OXIDASES ACTIVITIES AND ANTIOXIDANT CAPACITY OF MINIMALLY PROCESSED BABY ROMAINE LETTUCE (Lactuca sativa L. cv. DUENDE) CULTIVATED UNDER DIFFERENT SALINITY CONDITIONS

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ABSTRACT

Enzymatic browning is a main problem involved in minimal processing and further storage of leafy vegetables, leading to shorter shelf-life of the product. Changes in the two oxidative activities, polyphenol oxidase (PPO) and peroxidase (POD), as well as in total phenolic content, colour parameters and antioxidant capacity (assayed with ORAC method), were monitored during 10 days of storage at 4 °C of minimally processed Baby Romaine lettuce (Lactuca sativa L. cv. Duende) cultivated under 3 different salinity conditions (2,8, 3,8, 4,8 dS/m), in order to determine the most suitable condition for further processing. Increasing levels of salinity reduced both oxidases activities immediately after cutting and throughout 7 days of storage. Samples cultivated under high salinity had also the lowest change in colour, expressed as $\Delta E^* [(\Delta L^2+\Delta a^2+\Delta b^2)^{1/2}]$, and showed the lowest reduction in total phenolic content and antioxidant capacity after 3 days of storage.

INTRODUCTION

Minimally processed lettuce has become popular in recent years because of the increased consumption of fast food and prepared salads. Unfortunately, the damage inflicted to plant tissues by shredding or slicing accelerates reactions that lead to quality defects such as browning of cut edges, discoloration, and reduced turgidity (King et al., 1991). Enzymatic...
browning is a main problem which arises during minimal processing and further storage of lettuce (Loaiza-Velarde et al., 1997; Tomas-Barberan et al., 1997), caused by polyphenol oxidase (EC 1.14.18.1; PPO) and peroxidase (EC 1.11.1.7; POD). PPO is a copper-enzyme that catalyses the \( \alpha \)-hydroxylation of monophenols (monophenolase activity) and the oxidation of the \( \alpha \)-diphenols to \( \alpha \)-quinones (diphenolase activity) using molecular oxygen. The formed \( \alpha \)-quinones undergo a complex series of nonenzymatic chemical changes which ultimately yield melamins and cause a loss of nutritional value due to involvement in reactions with aminoacids. Peroxidase is mainly involved in lignification processes but it can also form melamins as previously described (Richard-Forget and Guillaud, 1997). Besides, the oxidation of phenolic compounds may also influence the antioxidant capacity since polyphenols are known to reduce oxidative stress and prevent chronic diseases (Mueller et al., 1999). The antioxidant properties of these compounds are responsible for their anticancer, antiviral and anti-inflammatory properties (Cao et al., 1997).

Aim of the present work was to value the variations in polyphenol oxidase and peroxidase, as well as in total phenolic content, colour parameters and antioxidant capacity, during 10 days of storage at 4 °C of minimally processed Baby Romaine lettuce (Lactuca sativa L. cv. Duende) cultivated under 3 different salinity conditions (2.8, 3.8, 4.8 dS/m), in order to determine the most suitable condition for further processing.

**MATERIALS AND METHODS**

**Plant materials.**

Baby Romaine lettuce (Lactuca sativa L. cv. Duende) was cultivated in three separated lots of the experimental field of DOFATA department (Catania, Italy), under three different salinity conditions (2.8, 3.8 and 4.8 dS/m). Lettuces were harvested at commercial maturity stage, transported to the laboratory and stored at 4 °C until they were processed the next day. External leaves were removed and next seven lettuce leaves were carefully excised and cut into 2x2 cm pieces. Midribs were excised and discarded. Excised tissues were dipped in a 100 ppm chlorine bath for 1 min at 4 °C and then hand centrifuged to remove surface moisture. Aliquots of about 150 g tissue were placed in perforated polypropylene bags and stored at 4 °C. Three samples for each condition were taken for analysis at 0, 3, 7, and 10 days.

**PPO and POD extraction and assay.**

Twenty grams of lettuce were added of 40 mL cold acetone (–20 °C) and homogenated for 10 minutes. The homogenate was filtered through Whatman 589² paper under vacuum on Buchner funnel; the acetone powder, after elimination of the acetone under vacuum, was collected and suspended in 30 mL 0,1 M citrate phosphate buffer pH 7,5 and kept over night at 4 °C, before being again filtered through Whatman 589² paper under vacuum on Buchner funnel. Clear solution was ultrafiltered in a Millipore stirred cell with 10 kDa membrane (Millipore 8050, Milan, Italy) and utilised as partially purified enzymatic extract.

