also shorten the lag phase associated with PPO activity (Kahn 1983). The relationship between oxidative enzymes and their phenolic substrate, and differentiation of their functional role has lead to considerable confusion in the literature. The current theory that litchi pericarp browning occurs as a result of PPO activity may therefore be incorrect and should be re-evaluated to determine the relative role of POD.

Materials and Methods

Plant Material

Mature red litchi fruit (*Litchi chinensis* Sonn. cvv. Tai So and Kwai May Pink) were obtained from the Maroochy Horticultural Research Station, south-east Queensland (Lat 27°S). Fruit were selectively harvested to ensure uniform maturity and size, and transported to the laboratory within 2 h.

The Localisation of Visual Browning

Mature red fruit were held under desiccating conditions (24°C, RH 60%) for 4 days to induce pericarp browning (Scott *et al.* 1982). The distribution of tissue browning was determined with an Olympus BH-2 light microscope. Tissue was hand sectioned, and mounted unstained in 30% glycerol.

Localisation of pericarp surface browning proved difficult due to the presence of anthocyanins which impeded injury visualisation. To overcome this problem, fruit were treated with sulfite (1% gaseous sulfur dioxide for 15 min) to decolourise the anthocyanins and inhibit latent PPO activity (Zauberman *et al.* 1991). Anthocyanin decolourisation due to the formation of a colourless anthocyanin-SO_3H complex has been previously reported in other species (Jurd 1964; Timberlake and Bridle 1968) and is a useful technique for visualising injury.

Polyphenol Oxidase Activity

PPO and POD activities were determined for each of the three pericarp tissue layers (epicarp, mesocarp and endocarp, as defined by Joubert 1986), prior to storage. Tissue (3 g) was ground in liquid nitrogen, and solubilised in 20 mM sodium phosphate buffer, pH 6.8, containing 1% PVP-40 (w/v) and 1% Triton X-100 (v/v) at 4°C for 1 h. The slurry was centrifuged at 13 000 *g* for 30 min at 4°C. The supernatant was concentrated (×10) in a Centricon-30 Microconcentrator at 4°C and used as the crude extract.

PPO activity was assayed polarographically using a Clark O_2 electrode (Rank Brothers, Bottisham, UK), calibrated daily with sodium dithionite. Activity was measured using a 0.05 mL aliquot of the extract in 2.60 mL 50 mM sodium phosphate buffer, pH 6.8. The solution was allowed to equilibrate at 28°C for 4 min. The reaction was initiated with 0.35 mL of 40 mM 4-methylcatechol in a phosphate buffer. Activity was determined based on the initial linear phase of oxygen consumption during the first 3 min of reaction. Protein concentration was determined using a DC BioRad protein assay, and specific activity expressed as a function of total protein content.

Peroxidase Activity

Pericarp tissue (3 g) was ground in liquid nitrogen, and solubilised in 47 mM sodium phosphate buffer, pH 6.5, containing 1% PVP-40 (w/v) at 1°C for 1 h. The slurry was centrifuged at 11 000 *g* for 30 min at 1°C, filtered and the supernatant used as the crude extract. POD activity was assayed spectrophotometrically based on a modified method developed by Kahn and Andrawis (1985*b*). Kahn (1985) and Kahn and Andrawis (1985*a*) demonstrated that tropolone plus hydrogen peroxide was both POD substrate and a highly effective PPO inhibitor, and could therefore improve POD and PPO differentiation. Therefore, guaiacol was replaced with tropolone plus hydrogen peroxide as the reaction substrate. A 0.4 mL aliquot of the extract was added to 2.6 mL of 47 mM sodium phosphate buffer, pH 6.5, containing 12 mM tropolone plus 3.3 mM hydrogen peroxide. The change in absorbance at 418 nm was recorded, with activity expressed as ΔOD_{418} min^{-1} mg^{-1} protein. Calculations were based on the linear portion of the curve during the first 60 s of the reaction.

Tissue Localisation of PPO and POD Activity

Tissue localisation of oxidative activity was determined using a method developed by Spruce *et al.* (1987) and modified according to Kahn (1985). Pericarp tissue was washed in distilled H_2O, thinly sliced, then lightly pressed against a nitrocellulose membrane (0.45 μm) pre-soaked for 5 min in either: 20 mM L-DOPA plus 420 units of catalase (PPO activity); 20 mM L-DOPA, 12 mM tropolone plus 60 mM hydrogen peroxide (POD activity); or 20 mM L-DOPA, 20 mM DIECA plus 420 units of catalase (PPO and POD inhibition). Control blots used sodium phosphate buffer, pH 6.5. Following staining, the membranes were washed in distilled H_2O and examined microscopically to determine the distribution of oxidative activity.

