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Physiological basis of sensitivity to enzymatic browning in 'lettuce', 'escarole' and 'rocket salad' when stored as fresh-cut products

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Abstract

In fresh-cut leafy vegetables, the operation of cutting may stimulate enzymatic browning, with important commercial consequences. In this work, a number of physiological and biochemical parameters, including the activities of key enzymes involved in the metabolism of phenols (such as PAL, PPO, and PODs) and ascorbic acid (ASA), were measured in three species: lettuce (*Lactuca sativa* var. *capitata* L.), escarole (*Cichorium indivia* var. *latifolium*) and rocket salad (*Eruca sativa*), upon cold storage as fresh cuts. The first two species are quite sensitive to leaf browning, which does not affect rocket salad.

The resistance of rocket salad to browning was associated with a much higher ASA content and a decrease in this compound upon storage, compared to other species in which ASA remained either constant (lettuce) or increased (escarole). It is proposed that the resistance of rocket salad to this disorder, which markedly affects other leafy species, e.g., lettuce and escarole, is a result of the inhibition of PPO activity and/or the reduction of quinones to phenols, which may both be induced by ASA.

Keywords: Ascorbic acid; Enzymatic browning; Peroxidases; Phenylalanine ammonia lyase; Polyphenol oxidase; Visual quality

1. Introduction

Fresh-cut (minimally-processed) horticultural products are subjected to simple operations soon after harvest, such as cleaning, washing, cutting and packaging, which make them ready-to-use. Cutting is the main factor responsible for the deterioration of these products during storage which, as a consequence, is more rapid than whole products.

Enzymatic browning in leafy vegetables is considered one of the most important disorders, since it is easily detected by consumers, evident consequences on marketing. Plant species react in different ways to cut-induced leaf browning; lettuce is one of the most studied species from this point of view as it shows a great sensitivity to this disorder (Saltveit, 2000). Altered phenol metabolism is thought to be involved in leaf browning of lettuce (Saltveit, 2000). The first step in phenol metabolism is the conversion of the amino acid L-phenylalanine to *trans*-cinnamic acid by the enzyme phenylalanine ammonia lyase (PAL, EC 4.3.1.5). The phenol compounds synthesized by PAL activity can be oxidized, by polyphenoloxidase (PPO, EC 1.10.32), to quinones, which spontaneously polymerize to brown pigments responsible for tissue browning (Ke & Saltveit, 1986, 1989). For this reason, some authors have suggested that the activity of PAL may be a marker for shelf-life in some fresh-cut products (e.g., Degl'Innocenti, Guidi, Pardossi, & Tognoni, 2005; Hyodo & Fujinami, 1989; Ke & Saltveit, 1986; Lopez-Galvez, Saltveit, & Cantwell, 1996; Ritenour, Ahrens, & Saltveit, 1995; Tavarini, Degl'Innocenti, Guidi, & Paradossi, in press). Indeed, an increased PAL activity was found to be correlated with the susceptibility to browning in lettuce fresh-cuts (Couture, Cantwell, Ke, & Saltveit, 1993). Moreover, the content of phenols is associated with the sensitivity to

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enzymatic browning in several vegetables, such as artichoke (Brecht et al., 2004).

In addition to the oxidation of phenols carried out by PPO, peroxidases (POD; E.C. 1.11.1.7) contribute to tissue browning. Indeed, mono- and di-phenol compounds may be substrates for POD activity (Degl'Innocenti et al., 2005; Nicolas, Cheynier, Fleuriet, & Rouet-Mayer, 1993; Robinson, 1991; Tavarini et al., in press). These enzymes catalyze the oxidation of phenols by the H_2O_2 produced during respiration or as a consequence of wounding (Amiot, Fleuriet, Veronique, & Nicolas, 1997).