Enzymatic assay was performed according to a reliable spectrophotometric method, using MBTH to trap the enzyme-generated ortoquinone (Pifferi and Baldassarri, 1973; Espin et al., 1996). PPO activity was assayed spectrophotometrically at 505 nm using 3,4-dihydroxysphenyl acetic acid as phenolic substrate with MBTH. The standard reaction mixture contained 0,9 mL of 40 mM phenolic substrate, 0,1 mL of 2% (w:v) MBTH in methanol, 0,05 mL of DMF, 1,5 mL of 50 mM sodium acetate buffer pH 7,0 and 0,5 mL of enzymatic extract. Reaction was stopped at different times with 0,5 mL of 5% H\(_2\)SO\(_4\). Blank was prepared by inverting the order between the enzymatic extract and H\(_2\)SO\(_4\). One unit of PPO activity is
defined as the amount of enzyme which produces 1 μmol of adduct per min at 25 °C under the conditions above described.

POD activity was determined spectrophotometrically as the change in absorbance at 470 nm. The reaction mixture contained 2 mL of 0.01 M citrate phosphate buffer (pH 7,0) containing 1,0% (v/v) guaiacol, 0.25 mL of 32 mM H2O2 and 0.1 mL enzyme extract (Flurkey and Jen, 1978). One guaiacol unit (U) is defined as the amount of enzyme which oxidizes 1 μmol of guaiacol per min at 25 °C and pH 7,0 under the conditions above described.

**Color of lettuce samples.**

The colour was determined with a compact tristimulus chromameter (Minolta CR-300, Ramsey, NJ, USA) with an 8 mm Ø viewing aperture, white plate reference (Y=94,3; x=0,3142; y=0,3211) and C illuminant (CIE, 2° observer) was used. Readings were expressed as L*, a* and b* parameters. ΔE* [((ΔL*2+Δa*2+Δb*2)1/2] parameter was calculated.

**Total phenolics determination.**

Aliquots of ground lettuce (25 g) were extracted with 50 mL of methanol, under continuous stirring for 1 h at room temperature. Samples were then centrifuged at 4000xg for 20 min at 4 °C and filtered through Whatman no. 42 paper under vacuum on Buchner funnel.

Total phenolics content was determined according to the Folin-Ciocalteau method (Singleton et al., 1999), using catechin as a standard. Results were expressed as μg catechin equivalents per gram of fresh weight.

**Antioxidant capacity.**

The total antioxidant capacity of lettuce methanolic extracts was determined by means of oxygen radical absorption capacity (ORAC) assay. The automated ORAC assay was carried out on a Wallac 1420 spectrofluorometric analyzer (Perkin Elmer, Turku, Finland; excitation wavelength = 485 nm and emission filter = 515 nm), basing on a slightly modified procedure proposed by Ou et al. (2001). Results were expressed as μmol of Trolox equivalents (TE) per 100 grams of fresh weight.

**RESULTS AND DISCUSSION**

**Oxidases activities.**

Polyphenol oxidase and peroxidase activities were monitored during 10 days of storage at 4 °C. Results are shown in Figures 1 and 2. Wounding of tissues due to minimal processing caused an activation of both enzymatic activities, much more evident after 7 days of storage. The increase of PPO activity seemed to be correlated with the low salt availability, whereas no marked increase was observed at higher salt concentrations. The increase of polyphenol oxidase activity ranged from 2,3% to 21,0% at day 3, reaching 59,8% at day 7 in samples cultivated under low salinity condition (2,8 dS/m). An increasing trend upon storage was also noticed relatively to peroxidase activity, with more similar activity among samples than PPO and a drastic increase up to day 7 (about 4 times higher than the initial value).

Again, POD extracts from samples cultivated under low salinity showed the highest activity (0,431 U/g fw at day 7) in comparison with the other two conditions (0,361 and 0,404 U/g fw at day 7 for medium and high salinity conditions, respectively). The increase of PPO activity after wounding is mainly due to activation process from latent to fully active form. Infact, as previously reported by Cantos et al. (2001), tissue wounding involves the decompartmentalization of cellular components with the subsequent release of proteases involving a cascade of reactions leading to the activation of latent PPO. On the other hand, Ke and Saltveit (1989) associated increased POD activity with cell wall lignification in response to wounding, because POD is involved in the lignin-specific pathway. The highest activity of oxidases extracts from samples cultivated under low salinity could be explained by the high availability of free water for the enzymatic reactions. Moreover, in earlier work (Spagna et al.,
we demonstrated the inhibition of PPO in presence of high concentrations of NaCl in the assay medium, due to the interaction of NaCl with the copper at the active centre of the enzyme.

**Browning of minimally processed lettuce.**

The color of Baby Romaine lettuce was measured during storage, taking \( \Delta E^* \) (\( \Delta E^*=(\Delta L^*^2+\Delta a^*^2+\Delta b^*^2)^{1/2} \)), a widely used parameter for determining color differences perceptible by human eye, as an index for colour changes. Results are reported in Table 1. It was possible to notice a sharp colour variation in all samples after 3 days of storage, more evident in samples cultivated under low salinity (\( \Delta E^*=9,92 \)) and 5,41 for samples cultivated under low and high salinity, respectively). Degradation of colour evidenced by \( \Delta E^* \) value was mostly due to variation in lightness (\( L^* \)) and green-red (\( a^* \)) values, whereas blue-yellow (\( b^* \)) chromatism did not influence significantly such parameter. Colour degradation was linear up to day 10 in samples cultivated under low salinity, while there was no significant difference between day 7 and 10 in samples treated with medium and high salinity.