Results and Discussion

Pericarp browning was first observed on the protuberance apices (Fig. 1). Surface browning, however, was initially masked by anthocyanin colour and could only be visualised by decolorising the anthocyanins using sulfites. Browning subsequently extended uniformly over the entire pericarp surface. Anatomic browning was highly localised and initially restricted to the upper epidermis (Fig. 2*A* and *B*). With further desiccation, browning extended into the underlying parenchymatous tissue via collenchyma intrusions into the sclerenchyma layer, to incorporate the entire epicarp and a portion of the upper mesocarp (Fig. 2*C*). While browning occurred uniformly throughout the epicarp, injury was initially more pronounced in the lenticels (Fig. 3*A*), stomata (Fig. 3*B*), and collenchyma (Fig. 2*C*). The distribution of browning in the outer pericarp tissue and in cells associated with water vapour and gas exchange further supports a causal relationship between pericarp browning and desiccation proposed by Scott *et al.* (1982).
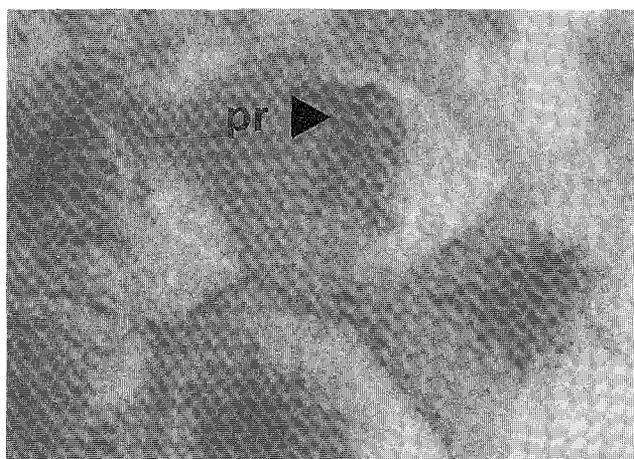
Fig. 1. Litchi pericarp surface (cv. Tai So) illustrating protuberance browning. To visualise injury, the anthocyanins have been decolourised using sulfite application to form a colourless anthocyanin-SO$_3$H complex. Prior to treatment there was no visible evidence of browning. Protuberance apex (**pr**). Magnification: ×20.

PPO and POD activity were significantly higher in the epicarp compared with the mesocarp or the endocarp (Table 1). Mesocarp and endocarp specific PPO activity were not significantly different (0.35 and 0.36 µmol O$_2$ min^{-1} mg^{-1} protein, respectively), while mesocarp POD activity was slightly higher than that in the endocarp (0.016 and 0.012 ΔOD$_{418}$ min^{-1} mg^{-1} protein, respectively).

Although total activity of tissue homogenates is a useful index of oxidative potential, it does not necessarily reflect *in vivo* enzymatic activity. Solubility, particularly of PPO, is an important criterion for enzymatic activity (Mayer and Harel 1979). PPO usually exists in a particulate latent form tightly bound to the membrane (Vaughn and Duke 1988), and it is only once the enzyme has solubilised that activity occurs. In the absence of solubilising agents such as detergents, it is usually difficult to assay PPO activity (Trémolières and Bieth 1984).

