Enzymatic Browning, Oleuropein Content, and Diphenol Oxidase Activity in Olive Cultivars (*Olea europaea* L.)

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The greatest browning potential of 10 olive cultivars (*Olea europaea* L.) occurred in green fruit and was still considerable when olives were picked. Browning capacity of olives results from complex interactions between diphenol oxidase (EC 1.10.3.2) activity and oleuropein content, the major ortho diphenolic compound in olive. Intervarietal comparison of olives picked at a similar stage of development showed there was a positive correlation between browning and oleuropein content ($r = 0.840$, $p = 1\%$) but no correlation with diphenol oxidase activity.

INTRODUCTION

Among the optimal organoleptics sought in green table olives, maintenance of color is an important parameter that can be considerably harmed by enzymatic browning of the fruit. Such browning occurs either after impact and wounding during harvesting or during subsequent technological treatment to remove bitterness to make olives edible. Comparison of five cultivars made it possible to establish relationships between browning, diphenol oxidase (DPO) activity and ortho diphenolic substrates (Sciancalepore and Longone, 1984; Sciancalepore, 1985). In addition, olive DPO is associated with chloroplast membranes (Shomer et al., 1979), becoming more and more soluble during maturation (Ben-Shalom et al., 1977a,b); it is thus probable that its involvement in fruit browning varies considerably with physiological stage, as has been shown in other cases (Harel et al., 1966; Macheix, 1970).

Oleuropein (Panizzi et al., 1960) and verbascoside (Fleuriet et al., 1984), a derivative of caffeic acid, are the major ortho diphenolics in olive fruit. Concentrations of these compounds vary considerably both during the life of the fruit and according to the cultivar (green or black/table or pressing) (Amiot et al., 1986). The degree of browning must depend on these variations.

We aimed to establish a relationship between the development of fruit browning potential and that of the main parameters in browning, i.e., DPO activity and phenolic content. We extended the study to 10 cultivars to compare them.

MATERIALS AND METHODS

Plant Material. Fruits of seven olive cultivars (*Olea europaea* L.) (Picholine, Lucques, Salonenque, Cailletier, Tanche, Verdale, and Bouteillan) and three clones being bred (L11, L365, and VP7) were picked from trees grown in the INRA horticultural orchard (Montpellier, France). The fruits used in these experiments were picked either during the commercial harvesting period (green table olives, i.e., cv. Lucques, Picholine, and Salonenque) or distinctly earlier for olives eaten black (Tanche, Cailletier, and Zrappola) and those grown for pressing (Bouteillan and Verdale). Sampling was carried out each week from mid-September until mid-November except for cv. Lucques, which was picked from mid-July.

After harvesting, the fruits from each cultivar were sorted into three batches of 100 fruits of homogeneous size and color. Half of each batch was then used for the determination of browning characteristics and half (after freeze-drying) for measurement of DPO activity and phenolic compounds.

Measurement of Browning. Browning was assayed by using three different approaches which have already been defined for grape (Sapis et al., 1983b) and which give three expressions of browning: BP_0, BP_1, and BP_2.

BP_0 was determined by immersing fresh plant material in liquid nitrogen and then reducing it to fine powder in a Dangoumau ball mill. This powder (1 g) was homogenized in 50 mL of 0.1 M phosphate–citrate buffer (pH 4.5) for 30 s at 20 °C, agitated at 20 °C for 30 min, and then centrifuged at 40000*g*. Supernatant was collected and filtered through a Millipore filter (0.65 μm) to remove lipids remaining in suspension. Absorbance of filtrate was measured by using a spectrophotometer at 400 nm against a standard prepared in a similar manner but with 0.5 mM mercaptoethanol (10% v/v) which prevents browning. Results are given in $A \times 10^{-3}$ g^{-1} DW at 400 nm.