Different treatments have been evaluated to reduce browning in fresh-cut products, such as the application of antioxidant compounds (e.g., sulfites), calcium salts to maintain membrane integrity, chemical inhibitors of PPO and/or POD, or the use of modified atmosphere packaging to exclude oxygen (Saltveit, 2000). Ascorbic acid (ASA) is a highly effective inhibitor of enzymatic browning in many tissues, primarily because it is able to reduce quinones to phenolic compounds, thus preventing the synthesis of the brown pigments (Walker, 1995). Moreover, at high concentration, ASA may inhibit PPO by decreasing the cytosol pH (Vamos-Vigyazo, 1981).

The aim of this work was to study the main biochemical processes involved in the enzymatic browning of minimally processed leafy vegetables; in particular, we tested the hypothesis developed in previous papers (Degl'Innocenti et al., 2005; Reyes, Villareal, & Cisneros-Zevallos, 2007) that a high constitutive ASA content is associated with the resistance of fresh-cut leaves to colour alteration upon storage. Three species were selected for their different sensitivities to this disorder, as assessed in preliminary work: lettuce (*Lactuca sativa* var. *capitata* L.), escarole (*Cichorium indivia* var. *latifolium*), and rocket salad (*Eruca sativa*); these species also differed with respect to their leaf constitutive content of ASA. Lettuce and escarole, in which browning is evident within few hours after cutting, had a much lower ASA content (less than 4 $\mu g\ g^{-1}$ FW) than had rocket salad, which contained up to 80 $\mu g\ g^{-1}$ FW and did not show any browning up to 8–10 days of cold storage.

2. Materials and methods

2.1. Plant material

Plant material was taken from a local market and processed in the laboratory within a few minutes. The plants had been cultivated in commercial greenhouses in the period between January and May 2005. The leaves were gently washed with chlorinated drinking water and excess water was removed with a manual salad spinner. Afterwards, leaves were cut (approximately 1 cm × 2 cm) perpendicular to the midrib with stainless steel scissors and stored for up to three days at 4 °C in plastic boxes (1.5 l). Whole heads of lettuce and escarole or intact leaves of rocket salad were stored under the same conditions as the fresh-cuts. Determinations were made of both fresh-cuts and intact leaves every day during storage and, for some parameters, also at different times during the first 24 h from processing.

2.2. Leaf browning assessment

To evaluate the occurrence of leaf browning, Image Tool 3.00 for Windows (University of Texas Health Science Center, San Antonio, USA) scanning software was used. A picture of each sample was captured with a digital camera Nikon Coolpix E4500, with a resolution of 118 pixel cm^{-2}. The images were imported by Image Tool, which created a grey copy to identify the brown spots on the leaves. The incidence of browning was determined as the percentage of colour-altered area.

2.3. Enzyme assay

2.3.1. PAL activity

PAL activity was measured as described by Degl'Innocenti et al. (2005). One unit of PAL activity equals the amount of PAL required to deaminate 1 μmol of L-phenylalanine to *trans*-cinnamic acid and NH_3 in 1 h/g fresh weight.

2.3.2. PAL inactivating factor

To measure PAL-Inactivating Factor (IF), 10 g of vegetable tissue were placed in tubes containing 16 ml of HEPES (hydroxyethylpiperazine ethanesulfonic acid) buffer, 0.1 M (pH 7.5), with 400 $\mu l\ l^{-1}$ of β-MeSH (mercaptoethanol) and 1 g PVPP (polyvinylpolyrrolidone) (Ritenour & Saltveit, 1996). The vegetable tissue was homogenized and centrifuged at 10 000*g* for 15 min. The supernatant was discarded and the pellet resuspended in 1 ml of HEPES buffer, 0.1 M (pH 6.0–8.8). Incubation of sample preparations and PAL enzyme were carried out by mixing 32 μU PAL from *Rhodotorula glutinis* (Sigma) with 1 ml of resuspended pellet and holding it at 30 °C. After 1 h of incubation, 0.3 ml of the incubation solution was microcentrifuged for 2 min and 0.2 ml of the resulting supernatant was used to measure PAL activity, as described in Degl'Innocenti et al. (2005).

