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Peroxidase Activities and Isoenzyme Profiles Associated with Development of Avocado (*Persea americana*, M.) Leaves at Different Ontogenetic Stages

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Abstract. Changes in four peroxidase activity fractions (soluble, membrane-bound, as well as ionically and covalently bound) were studied during development of juvenile and adult avocado leaves. Greater differences were found in the soluble fraction with an increase in total activity at the end of the growth phase. In relation to the ontogenetic stages, there were significant variations in the soluble peroxidase activity of both stages, especially in leaves which have already detained their growth, 263 U/g fresh wt in adult leaves vs. 70 U/g fresh wt juvenile leaves. Moreover, the isozyme profile of this fraction revealed the appearance of an anionic band, R_f 0.35, at much earlier stages in juvenile than in adult leaves. Concerning the other three fractions, there were no marked changes in total activity of either membrane-bound or ionically and covalently bound peroxidases. However, in the isoenzyme profiles of the ionically bound fraction of juvenile leaves, three highly cationic bands appeared at much earlier stages than in adult leaves. In avocado, attempts to use leaf peroxidase activity as marker of ontogenetic age must be taken with caution, since great fluctuations related with developmental stages occur in juvenile and adult leaves.

Various biochemical, physiological, and morphological traits are associated with the different ontogenetic stages of plant development (Bonga 1982). During the transition from juvenile to adult tissue, gradual changes occur in these traits (Meier-Dinkel and Kleinschmidt 1990). However, unless morphological features are evident (e.g., disappearance of thorns in *Citrus*), the juvenile and transition periods are difficult to distinguish and plants in the transition phase are considered as juvenile (Zimmerman 1972). Various efforts have been made searching for biochemical markers which could be used as indicators of phase change. Differences in nucleic acids (Monteuuis and Gendraud 1987) and protein content (Bon and Monteuuis 1987) have been found among plants in the juvenile and adult phases. More recently, Bon (1988) has found a membrane-associated polypeptide which appears only in juvenile material of *Sequoiadendron giganteum*, so it could be used as a maturation marker in this species. Peroxidases have also been associated with ontogenetic age. Verschoore-Martouzet (1985), working with *Sequoia sempervirens*, found higher peroxidase levels in juvenile than adult material. Furthermore, when adult material was subjected to rejuvenation treatments, peroxidase levels increased, reaching values similar to those of juvenile material. Peroxidase activity has also been related to growth and development process (Wolter and Gordon 1975, Patra and Mishra 1979). In this investigation, peroxidase activity from juvenile and adult avocado leaves at different developmental stages was measured. Zymograms of the different peroxidase fractions at each stage were also obtained.

Materials and Methods

Plant Material

Avocado (*Persea americana*, M.) leaves from an adult tree and 1-year-old seedlings of the Topa-Topa cultivar were used as experimental material. On a given branch, the first 12 leaves starting from the apex were chosen (spring growth). Leaf number one was always a fully extended leaf, at least 1 cm in length. Another set of leaves (P) from the previous growth season (autumn), and located proximal to the spring growth, was also chosen. These leaves had already completed their growth at the time of sampling. Several parameters—weight, length, and surface area—were measured from each sample, and different growth curves were obtained.

Enzyme Extraction and Assay

Leaf tissue was excised and macerated with 50 mM sodium phosphate buffer at pH 6.0 (tissue:buffer ratio, 1:6 w/v). The homogenate was centrifuged for 20 min at 26,000 *g* and the supernatant used for determination of soluble peroxidase activity. The pellet was extracted three more times with the same buffer and then the pellet was incubated with 1% (w/v) Triton X-100 in the 50 mM sodium phosphate buffer, pH 6.0, for 10 min and centrifuged as above. This was considered the membrane-bound peroxidase fraction. After a new extraction with 1% (w/v) Triton X-100, the residue was repeatedly extracted with the buffer solution at pH 6.0 until peroxidase was no longer detected in the extract (three times). The remaining residue was extracted by incubation for 2 h at 4°C with 1 M KCl in 50 mM sodium phosphate buffer at pH 6.0. The extract was centrifuged as above and dialyzed against the same buffer for 24 h with three changes of buffer. This was considered the ionically bound peroxidase fraction. After three washes, the residue was incubated overnight at room temperature with 0.1 M sodium phosphate buffer solution, pH 5.5, containing peroxidase-free cellulase (500 mg/100 ml Sigma Chemical Co., St. Louis, MO, USA) and pectinase (2500 mg/100 ml Sigma Chemical Co., St. Louis, MO, USA). After incubation, the mixture was centrifuged and dialyzed as described for the ionically bound fraction. This was considered the covalently bound peroxidase fraction.