To improve our understanding of the distribution of PPO and POD activity in the pericarp, tissue localisation using tissue blots was investigated (Fig. 4). Significant PPO activity was detected in the epicarp and upper mesocarp tissue and minor activity in the vascular tissue and the endocarp (Fig. 4A). Heavy staining on the protuberance apex correlated with localised pericarp surface browning (Fig. 1). Although this may confirm a distributional correlation between protuberance browning and high PPO activity, similar protuberance staining was also observed in the control (Fig. 4D). Based on additional control blots, protuberance staining (in the control) seems to stem from residual brown pigmentation being transferred to the membrane.
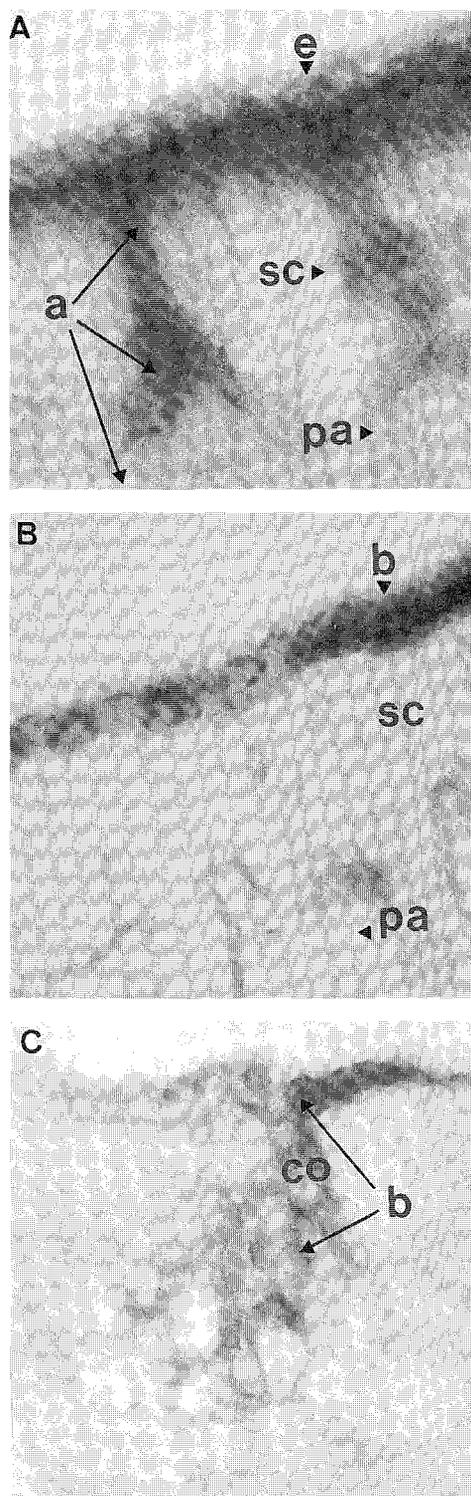
Fig. 2. Light microscope sections of the epicarp (cv. Tai So) of whole fruit kept under desiccating conditions (25°C, 60% RH) to induce browning. Sections represent a time course of injury development. (*A*) Epicarp tissue immediately after harvest (prior to browning); (*B*) localised epidermal browning 12 h after storage; (*C*) browning of the epicarp 72 h after storage. Epidermis (**e**); sclerenchyma (**sc**); parenchyma (**pa**); collenchyma (**co**); browning (**b**); anthocyanin (**a**). Magnification: (*A*) ×600, (*B* and *C*) ×700.
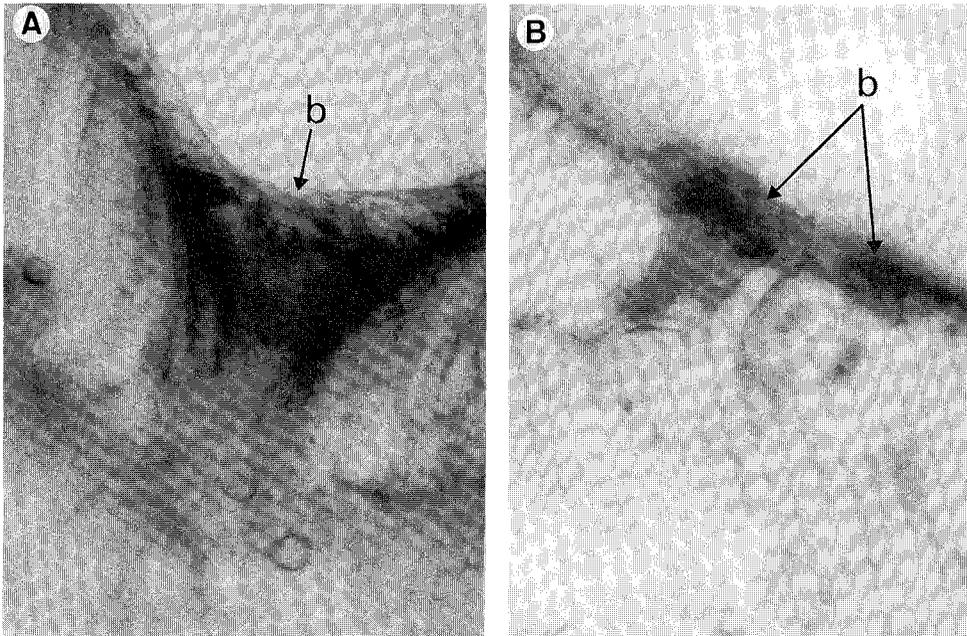
Fig. 3. Light microscope sections of the epicarp (cv. Tai So) illustrating localisation of browning within cells associated with water vapour and gas exchange. (*A*) Single lenticel; (*B*) stoma. Browning (**b**). Magnification: ×800.
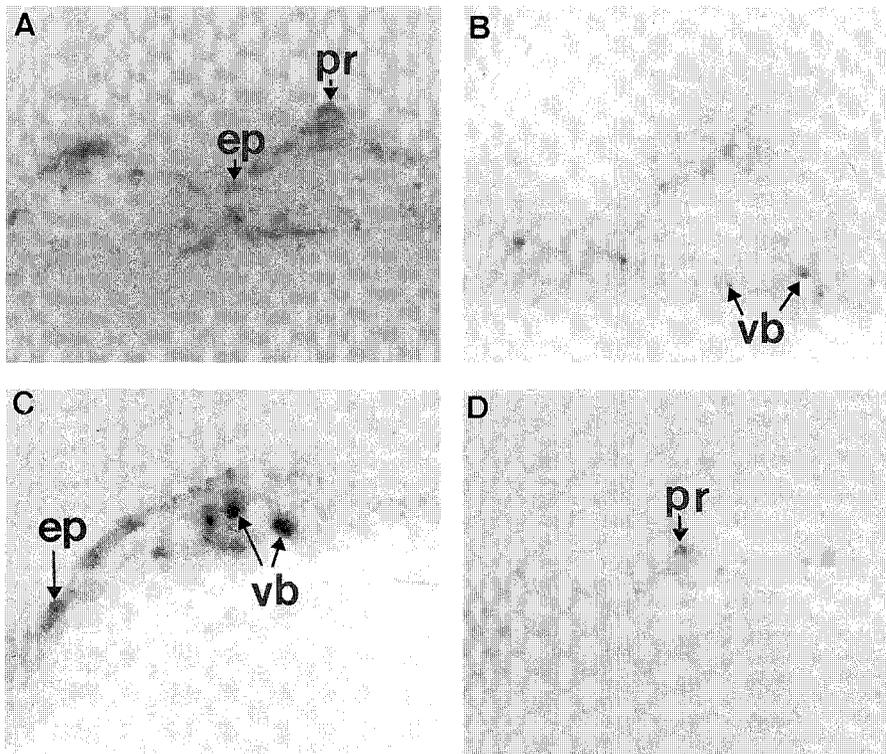
Fig. 4. Tissue localisation of PPO and POD activity (cv. Tai So) using tissue blots of the mature red pericarp. (*A*) PPO activity; (*B*) POD activity; (*C*) POD activity in cv. Kwai May Pink; (*D*) control (PPO and POD selectively inhibited). Vascular bundles (**vb**), protuberance apex (**pr**), epicarp (**ep**). Magnification: ×10.