BP_1 and BP_2 were determined by using the same procedure but with the addition of either (BP_1) 0.3 mL of Triton X-100 (a nonionic detergent) or (BP_2) the same detergent and an excess of phenolic substrate (2 mL of 0.2 M 4-methylcatechol). The values are means of triplicate determinations; coefficients of variation were 1, 3, and 5.5% for BP_0, BP_1, and BP_2, respectively.

Extraction and Purification of DPO. Several types of extraction of DPO were compared. Maximum activity was obtained by using acetone powder which was prepared with 4 g of freeze-dried fruit homogenized for 2 min in 100 mL of cold (−20 °C) acetone (80% v/v) containing poly(ethylene glycol) (100 mg g^{-1} of powder). This mixture was filtered under vacuum and the residue mixed three times with 80% acetone; the white powder obtained was dried under vacuum for 2 h to remove acetone. Acetone powder (1 g) was then homogenized for 30 s with an Ultraturrax in 50 mL of 0.1 M phosphate buffer (pH 7.2), shaken for 15 min, filtered through muslin, and centrifuged for 20 min at 40000*g*. All these operations were carried out at 0–4 °C. Filter residues and centrifugation pellets were treated twice under the same conditions and the three supernatants combined to form the enzyme extract.

DPO Assay. DPO activity was assayed by O_2 consumption measured with an oxygen electrode at 30 °C. The incubation medium consisted of 0.5 mL of enzyme extract, 100 μL of 0.2 M 4-methylcatechol, and 1.1 mL of 0.1 M phosphate–citrate buffer (pH 5). Results are expressed in moles of O_2 consumed min^{-1} g^{-1} DW. The values are means of three different samples.

Extraction of Phenolic Compounds. This was carried out at 4 °C with an ethanol–water mixture (80/20 v/v) according to a method described elsewhere (Amiot et al., 1986). Oleuropein and verbascoside were separated and assayed by reverse-phase high-performance liquid chromatography (Fleuriet et al., 1984; Amiot et al., 1986). The results are expressed in mg g^{-1} DW; the values are average of three determinations with variability of less than 6%.

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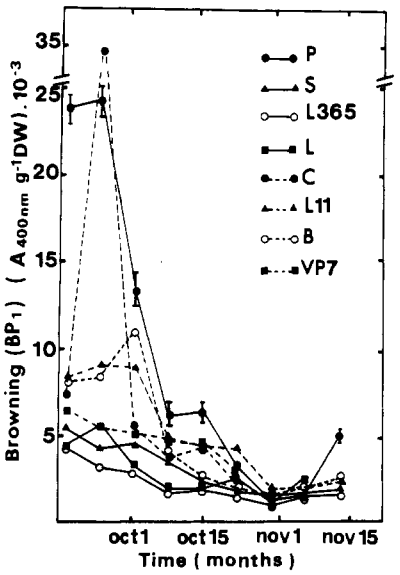

Figure 1. Changes of browning (BP_1) during maturation in various olive cultivars: P, Picholine; B, Bouteillan; S, Salonenque; C, Cailletier; L, Lucques; L365, L11, and VP7, see text. Verdale and Tanche present the same variations as L365. Values are means ± SE of three replicates.

RESULTS AND DISCUSSION

Development of Browning. Potential browning (BP_1) was high in green fruits (Figure 1), and a maximum was observed in five cultivars; it then fell during maturation. In addition, browning intensity was very different according to the cultivar since maximum values were between 4 and 35 (Table I).

Variations in BP_0 and BP_2 were very similar to those of BP_1 (data not shown). Values fell strongly in early maturation in all cases. BP_1 values were always greater than those of BP_0 (Table I), with the exception of cv. Cailletier, in which BP_0 and BP_1 were identical. In addition, excess exogenous substrate (BP_2) always gave distinctly higher values (Table I).