Enzyme activity was measured by following absorbance at 470 nm after incubating the extracts with 6 mM guaiacol and 6 mM H_2O_2 in 50 mM acetate buffer pH 4.5 at 30°C. A unit represents a one increment increase in absorbance (470 nm) per minute at these assay conditions. Each point of the growth curves is the mean of three extractions with three different measurements/extract.

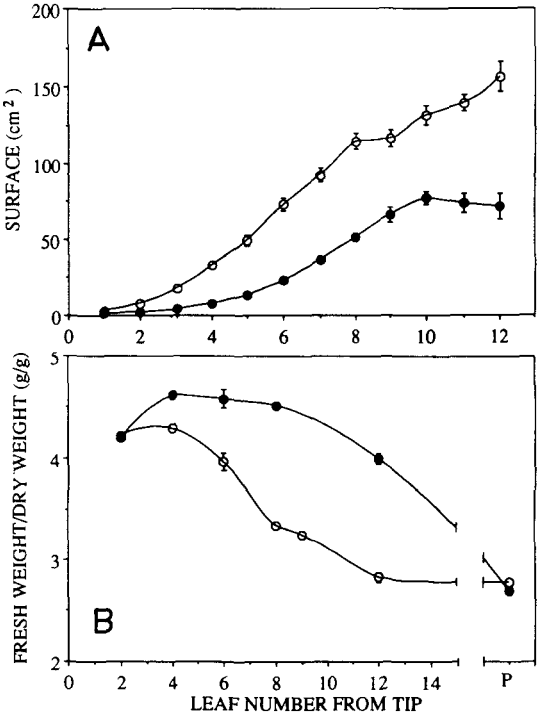

Fig. 1. (**A**) Surface variation during development of juvenile (○) and adult (●) avocado leaves. (**B**) Fresh wt/dry wt ratio in juvenile (○) and adult (●) avocado leaves. Numbers and P indicated position of leaves starting from the stem tip.

Electrophoresis

Anionic polyacrylamide gel electrophoresis (PAGE) was performed following the method described by Davis (1964) using 7.7% polyacrylamide for the separating gel and 4.6% for the stacking gel.

Cationic separation was achieved using the procedure of Reisfield et al. (1962) with 7.7% polyacrylamide for the separating gel and 4.6% for the stacking gel.

Gels were stained to visualize peroxidase activity by incubation in the presence of 12 mM guaiacol and 17 mM H_2O_2 in acetate buffer, pH 4.5. After incubation, the gels were immersed in a 7% acetic acid solution for 3 min and washed thoroughly with water.

Results

As expected, the obtained growth patterns for the different parameters (e.g., weight, surface area, and length) were of sigmoidal type. The growth curve for leaf surface is shown in Fig. 1A. Those of weight and length showed similar trends. With each case, a lag phase could be observed; this phase was more clearly distinguishable in adult than in juvenile material. During the exponential phase, differences between both types of leaves were clearly noticeable, with a higher growth rate on the juvenile leaves. The fresh wt:dry wt ratio is shown in Fig. 1B.

Soluble peroxidase activity for juvenile and adult avocado leaves during their growth phases is indicated in Fig. 2A. Almost until the end of the exponential phase, peroxidase activity was relatively low for both leaf types, although it showed a sharp increase in adult leaves at the 12th position. These differences were even more drastic when leaves of the previous growth season were considered (e.g., 263 U/g fresh wt in adult leaves vs. 70 U/g fresh wt for juvenile leaves).