2.3.3. PPO enzyme

PPO activity was determined according to the method reported by Degl'Innocenti et al. (2005). One unit of PPO activity was defined as the amount of enzyme causing a change of 0.01 in absorbance (480 nm) per min per mg of soluble protein. Protein determinations were performed using the Protein Assay Kit II (Bio Rad).

2.3.4. Peroxidases (POD)

The determination of POD activity was carried out as reported in Degl'Innocenti et al. (2005). Absorbance was recorded at different wavelengths, depending on the substrate utilized: 470 nm for guaiacol substrate, 654 nm for tetrametylbenzidine (TMB), 410 nm for chlorogenic acid and 450 nm for caffeic acid. POD activity was expressed as Δ_{ABS} per min per g fresh weight.

2.4. Phenolic compounds

The extraction of phenolic compounds was performed as described by Degl'Innocenti et al. (2005). The total phenolic content in the methanol extracts was determined according to Folin-Ciocalteu by Singleton and Rossi (1965). A 100 µl aliquot of the supernatant was combined with 500 µl of Folin-Ciocalteu's reagent and 400 µl of sodium carbonate (7.5%). The tubes were mixed for 15 s and then allowed to stand for 30 min at 20 °C. Absorbance was measured at 765 nm, using a UV–Vis spectrophotometer (Ultrospec 2100, Pharmacia).

2.5. o-Quinone content

Soluble *o*-quinones of leaf vegetable tissues were extracted as described by Ke and Saltveit (1986): 10 g of tissue were homogenized with 20 ml of methanol. The homogenate was filtered through four layers of cheesecloth and centrifuged at 15000*g* for 15 min. The supernatant was used directly to measure the soluble *o*-quinones at a wavelength of 437 nm.

2.6. Ascorbic acid content

ASA was determined spectrophotometrically as described by Kampfenkel, Montagu, and Inzè (1995). Ascorbic acid was reported as mg/100g fresh weight.

2.7. Statistical treatment

The experiment was repeated three times with similar results. Two-way ANOVA was used to assess the influence of storage and plant genotype on the measured quantities. Mean comparison was conducted using the LSD test.

3. Results

No significant difference in any of the measured parameters was observed during cold storage (up to 72 h) in whole heads of lettuce and escarole or in intact leaves of rocket salad. Therefore, any change in the fresh-cuts observed during conservation is reasonably interpreted as a genuine effect of simulated processing, in particular due to cutting.

As expected, the species differed markedly in their sensitivity to browning upon storage. Lettuce began to show marked alterations of leaf colour, as measured by image analysis, during the first day; by the end of storage, the darkened area was about 6% of total leaf area, which indicated severe damage from the commercial point of view (Fig. 1). The disorder also occurred in escarole, albeit to a lesser extent than in lettuce (Fig. 1), but it was not observed in rocket salad until 72 h (Fig. 1). In the latter species, toward the end of storage, computerized image analysis revealed a slight colour alteration that was due to leaf wilting, not to enzymatic browning.

In lettuce, PAL activity significantly increased in the first hours after cutting and a peak was observed by 6 h (Fig. 2). In escarole, PAL activity did not change until 48 h of storage, when it increased significantly reaching the highest level after 72 h (Fig. 2). Conversely, in rocket salad, PAL activity reached a maximum level by 12 h after cutting; then it decreased significantly and very low values were detected at the end of storage.

The activity of PAL-IF did not change significantly during storage in lettuce, while an opposite trend was observed in escarole and rocket salad (Fig. 3). In escarole, activity decreased markedly after 24 h and then remained constant, whereas, in rocket salad, activity, which was much lower than that in the other two species, tended to increase after