Comparison of BP_0, BP_1, and BP_2 (Table I) showed two main features: (a) endogenous substrates were in all cases very limiting factors in browning since BP_2 (measured after saturation of DPO by an excess of substrate) was always higher than BP_1; (b) solubilization of DPO by Triton X-100 was determinant in browning intensity (see ratio of BP_1 to BP_0). This is probably related to the localization of DPO in olive, mainly on the membrane (Shomer et al., 1979); in cv. Cailletier ($BP_0 = BP_1$), early maturation is probably responsible for natural disorganization of chloroplast structures and solubilization of DPO (Ben-Shalom et al., 1977a).

Changes in the Main Parameters of Browning. Variations of the amounts of ortho diphenolic substrates and DPO activity were studied in cv. Lucques throughout growth and maturation. In the olive, maturation proceeds in two successive stages (Shulman and Lavee, 1976): green maturation, characterized by a fall in chlorophyll content, and black maturation, when anthocyanin pigments accumulate. The two major ortho diphenolic substrates of DPO are oleuropein and verbascoside (Fleuriet et al., 1984), whose variations are quite different during growth and maturation (Amiot et al., 1986). Nevertheless, verbascoside concentrations were always very low in comparison to those of oleuropein, and the contribution of the former to browning was probably negligible. Oleuropein content was high in young fruits, fell considerably at the beginning of the green maturation, and then stabilized (Figure 2).

DPO displayed two successive peaks (Figure 2): one during green maturation and the second at the beginning of black maturation (mid-October). Such increase in DPO activity during maturation has already been reported for several other fruits (Harel et al., 1966, 1970; Sapis et al., 1983a).

The relative importance of DPO activity and ortho diphenolic substrate content in the intensity of browning in fruits has already been discussed extensively (Macheix et al., 1990). Some authors show that the limiting factor is not always the same at different physiological stages of the fruit (Harel et al., 1966; Macheix, 1970).

In the case of olives there was a strong parallel during maturation between development of the different types of browning and of DPO activity (Figure 2). Indeed, during maturation DPO activity is maximal at the same time as browning (BP_0, BP_1, BP_2), whereas oleuropein content is low. In contrast, DPO activity remained fairly constant and high during growth, whereas browning was initially very weak in spite of high oleuropein content. Thus, there is no similarity between variation in oleuropein content and browning during development of fruit.

Hence, although ortho diphenolic substrates are always limiting for browning (see comparison of BP_1 and BP_2, Table I), their effect may vary from one physiological stage to another because of interference with other factors, as was shown previously in apple (Harel et al., 1966; Macheix, 1970). As already reported with other fruits, variations in DPO activity (see above), the presence of endogenous inhibitors (Walker, 1976), the existence of latent or inactive forms of DPO and their degree of linkage to membranes (Harel et al., 1970; Sato and Hasegawa, 1976; Ben-Shalom et al., 1977a), and the presence of reducing agents such as ascorbic acid are the main factors that can mask the limiting nature of oleuropein and other ortho diphenolic substrates during physiological development of fruit.

Intervarietal Comparison. This comparison was carried out for each cultivar at the stage of maturation corresponding to maximum browning (Table I). Fruits with the greatest browning (cvs. Cailletier and Picholine) were those in which oleuropein contents were highest, whereas the same fruits had relatively weak DPO activity. In contrast, several cultivars with a low oleuropein content (i.e., cv. Salonenque or L11) displayed only slight browning although they possessed very high DPO activity.

Statistical analysis of the data (Table I) showed a positive correlation ($r = 0.840$, $p = 1\%$) between browning and oleuropein contents if cv. Cailletier was not taken into account (Figure 3). The particular behavior of this cultivar was certainly connected with the accumulation during maturation of demethyloleuropein, a derivative of oleuropein (Amiot et al., 1986), which may also be a substrate for DPO but has not been taken into account. These preliminary observations suggest that phenolic compounds play a determinant role in the expression of browning in the various cultivars.