In relation to the isozyme profile, anodic electrophoresis showed that new bands appeared as leaf development went on. It is noticeable, however, that an anionic band, $R_f = 0.35$ (Fig. 3), appeared at much earlier stages in juvenile (initiation of exponential phase) than in adult leaves (end of the growth phase). In relation to cathodic electrophoresis, one band, $R_f = 0.43$, was observed in both types of material, on leaves which had already detained their growth (P).

In relation to membrane-bound peroxidases, no clear differences were detected between both leaf types, with low levels of activity in both cases;

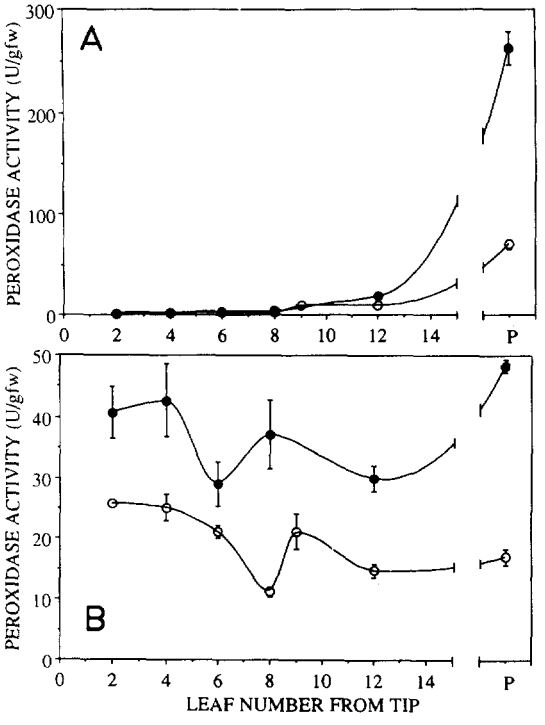

Fig. 2. Peroxidase activities in **A** soluble fraction and **B** ionically bound fraction obtained from growing juvenile (○) and adult (●) leaves. Numbers and P correspond to progressive developmental stages of the leaves. Values are mean ± SEM (represented by vertical bars) for three determinations from three extracts.

however, values observed in adult leaves remained almost constant (8 U/g fresh wt throughout the growth period), while those of juvenile leaves increased from 5 U/g fresh wt on leaf number 2–13 U/g fresh wt on leaf number 12. However, as it was shown for the soluble fraction, values for adult leaves which had already detained their growth were much higher (61 U/g fresh wt) than those of juvenile leaves (34 U/g fresh wt).

The electrophoretic profile showed the same band types observed for the soluble fraction, although the relative importance of the various isozymes seemed to differ (data not shown) in each stage.

The values obtained for the ionically bound peroxidase fraction are indicated in Fig. 2B. All throughout the growth phase, the adult material showed greater activity than the juvenile. This trend was also true for leaves which had already finished their growth.

In relation to the isozyme pattern, no bands could be detected on the anionic electrophoresis, while clearly distinguishable isozymes could be seen in the cathodic electrophoresis (Fig. 4). On juvenile leaves, there were bands with very high R_f values (e.g., 0.88, 0.92, and 0.95). These bands appeared all throughout the growth curve with different degrees of intensity. In adult leaves, these bands did not appear until the end of the growth period (leaf number 12). At earlier stages, other isozymes of intermediate mobility $R_f = 0.63$ and 0.68 were also detected.

The covalently bound peroxidase fraction showed relatively high activity values for both leaf types (58 U/g fresh wt) at the beginning of the growth phase. In both cases, a decrease in activity accompanied leaf development, although higher values were always observed in adult material. Moreover, in leaves which had already detained their growth, the observed values were 30 U/g fresh wt for juvenile leaves vs. 63 U/g fresh wt for adult leaves.

The electrophoretic pattern of this fraction was drastically different from that of ionically bound peroxidases. Anodic isozymes were more common here, while only two bands were observed in the cathodic fraction (data not shown) and only on juvenile and adult leaves from the previous growth season.

In the anodic fraction, a band, $R_f = 0.35$, could be observed. The band was more noticeable in juvenile than in adult leaves. Another isozyme, $R_f = 0.27$, was also present in juvenile and adult leaves of the previous growth season.