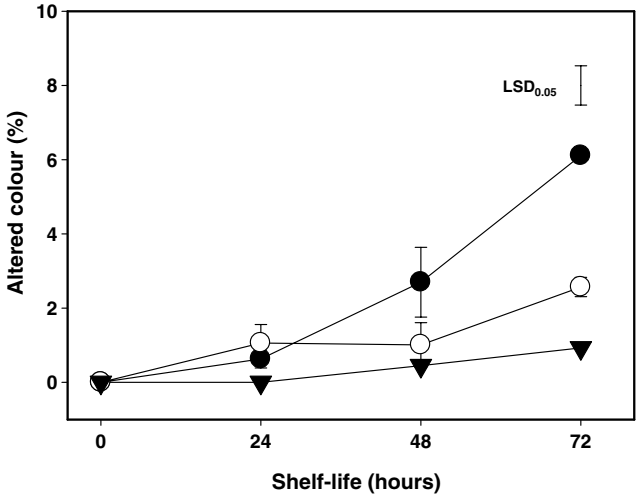

Fig. 1. The incidence of browning in fresh-cut leaves of lettuce (closed circle), escarole (open circle) and rocket salad (closed triangle down) stored at 4 °C. Each value is the mean of ten replicates (±S.E.). The bar inside the graph indicates LSD.

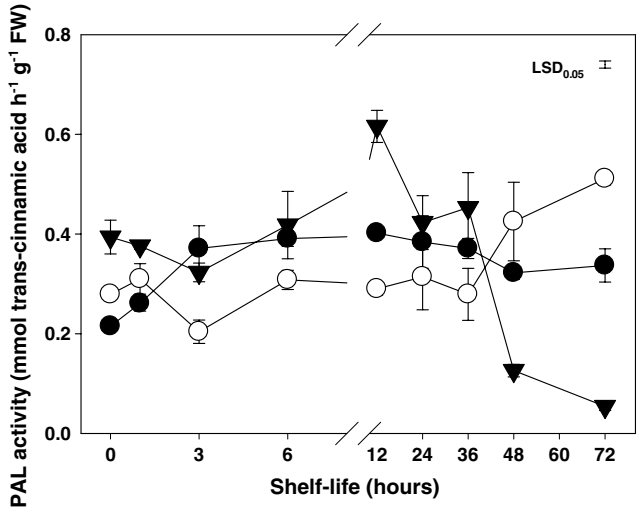

Fig. 2. The activity of phenylalanine ammonia lyase (PAL) in fresh-cut leaves of lettuce (closed circle), escarole (open circle) and rocket salad (closed triangle down) stored at 4 °C. Each value is the mean of six replicates (±S.E.). The bar inside the graph indicates LSD.

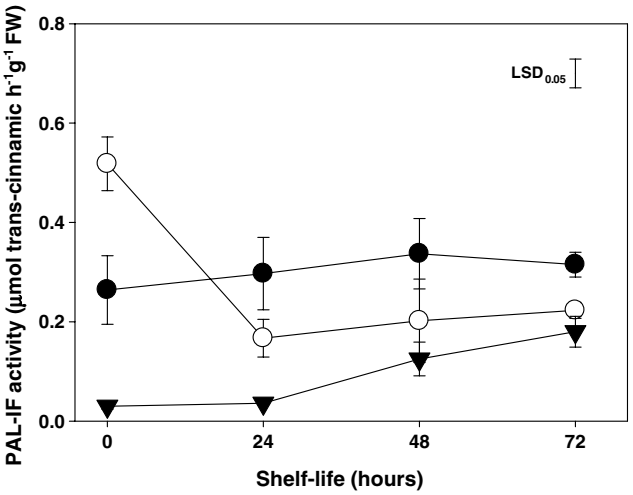

Fig. 3. The activity of phenylalanine ammonia lyase inactivating factor (IF-PAL) in fresh-cut leaves of lettuce (closed circle), escarole (open circle) and rocket salad (closed triangle down) stored at 4 °C. Each value is the mean of six replicates (±S.E.). The bar inside the graph indicates LSD.