These results illustrate the complexity of the relationships between the various parameters involved in browning. Whereas no correlation has been found between oleuropein content and browning according to the different physiological stages of fruit development (Figure 2), there is a correlation in the comparison of olive cultivars (Figure 3). Similar results have already been reported for ortho diphenolics in five different olive cultivars (Sciancale-

Table I. Intervarietal Comparison of the Main Parameters of Browning[a]

cv.	browning, (A_{400nm} g^{-1} DW) × 10^{-3}			BP_1/BP_0	BP_2/BP_1	DPO activity, μmol of O_2 min^{-1} g^{-1} DW	oleuropein, mg g^{-1} DW
	BP_0	BP_1	BP_2				
Cailletier	35	35	134	1.0	3.8	43.0	44
Picholine	15	24	77	1.6	3.2	29.8	67
Bouteillan	5	11	48	2.2	4.4	28	33
L11	4	9	45	2.2	5.0	135.0	20
Tanche	4	8	37	2.0	4.6	28.0	36
Lucques	3	6	34	2.0	5.7	47.0	22
VP7	4	6	33	1.5	5.5	57.0	30
Salonenque	3	5	37	1.7	7.4	162.0	6
L365	2	4	36	2.0	9.0	102.0	32
Verdale	2	4	32	2.0	8.0	47.0	24

[a] Values are means of three replicates. All the values are for maximum browning of each cultivar.

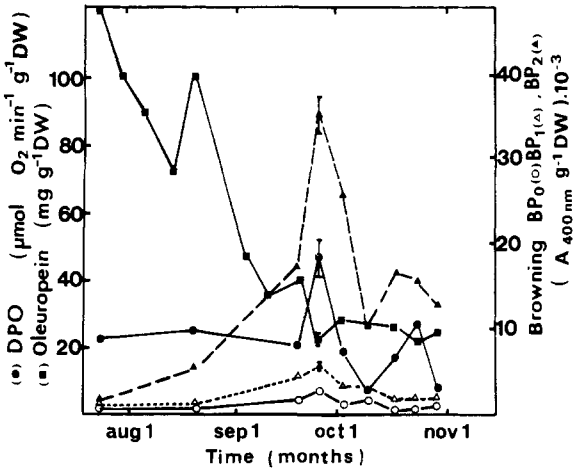

Figure 2. Evolution of different parameters of browning in the fruit of cv. Lucques during development and maturation. Values are means ± SE of three replicates.

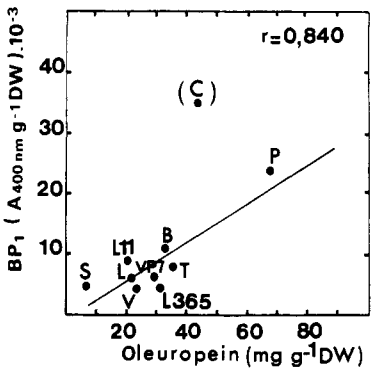

Figure 3. Correlation between BP_1 and oleuropein for nine olive cultivars. Cultivar Cailletier (C) was not taken into account. (Abbreviations are as in Figure 1.)

pore and Longone, 1984; Sciancalepore, 1985) or other fruits (Macheix et al., 1990), but correlations become less distinct or nonexistent as the number of cultivars increases (Romeyer et al., 1985).

Whereas the rate of browning is limited principally by DPO activity during the final period of growth and maturation of the olive, no correlation was found between the two parameters in intercultivar comparison, in contrast with previous data on other cultivars (Sciancalepore and Longone, 1984).

CONCLUSION

Browning capacity of olive is the result of complex interactions involving DPO activity, oleuropein content, and many other factors whose relative importance may vary with the physiological development of the fruit. Nevertheless, when fruits of different cultivars are picked during maturation, endogenous substrates seem to be the main limiting factor in browning. Harvesting of green olives occurs during a period when their browning capacity is still substantial and is responsible for loss of quality occurring particularly during mechanical harvesting. Knowledge of the behavior of cultivars, as shown in this work, should make it possible to determine optimum picking dates for each of them.