Discussion

Plant material of different ontogenetic ages shows very specific traits (Bonga 1982). In avocado, juvenile and adult leaves showed sigmoidal-type curves for several growth parameters (e.g., length, surface, and fresh weight). Similar growth curves have been reported when measuring leaf development in other species (Kennedy and Johnson 1981). The higher growth rates detected during the exponential phase for the juvenile leaves coincide with previous observation in other species, where leaf area (Borzenkova and Nefedova 1981) and length (Rogan and Smith 1975) were also greater in juvenile material. In callus cultures of *Hedera helix*, those obtained from juvenile explants showed a higher growth rate than their adult counterparts (Robbins and Hervey 1970). Furthermore, when rejuvenation treatments were applied to adult explants, a progressive increase in cellular growth rate could be observed (Miller and Goodin 1976).

In relation to fresh wt/dry wt ratio, lower values were found on juvenile than in adult leaves, which may indicate a higher degree of metabolic activity in the former.

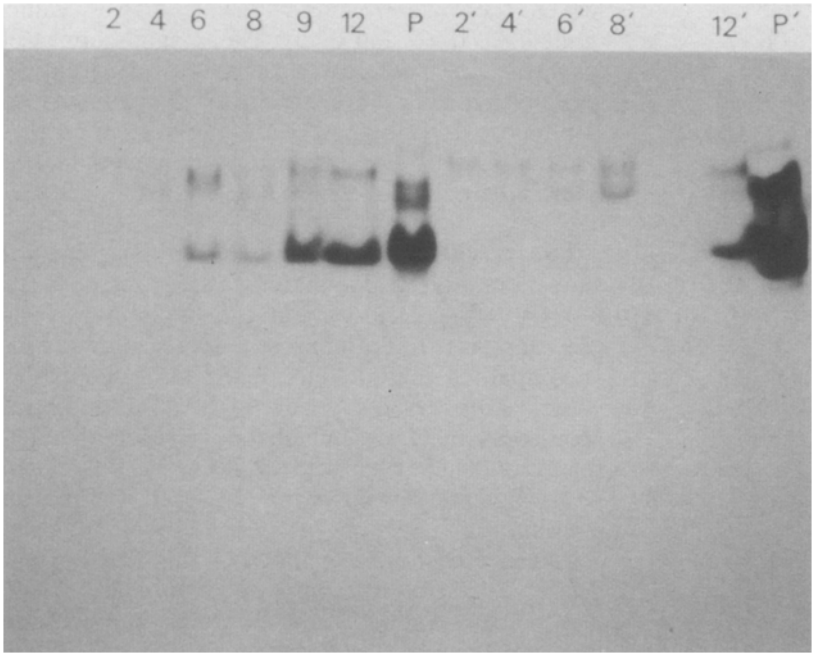

Fig. 3. Anionic electrophoretic profile in polyacrylamide gel staining for peroxidase activity of soluble fraction from juvenile and adult (') leaves. Numbers and P correspond to leaf position from tip and leaf of the previous growth season, respectively.

Peroxidase activity has been linked to growth and development process in aspen callus (Wolter and Gordon 1975). More recently, Verschoore-Martouzet (1985) reported a higher peroxidase activity in juvenile than in adult plants of *Sequoia sempervirens*. In avocado, however, higher activity values were always obtained in adult material. Furthermore, the increase found at the end of the growth phase in both types of leaves, although more drastic in adult material, could indicate a role of peroxidases on the senescence process. Moreover, leaves from the previous growth season, showed a green–brown color rather than intense dark green. Trippi et al. (1989) and Thomas (1990) have also associated a decrease in chlorophyll content with the beginning of the senescence process. The link between peroxidase activity and senescence has been previously reported by other authors (Thompson et al. 1987). Probably, peroxidase might control the levels of hydroperoxide, which increase during senescence (Benson 1990), acting then as a detoxifying agent (Gaspar et al. 1985). Interestingly, and in spite of the lower peroxidase activity detected in juvenile leaves, an anionic band, $R_f = 0.35$, was present in this material at early developmental stages. Apparently, this enzyme is not involved in control of endogenous auxin levels (data not shown), although it shows a high specific activity against lignin-related substrates. Thus, its function could be related to the lignification or detoxification process.