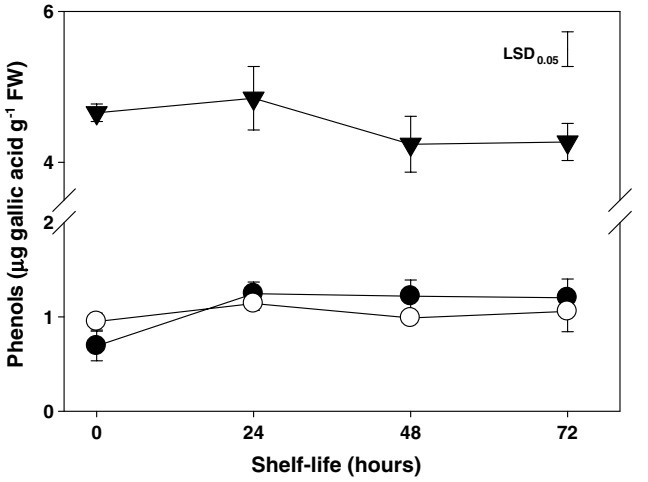

Fig. 5. The concentration of phenols in fresh-cut leaves of lettuce (closed circle), escarole (open circle) and rocket salad (closed triangle down) stored at 4 °C. Each value is the mean of six replicates (±S.E.). The bar inside the graph indicates LSD.

the first day of storage (Fig. 3). Regression analysis shows a significant negative relationship between PAL and IF-PAL activity only in rocket salad ($r = -0.86$; Fig. 4).

The content of phenols did not show important changes during storage; it was 4–5 times higher in rocket salad than in the other species (Fig. 5).

The activity of PPO, the key enzyme responsible for the oxidation of phenols to quinones, was quite low in lettuce and increased significantly only after 48 h (Fig. 6). In rocket salad, the activity of PPO was much higher than that in lettuce and tended to decrease during storage (Fig. 6). In escarole, the activity was significantly higher than those in the other two species and, after a transient peak by 3 h, showed a tendency to decrease (Fig. 6).

The study also considered the activity of POD, which was determined using different substrates with very similar results. For the sake of brevity, Fig. 7 shows only the results obtained using chlorogenic or caffeic acid. POD

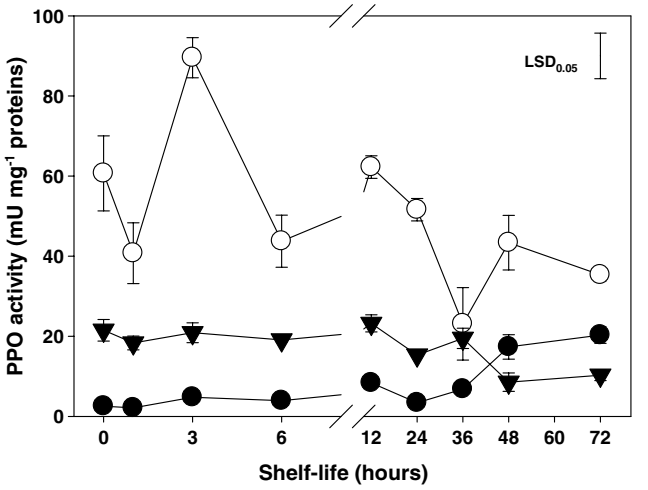

Fig. 6. The activity of polyphenol oxidase (PPO) in fresh-cut leaves of lettuce (closed circle), escarole (open circle) and rocket salad (closed triangle down) stored at 4 °C. Each value is the mean of six replicates (±S.E.). The bar inside the graph indicates LSD.

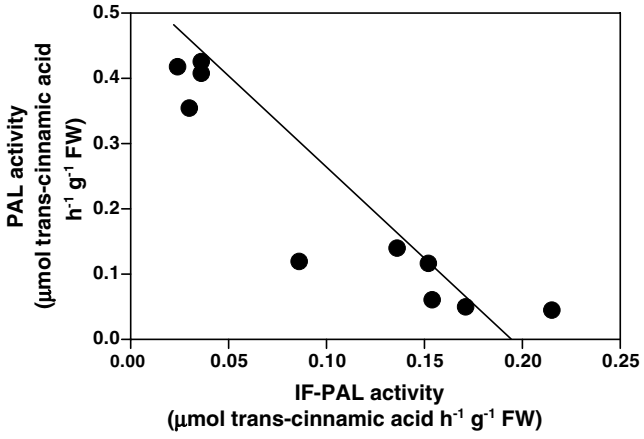

Fig. 4. The relationship between IF-PAL and PAL activity in leaf tissues of minimally processed rocket salad stored at 4 °C.

activity remained quite stable in lettuce and escarole while, in rocket salad, it was much higher with a tendency to rise during storage (Fig. 7).