Table 1. Polyphenol oxidase and peroxidase activity assayed from the epicarp, mesocarp and endocarp tissue of the litchi pericarp immediately after harvest
Values are the means of three independent extractions ± s.e.

Tissue	Specific PPO activity (μmol O_2 min^{-1} mg^{-1} protein)	Specific POD activity (ΔOD_{418} min^{-1} mg^{-1} protein)
Epicarp	0.61 ± 0.06	0.055 ± 0.002
Mesocarp	0.35 ± 0.04	0.016 ± 0.002
Endocarp	0.36 ± 0.04	0.012 ± 0.001

Although little is known of PPO cellular distribution in other tissue, Vaughn and Duke (1984) considered PPO to be a plastidic enzyme primarily associated with the thylakoid membrane. If this is so, PPO localisation may reflect chloroplast distribution. The findings that photosynthetic tissue in the immature pericarp was concentrated in the epicarp and upper mesocarp, where tissue blots showed significant PPO activity; that there was a concomitant reduction of PPO activity and chlorophyll content during ontogeny; and that green immature fruitlets browned more rapidly during anatomical sectioning (Underhill and Critchley 1992), all supported this suggestion.

POD activity occurred primarily in the vascular bundles of the mesocarp and endocarp, and only minor activity was seen in the epicarp (Fig. 4B). POD localisation in other tissue has been closely associated with lignin distribution. Kahn (1985) suggested that tissue lignification could be accurately determined on the basis of histochemical localisation of POD activity. Vascular tissue, particularly xylem and bundle sheath cells together with subepidermal sclerenchyma, is known to be lignified so that heavy POD staining of this tissue would be expected and was indeed found. Localisation of POD activity in other litchi cultivars (cv. Kwai May Pink) also showed significant staining of the vascular tissue, particularly in the upper mesocarp (Fig. 4C). In that cultivar, however, epicarp staining was far more significant than in cv. Tai So (Fig. 4B), indicating possible cultivar variability.

We believe that litchi pericarp browning during ambient postharvest storage of the mature fruit is due to highly localised oxidative activity restricted to the epicarp and upper mesocarp. PPO and POD activities were significantly higher in this tissue with tissue blots confirming this distributional relationship. While further work is needed to confirm the relative significance of POD in litchi pericarp browning, the current focus on PPO as the sole mediator of browning should be re-appraised. It is postulated that both PPO and POD are involved in litchi pericarp browning caused by ambient desiccation.

Much of the current confusion in the literature regarding the importance of PPO may be due to the localised distribution of oxidative activity and inadequate differentiation of PPO and POD activities. The localised distribution of cellular browning has not been previously reported in the literature and consequently PPO activity appears to have been derived from whole pericarp extractions. It is postulated that this led to an inaccurate assessment of the importance of POD activity.

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