The cathodic band, $R_f = 0.43$, is much more intense in adult than in juvenile leaves. Several authors (Gaspar et al. 1985) have found basic isoenzymes to be related to IAA degradation. In this sense, we found higher endogenous free IAA levels in juvenile leaves (data not shown).

No clear differences could be detected between juvenile and adult leaves in membrane-bound peroxidases, although activity in fully developed leaves from previous growth season was higher in adult material.

In relation to ionically bound peroxidase, activities were clearly higher in the adult material, both during the growth season and when leaves had attained their growth. The fact that only cathodic peroxidases were clearly distinguishable in this fraction coincides with previous observations by Masuda et al. (1983) and Goldberg et al. (1986) in other species. Abeles and Biles (1991) have pointed out that the positive charge of these proteins could facilitate their linkage to the negatively charged cell wall. Their role could then be related to lignification in the cell wall (Masuda et al. 1983, Goldberg et al. 1986). In our experimental system, the higher activity observed in adult leaves could explain their slower growth rate in relation to juvenile leaves.

Peroxidases covalently linked to cell wall also showed higher activity values in adult than in juvenile leaves. The two bands, $R_f = 0.27$ and $R_f = 0.35$, observed in the anodic fraction coincide with that observed in soluble peroxidases, which could indicate that both isozymes are of cytoplasmic origin and could be excreted to the cell wall. More-

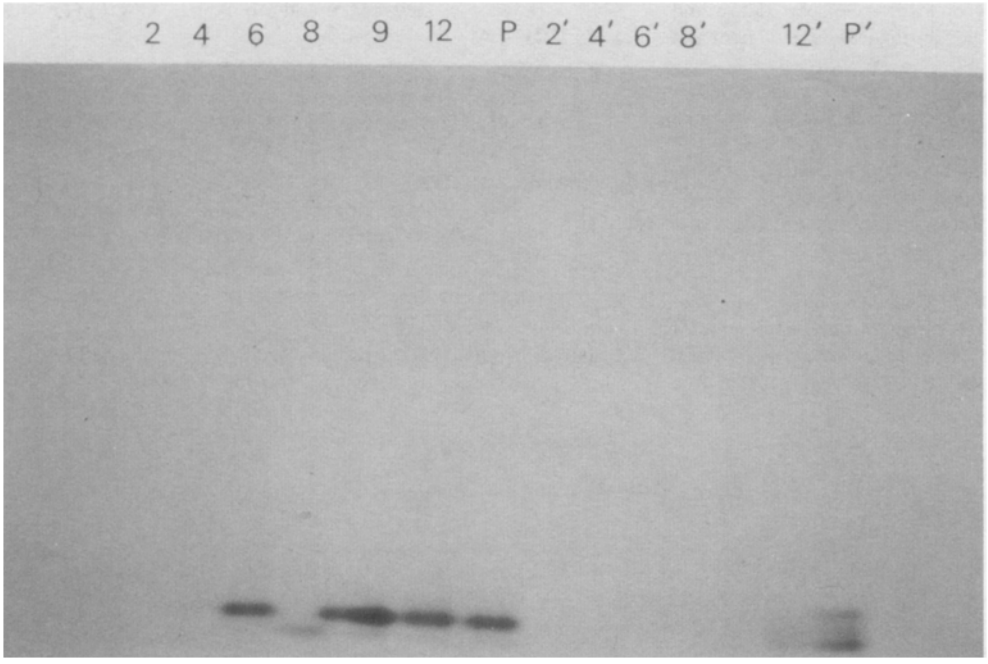

Fig. 4. Electrophoretic profile of fraction ionically bound to cell wall from juvenile and adult (') leaves, obtained by cationic electrophoresis and staining for peroxidase activity. Numbers and P correspond to leaf position from tip and leaf of the previous growth season, respectively.

over, experiments carried out with lignin-related substrates have shown a high specific activity for the isozyme $R_f = 0.35$.

It seems that attempts to use peroxidases as markers for phase change must be taken with caution. Our results in avocado clearly show more activity in adult than in juvenile leaves, in contrast to previous observations in *Sequoia sempervirens*. Moreover, great fluctuations in activity have been found related to the different developmental stages, with drastic increases in mature, fully developed leaves, probably linked to initiation of the senescence process.

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