The changes, upon storage, in the content of *o*-quinones are illustrated in Fig. 8. The content was low and relatively constant in escarole, whereas an initial decrease to values that remained stable until the end of storage was observed in rocket salad; in this species, on average, the content was much higher than that in escarole. In lettuce, the concentration of *o*-quinones was quite variable, as it decreased markedly by 3 h, then increased, reaching a peak by 12 h and finally decreased to very low values at the end of storage, as compared to the contents recorded in the other two species.

Tissue ASA content was much lower in lettuce and escarole than in rocket salad (Fig. 9). In the latter species, the

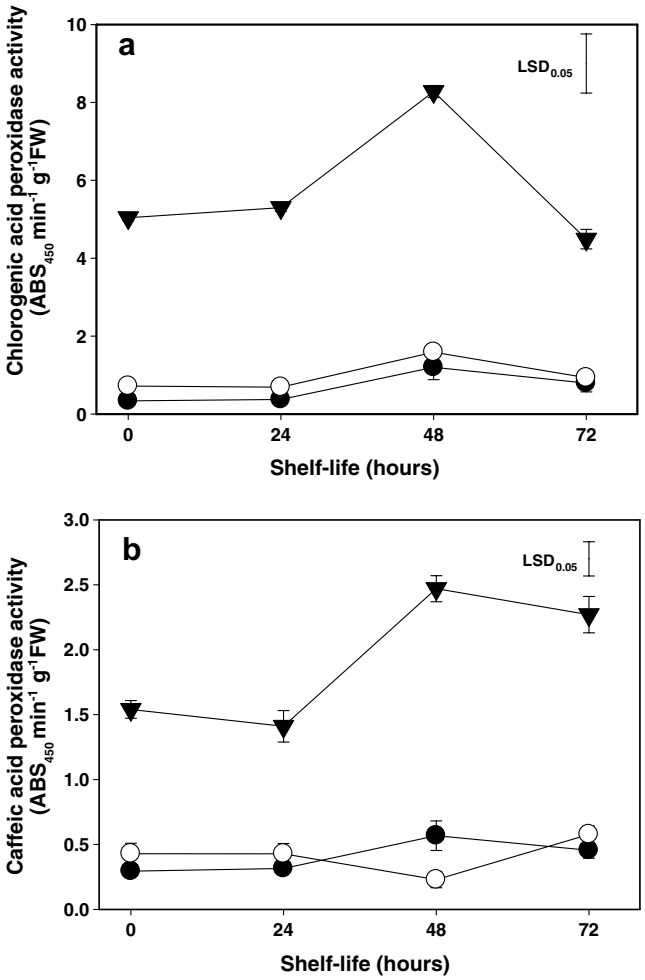

Fig. 7. The activity of peroxidases (POD) in fresh-cut leaves of lettuce (closed circle), escarole (open circle) and rocket salad (closed triangle down) stored at 4 °C. The activity was measured on different substrates: chlorogenic acid (a) and caffeic acid (b). Each value is the mean of six replicates (±S.E.). The bar inside the graph indicates LSD.

