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Effect of cutting on ascorbic acid oxidation and recycling in fresh-cut baby spinach (*Spinacia oleracea* L.) leaves

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ABSTRACT

Fresh-cut spinach during processing undergoes several mechanical procedures such as cutting, which may induce stress responses. These stresses may trigger the accumulation of harmful reactive oxygen species (ROS). Plants respond through a wide range of mechanisms and ascorbic acid (AsA) has an important role. The combined effect of cutting, temperature and storage time on AsA recycling route in spinach fresh-cut leaves was studied. AsA, gene expression and activities of the enzymes involved in the AsA oxidation and recycling were considered. Spinach leaves were cut in six pieces and stored at 4 °C or 20 °C. AsA content and enzymes activities were measured over six days of storage, while gene expression analyses were performed in a time-point experiment within 24 h after cutting. Results showed that AsA decreased after cutting (from 19.41 mg/100 g FW to 15 mg/100 g FW) and generally was higher in samples stored at 4 °C. After six days, AsA was 10 mg/100 g in control and 5 mg/100 g FW in cut leaves. The expression of genes and activities of the enzymes involved in the AsA oxidation and recycling route were, for some enzymes, in accordance with AsA levels. The APX (EC 1.11.1.11) activity after cutting increased up to 290 nmol AsA mg^{-1} prot min^{-1} compared to the control with 190 nmol AsA mg^{-1} prot min^{-1}. AsA reduction is firstly affected by temperature and aggravated by cutting procedures. AsA represents a valuable postharvest quality indicator of freshness in spinach leaves.

1. Introduction

Recent years have been characterized by an increase in the demand for *ready to use* baby spinach leaf among consumers, and consequently production has strongly increased. Before the development of ready to eat products, spinach had been commonly eaten as adult leaves after cooking. The growing interest toward *ready to use* products is due to the ease of use and practicality, reducing the timing for meal preparations. Moreover, health-oriented consumers are attracted by these products as they represent a good source of phytochemicals and antioxidants involved in some disease prevention (Ames et al., 1993; Gil et al., 1999).

Fresh-cut baby spinach leaves are usually marketed after minimal processing consisting mainly of cutting and washing procedures, and they have a shelf-life of 5–6 days. Even though the processing is mild, fresh-cut spinach leaves undergo a loss of nutritional and quality appeal because tissues are damaged. A preliminary investigation of the effects of cutting concentrated on cell membrane degradation and phospholipases C and D. with results showing that phospholipases were not involved in the primary responses to cutting (Antonacci et al., 2011). However, it is well known that cutting procedures during fresh-cut preparation can trigger or speed up senescence processes and oxidative stresses that lead to accumulation of reactive oxygen species (ROS), which are important in physiological processes and abiotic stress responses in plants (Halliwell, 2006; Gill and Tuteja, 2010). Normally, ROS levels are controlled by adequate protective systems, but in the case of oxidative stress, those systems are no longer able to limit ROS accumulation and this could lead to cell damage, cell death and limit normal cell functions (Noctor and Foyer, 1998; Mittler, 2002).

Ascorbic acid (AsA) is an effective radical scavenger that is able to interact with ROS that are produced during oxidative stress responses (Davey et al., 2000; Foyer and Noctor, 2003; Maggio et al., 2002; Barbieri et al., 2012). AsA is involved in the ascorbate-glutathione cycle (Fig. 1), together with glutathione and with the enzymes ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR) and glutathione reductase (GR) (Nakano and Asada, 1981). This cycle represents an efficient way of H_2O_2 scavenging and has been found to be active in several plant cell organelles (Shigeoka et al., 2002).

Studies conducted on the effect of cutting on the ascorbate-glutathione cycle has allowed understanding of the mechanism of stress response and the metabolic changes in several plant species.

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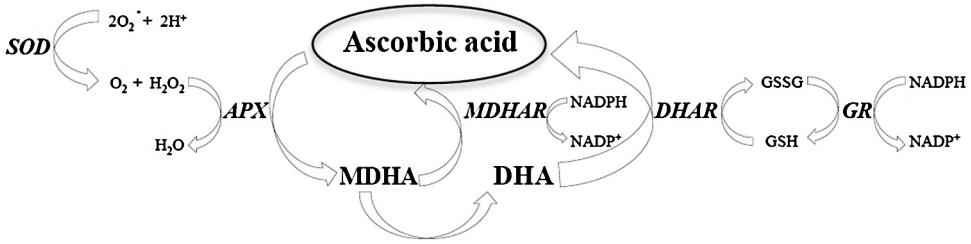

Fig. 1. The main AsA oxydation and recycling pathway. The superoxide radicals ($O2^{\bullet}-$) are dismutated by superoxide dismutase (SOD) with the contemporary production of hydrogen peroxide (H_2O_2). H_2O_2 is converted to water by the reaction catalyzed by ascorbate peroxidase (APX) with the production of oxidized forms of AsA monodehyroascorbate and dehyroascorbate (MDHA and DHA). Monodehyroascorbate reductase (MDHAR) reduces the MDHA to AsA. However, part of the MDHA may be converted to AsA and DHA by non-enzymatic reactions. The DHA is converted to AsA by dehydroascorbate reductase (DHAR), contemporary the enzyme glutathione reductase (GR) catalyzes the last step of the cycle in which the oxidized form of glutathione (GSSG) produced by the action of DHAR is converted to the reduced form (GSH) with consumption of NADPH.