**Antioxidant capacity.**

Since the polyphenolic content is an important source of antioxidant power of leafy vegetables, as widely reported in literature (Caldwell, 2003; Llorach et al., 2004), the variation of both total phenolics and antioxidant capacity was monitored throughout the cold storage of minimally processed lettuce. A general decrease of phenolic content was noticed in all samples (Fig. 3), reaching minimum values at day 7, in correspondence of maximum values of PPO and POD activity. Extracts from samples cultivated under low salinity showed the highest phenolics degradation after 7 days of storage (-65,3% of the initial value). Such decrease could be explained by the oxidation associated with the browning reaction, considering the increasing trend of oxidases activity upon storage. A very similar decreasing course was noticed relatively to antioxidant capacity of lettuce methanolic extracts (Fig. 4). Infact, also in this case, the highest variation was found after 7 days of storage at 4 °C in samples cultivated under low salinity (-57,3% of the initial value), although a general diminution of ORAC units was observed in all samples. As far as we know, no work in literature reports the changes of antioxidant capacity (measured with the ORAC method) during storage of fresh-cut lettuce.

In order to verify the role of polyphenols in determining the antioxidant capacity of the product, the correlation between ORAC values and total phenolics was calculated (Fig. 5). The different phenolic contents of methanol extracts were positively correlated with the antioxidant capacity, with \( r^2 \) values ranging from 0,91 to 0,98. These results agree with those reported in literature relatively to lettuce (Llorach et al., 2004). In samples cultivated under high salinity (4,8 dS/m), it was noticed the highest ratio between ORAC values and total phenolics throughout the storage period.

**CONCLUSIONS**

Cultivating Baby Romaine lettuce under conditions of low salinity showed to be unappropriate for further minimal processing, due to exposure of the product to oxidases activities and browning phenomena throughout the cold storage. Moreover, the oxidation of phenolic compounds inevitably leads to a drastic decrease of the antioxidant capacity, since the two parameters are positively correlated. Increasing levels of salinity (up to 4,8 dS/m) were effective in reducing PPO and POD activities, color changes and phenolics degradation, preserving then the antioxidant capacity of the product.
REFERENCES


Table 1. ΔE* values of minimally processed Baby Romaine lettuce during 10 days of storage at 4 °C. Salinity conditions: B1=2,8 dS/m; B2=3,8 dS/m; B3=4,8 dS/m.

<table>
<thead>
<tr>
<th>Days of storage at 4 °C</th>
<th>B1</th>
<th>B2</th>
<th>B3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 3</td>
<td>9,92a ± 1,11</td>
<td>7,75a ± 0,67</td>
<td>5,41a ± 0,32</td>
</tr>
<tr>
<td>Day 7</td>
<td>15,01b ± 1,43</td>
<td>14,82b ± 1,34</td>
<td>12,25b ± 1,32</td>
</tr>
<tr>
<td>Day 10</td>
<td>20,18c ± 1,65</td>
<td>14,06b ± 1,11</td>
<td>12,77b ± 1,10</td>
</tr>
</tbody>
</table>

Z Means in a same column followed by the same letter are not significantly different at the P≤0.05 level according to Duncan’s Multiple range test.

Figure 1. Polyphenol oxidase activity in minimally processed Baby Romaine lettuce during 10 days of storage at 4 °C. Salinity conditions: B1=2,8 dS/m; B2=3,8 dS/m; B3=4,8 dS/m.
Figure 2. Peroxidase activity in minimally processed Baby Romaine lettuce during 10 days of storage at 4 °C. Salinity conditions: B1=2.8 dS/m; B2=3.8 dS/m; B3=4.8 dS/m.
Figure 3. Total phenolic content of minimally processed Baby Romaine lettuce during 10 days of storage at 4 °C. Salinity conditions: B1=2,8 dS/m; B2=3,8 dS/m; B3=4,8 dS/m.
Figure 4. Antioxidant capacity of minimally processed Baby Romaine lettuce during 10 days of storage at 4 °C, expressed as ORAC units (µmol Trolox equivalent/100g fw). Salinity conditions: B1=2,8 dS/m; B2=3,8 dS/m; B3=4,8 dS/m.
Figure 5. Dependence of antioxidant activity (ORAC) on phenolic content in minimally processed Baby Romaine lettuce during 10 days of storage at 4 °C. Salinity conditions: B1=2,8 dS/m; B2=3,8 dS/m; B3=4,8 dS/m.