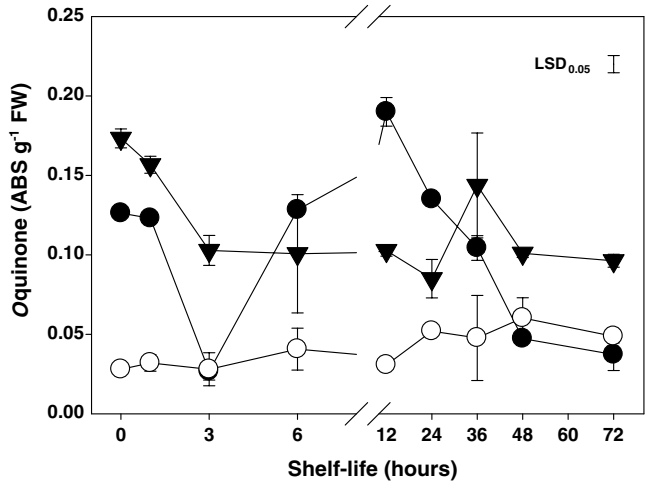

Fig. 8. The concentration of *o*-quinones in fresh-cut leaves of lettuce (closed circle), escarole (open circle) and rocket salad (closed triangle down) stored at 4 °C. Each value is the mean of six replicates (±S.E.). The bar inside the graph indicates LSD.

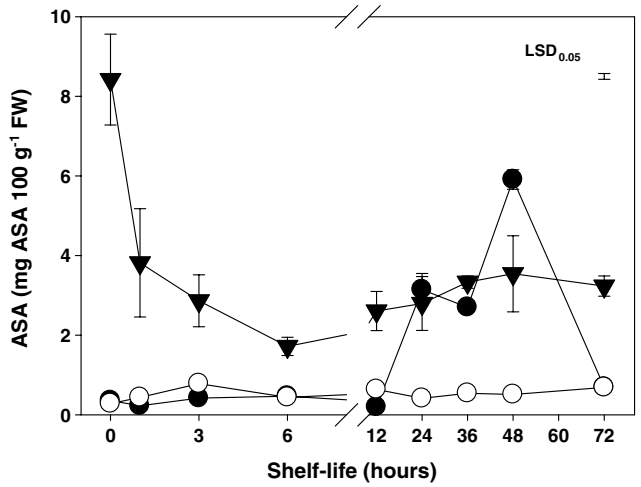

Fig. 9. The concentration of ascorbic acid (ASA) in fresh-cut leaves of lettuce (closed circle), escarole (open circle) and rocket salad (closed triangle down) stored at 4 °C. Each value is the mean of six replicates (±S.E). The bar inside the graph indicates LSD.

content decreased markedly from approximately 8 mg/100 g FW within 1 h of cutting; afterwards it remained rather constant (Fig. 9). By contrast, in the other species, ASA content increased during storage, in particular in lettuce, in which the value by 48 h was even higher than that measured in rocket salad.

4. Discussion

It has been suggested that, in fresh-cut leafy vegetables, the operation of cutting stimulates the activity of PAL, which controls the metabolism of phenols, as well as of PPO and POD, and this results in the synthesis of brown pigments from the oxidation of phenols (e.g., Ke & Saltveit, 1989; Lopez-Galvez et al., 1996; Peiser, Lòpez-Galvez, Cantwell, & Saltveit, 1998; Tomàs-Barberàn, Gil, Castaner, Artes, & Saltveit, 1997).

Only few results obtained in this study with lettuce and escarole, which showed an evident susceptibility to browning, are in accordance with this biochemical model. In fact, cutting induced an increase in PAL activity in both species, but it did not have any important effect on PPO and POD. Other authors have reported that browning is not necessarily related to phenolic content in fruit species such as apples (Couture et al., 1993; Weurman & Swain, 1955). The correlation between the incidence of browning (as assessed by image analysis), and the changes in phenol content upon storage showed a significant result only for the sensitive species lettuce ($y = 4.139 \times -2.159$; $r = 0.752$ $P < 0.05$). Therefore, other factors are likely to be involved in the occurrence of enzymatic browning in fresh-cut leaves.

The possibility of preventing enzymatic browning in fresh-cut products through the application of exogenous ASA suggests the possible involvement of this antioxidant compound in the occurrence of this disorder in wounded tissues (Sapers, 1993). So far, however, there is not a clear relationship between ASA and this disorder, notwithstand-

ing a number of physiological studies conducted in many fruits and horticultural products. For example, Sawamura, Nakagawa, Katsuno, Hamaguchi, and Ukeda (2000) reported that the process of browning in citrus juice, proceeded slowly because of its higher content of ASA. The same was true in apples (Pointing & Joslyn, 1947; Walker, 1962), and in several vegetables (Campos-Vargas, Nonogaki, Suslow, & Saltveit, 2005; Degl'Innocenti et al., 2005; Heimdal, Kuhn, Poll, & Larsen, 1995; Reyes et al., 2007).