For example, senescent leaves of *Pisum sativum* L. showed an increment in H_2O_2 levels and, in parallel, a significant reduction in the activity of DHAR and GR in the mitochondrial matrix and of APX and MDHAR in the mitochondrial membrane; the authors indicate that this phenomena is a specific response to senescence (Jiménez et al., 1997, 1998a). Considering different approaches, several studies have demonstrated the influence of cutting on the transcriptional regulation of genes involved in AsA biosynthesis, oxidation and recycling and on the accumulation of this important vitamin in crops. A recent publication reports that cutting procedures lead to an induction of the expression of genes encoding for the main enzymes of the ascorbate-glutathione cycle and to an increment in AsA levels in tomato (Ioannidi et al., 2009). These data disagree with those reported with sliced potatoes (Liso et al., 1985), suggesting that the mechanisms that regulate cutting stress responses could differ among different species. In another paper Lee and Kader (2000) reported that losses in AsA depend on the severity of cuts. Cutting could induce a total consumption of AsA in tomato but not in *Arabidopsis* (Suza et al., 2010). The authors hypothesized the presence of alternative antioxidants able to scavenge ROS in *Arabidopsis*. In *Arabidopsis*, wounding induced the expression of genes involved in the main AsA biosynthetic pathway as well as in those of the alternative myo-inositol pathway and consistently determined an increment in AsA levels after the treatment (Suza et al., 2010). However, gene expression is not always a good predictor of AsA levels. For example, in acerola, it has been observed that some genes in the AsA biosynthetic pathway appeared to be overexpressed 24 h after cutting, while after 48 h the same genes were down-regulated, but in both cases AsA levels were higher in control compared with cut fruit (Badejo et al., 2009).

The effect of cutting on AsA metabolism is therefore influenced by plant species and tissues and it depends on the extent of the mechanical injury. Application of different combinations of time and temperature during storage represent a useful strategy to better understand the mechanisms of stress response in minimally processed leafy vegetables. The aim of this work was to study the effects of cutting and storage temperature on the expression of genes encoding for the enzymes involved in ROS detoxification as well as on the specific activity of the corresponding enzymes. Moreover, data obtained from the molecular and biochemical analyses have been compared with AsA levels. This study may give a better indication of the physiological stage of spinach leaves during storage, evaluating the combined effect of time, temperature and cutting.

2. Materials and methods

2.1. Plant material

Spinach (*Spinacia oleracea* L., fam. *Amaranthaceae*) plants of the cultivar 'Bella' were hydroponically grown in a growth chamber. The environmental factors were set to 16 h photoperiod, relative humidity 60–70% and light intensity 400 W m^{-2}. Leaves were harvested as they reached the fully expanded stage as required for the fresh-cut industry.

Wounds were performed by cutting freshly harvested leaves into 4–6 pieces, while intact leaves were used as controls. Samples were collected in sealed plastic bags and stored in dark conditions at 4 or 20 °C for six and three days respectively. Relative humidity inside the bags ranged between 95% and 98%. Five bags for each treatment were prepared.

Samples were collected 1 h after cutting (T0) as well as after one, three, and six days for analyses of AsA content and enzymatic assays. At 20 °C, the sampling at six days was not performed since the spinach leaves were in advanced stage of deterioration. For molecular analyses, samples stored at 20 °C were collected 1 h (T0), and 3, 6, 9 and 24 h after cutting procedures.

2.2. Total RNA isolation

Total RNA was extracted from leaves (about 1 g) according to Wan and Wilkins (1994) with some modifications. Spinach leaves were grounded in a mortar under liquid N_2 and the powder transferred to 5 volumes of a 80 °C preheated extraction buffer (0.2 M sodium borate decahydrate/30 mM [ethylenebis(oxyethylenenitrilo)] tetraacetic acid/1% (w/v) sodium lauryl sulfate/1% (w/v) deoxycholic acid/10 mM dithiothreitol (DTT)/1% (w/v) Igepal/2% (w/v) polyvinylpyrrolidone-40). The extracts were vortexed for 30 s to thoroughly mix, then 0.015% (w/v) proteinase K (Sigma, Italy) was added before the tubes were gently inverted and placed horizontally in a shaking incubator at 42 °C for 1.5 h. Then 0.08 volumes of 2 M KCl were added and the extracts were incubated on ice for 30 min, then centrifuged at 26,000 × *g* for 20 min at 4 °C. The supernatant was transferred into a new tube and an equal volume of 4 M LiCl (Sigma, Italy) was added and precipitated overnight. The precipitate was then pelleted by centrifugation at 26,000 × *g* for 30 min at 4 °C, resuspended in 800 μL of sterile water and an equal volume of chloroform was added, vortexed and centrifuged for 5 min at room temperature. The upper part of the surnatant was transferred into a new tube (400 μL) and 500 μL of diethyl pyrocarbonate-treated water (DEPC water) was added to the chloroform and the mixture was vortexed and centrifuged again. Another 400 μL were taken from the upper layer and transferred into the tubes with the previous supernatant. Total supernatant was 800 μL and 160 μL of 3 M sodium acetate were added plus 800 μL isopropanol. The mixture was left on ice for 30 min. The resulting turbid solution was centrifuged at 16,000 × *g* for 10 min at 4 °C, the RNA pelleted was washed with 80% (v/v) ethanol and resuspended in 100 μL sterile water. The RNA was quantified at A_{260} nm.