ABBREVIATIONS USED

DPO, diphenol oxidase (EC 1.10.3.2); BP_0, BP_1, BP_2, different expressions of browning (see Materials and Methods); DW, dry weight.

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LITERATURE CITED

Amiot, M. J.; Fleuriet, A.; Macheix, J. J. Importance and evolution of phenolic compounds in Olive during growth and maturation. *J. Agric. Food Chem.* **1986**, *34*, 823–826.

Ben-Shalom, N.; Kahn, V.; Harel, E.; Mayer, A. M. Olive catechol oxidase—Change during fruit development. *J. Sci. Food Agric.* **1977a**, *28*, 545–550.

Ben-Shalom, N.; Kahn, V.; Harel, E.; Mayer, A. M. Catechol oxidase from green Olives properties and partial purification. *Phytochemistry* **1977b**, *16*, 1153–1158.

Bureau, D.; Macheix, J. J.; Rouet-Mayer, M. A. *Lebensm. Wiss. Technol.* **1977**, *10*, 211–216.

Fleuriet, A.; Macheix, J. J.; Andary, C.; Villemur, P. *C.R. Acad. Sci. Ser. III* **1984**, *7*, 253–256.

Harel, E.; Mayer, A. M.; Shain, Y. Catecholoxidases, endogenous substrates and browning in developing apples. *J. Sci. Food Agric.* **1966**, *17*, 389–392.

Harel, E.; Mayer, A. M.; Lerner, H. R. Changes in the levels of catechol oxidase and laccase activity in developing Peaches. *J. Sci. Food Agric.* **1970**, *21*, 542–544.

Macheix, J. J. *Physiol. Veg.* **1970**, *8*, 585–602.

Macheix, J. J.; Fleuriet, A.; Billot, J. Phenolic compounds in fruit processing. In *Fruit Phenolics*; CRC Press: Boca Raton, FL, 1990.

Panizzi, L.; Scarpati, M. L.; Oriente, E. G. *Gazz. Chim. Ital.* **1960**, *90*, 1449–1485.

Romeyer, F. M.; Sapis, J. C.; Macheix, J. J. Hydroxycinnamic esters and browning potential in mature berries of some grape varieties. *J. Sci. Food Agric.* **1985**, *36*, 728–732.

Sapis, J. C.; Macheix, J. J.; Cordonnier, R. E. The browning capacity of grapes. I. Changes in polyphenol oxidase activities during development and maturation of the fruit. *J. Agric. Food Chem.* **1983a**, *31*, 342–345.

Sapis, J. C.; Macheix, J. J.; Cordonnier, R. E. The browning capacity of grapes. II. Browning potential and polyphenol ox-

idase activities in different mature grapes varieties. *Am. J. Enol. Vitic.* **1983b**, *34*, 157–162.
Sato, M.; Hasegawa, M. The latence of Spinach chloroplast phenolase. *Phytochemistry* **1976**, *15*, 61–65.
Sciancalepore, V. Enzymatic browning in five olive varieties. A research note. *J. Food Sci.* **1985**, *50*, 1194–1195.
Sciancalepore, V.; Longone, V. Polyphenoloxidase activity and browning in green olives. *J. Agric. Food Chem.* **1984**, *32*, 320–321.
Shomer, I.; Ben-Shalom, N.; Harel, E.; Mayer, A. M. The intracellular location of catechol oxidase in the Olive fruit. *Ann. Bot.* **1979**, *44*, 261–263.
Shulman, Y.; Lavee, S. Endogenous cytokinins in maturing Manzanillo Olive fruits. *Plant Physiol.* **1976**, *57*, 490–492.
Walker, J. R. L. The control of enzymic browning in fruit juices by cinnamic acids. *J. Food Technol.* **1976**, *11*, 341–345.

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