In the present work, it was found that rocket salad contained very high amounts of ASA, as compared to lettuce or escarole and, in contrast to these two species, did not show any symptoms of tissue browning until many days after cutting. Contrary to the current biochemical model, earlier described, the resistance of rocket salad to browning was associated with higher activities of PAL, PPO and POD, also compared to the lettuce, that it was quite sensitive to browning. The high activity of PAL detected in rocket salad was matched by a low activity of IF-PAL. Ritenour and Saltveit (1996) also found a negative correlation between the activity of PAL and IF-PAL in fresh-cut leaves of Iceberg lettuce. Rocket salad also had a very high content of phenols, compared to lettuce and escarole.

The high tissue concentration of ASA could determine the reduction of *o*-quinone and/or inhibit the catalytic action of PPO (Sapers, 1993) by decreasing the pH to values below the optimum for PPO activity (pH 5–7), chelating metal ions and/or reducing the availability of oxygen, which are both needed to oxidize phenols (Vamos-Vigyazo, 1981).

In rocket salad, ASA may have prevented the occurrence of browning by means of these mechanisms, since it decreased significantly during storage, while phenol and quinone concentrations did not vary, possibly as result of continuous conversion between these molecules. In the conversion of quinones to phenols, ASA is converted to DHA through a non-enzymatic spontaneous reaction. The reaction in the opposite direction, which would regenerate ASA, is not thermodynamically the most favoured, so that ASA concentration tends to decrease during shelf-life. Agar, Massantini, Hess-Pierce, and Kader (1999) reported that, in kiwifruit fruit, the cutting caused a reduction in ASA levels and a concomitant increase in DHA content. Other authors have reported a low content of ascorbic acid in cut plant tissues (Moretti, Baldwin, Sargent, & Huber, 2002). Diplock et al. (1998) found that, in spinach green leaves, ASA concentration fell down to 10% of the initial content within three days of storage at room temperature. Wills, Wimalasiri, and Greenfield (1984) found that DHA increased in intact fruits and vegetables stored at room temperature. By contrast, in rocket salad fresh-cuts, DHA content decreased upon storage (data not shown), possibly because of spontaneous conversion of DHA to diketogulonic acid, a biologically inactive compound.

Finally, the increase of POD activity observed in rocket salad upon storage may be interpreted to result from the increase in hydrogen peroxide produced by cutting. POD can scavenge hydrogen peroxide by utilizing phenols (Bestwick, Brown, & Mansfield, 1998; Pèrez, Villegas, & Mejia, 2002), which occur in large amounts in this species; this reaction leads to the generation of a phenoxy radical, which can accept electrons from ASA more readily than from other radicals (Nijs & Kelly, 1991; Pèrez et al., 2002).

In this context, the major role of ASA in rocket salad fresh-cut tissues is devoted to control of the radical species formed after cutting. On the other hand, Reyes et al. (2007) recently reported that high levels of reduced ASA in leaf tissues permits the control of reactive oxygen species generated by cutting, while phenols are used for other purposes (e.g., lignin and suberin synthesis). Conversely, in tissues with low levels of reduced ASA, this is consumed readily and phenolics are possibly synthesized partly to control reactive oxygen species.

In conclusion, our findings suggest the involvement of ASA in the protection of rocket salad fresh-cuts against enzymatic browning. This disorder did not appear to be related to the endogenous content of phenols, as suggested by the observation of lettuce and escarole, which were quite sensitive to colour alteration during cold storage. The mechanism by which ASA exerts its protection may be through the direct inhibition of PPO and/or the reduction of quinones to phenols. Work is in progress for further investigation of the possible role of ASA in the tolerance of plant tissues to cut-induced browning.

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