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Use *In Vivo* of Natural Anti-Browning Agents Against Polyphenol Oxidase Activity in Minimally Processed Eggplant

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Plant polyhenol oxidase (PPO, EC 1.14.18.1) is responsible along with other oxidases for the enzymatic browning reaction occurring during handling, storage and processing of vegetables. This work aimed at assessing the efficacy in vivo of some natural anti-browning agents in minimally processed eggplants. They were collected, washed, diced, submitted to dipping with inhibitors (L-ascorbic, benzoic, citric, ferulic and L-glutamic acid) at three concentrations (0.2, 0.5 and 1 %) and packed in ordinary atmosphere bags (PET), covered by a double barrier film and refrigerated for 7 days (4.0 ± 0.5 °C, 95 % RH). The enzymatic activity was inhibited by addition of all anti-browning agents tested. At t=7 the greatest reduction in PPO activity was observed at the highest concentrations (0.5 and 1 %) of the inhibitors in the following order: ferulic acid (-43 %), L-glutamic acid (-32 %), citric acid (-27 %), L-ascorbic acid (-21 %) and benzoic acid (-15 %). These positive effects were also translated in terms of browning index, demonstrating the efficacy of the anti-browning treatments studied to extend the shelf-life of minimally processed eggplants.

1. Introduction

Polyhenol oxidase (PPO, EC 1.14.18.1) is an enzyme responsible, along with other oxidases, for the browning reaction occurring during handling, storage and processing of vegetables and fruits, since after cutting, the phenolic substrates come in contact with this enzyme causing a cascade of reactions (Espìn et al., 1995; Castaner et al., 1999; Barbagallo et al., 2009).
PPO is predominantly located in cell organelles such as chloroplasts thylakoid, mitochondria and peroxisomes where it is firmly bound to the membrane and may even be found in the soluble fraction of the cell (Barbagallo et al., 2009; Scuderi et al., 2011). In young and unripe fruits, it is mostly present under conjugated form, while in those ripe in the soluble fraction (Conforti et al., 2007).
Within *Solanaceae*, the eggplant is one of vegetables mostly affected by PPO, due to the extensive chlorogenic acid content, the main substrate for activating these biochemical reactions (Morishita and Ohnishi, 2001; Kwak and Lim, 2005).
Recently, along as fresh product, eggplants are consumed as minimally processed vegetables, which represents an increasing sector of market, favored by the consumers who look for healthy food that

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requires a minimum preparation time. These minimally processed vegetables are precut, washed and conditioned under plastic packaging of household or collective size.
To prevent the rapid browning of the product, many strategies are proposed to contrast the PPO activity. The traditional use of sulphites and their derivatives as inhibitors of quinones polimerisation and following production of uncoloured compounds, is a good solution but their use may cause allergic reactions in asthmatics (Sapers, 1993; Martinez and Whitaker, 1995).
Consequently, the current researches are addressed towards different solutions than sulphites, introducing the use of natural inhibitors (Nicoli et al., 1999; Raju and Bawa, 2006).
Among these, the most widespread are organic acids and ascorbic acid. The phenolic compounds extracted from plants, able as well to contrast the microbial spoilage, due to their repulsive action (Kwak and Lim, 2005) are of great interest.
This research aimed at evaluating the PPO activity in fruits of eggplant cv. 'Birgah', cultivated under cold green house, cut as cubes and treated *in vivo* with inhibitors (L-ascorbic, benzoic, citric, ferulic and L-glutamic acids) at different concentrations, in order to extend the shelf-life of this minimally processed vegetable.

2. Material and Methods

2.1. Minimally processed eggplant preparation

Fruits of 'Birgah' eggplant were obtained under cold greenhouse conditions in Pachino (Siracusa, Italy). They were uniformly sized, round shaped and light purple coloured.
In laboratory, the fruits were sanitized into a cold 100 ppm chlorine solution for 30 sec, dried, cut as cubes (2.5x2.5x2.5 cm), submitted to dipping for 2 min in a bath with five different inhibitors, L-ascorbic acid (Sigma, ≥99.0% purity), benzoic acid (Sigma-Aldrich, ≥99.5% purity), citric acid (Sigma-Aldrich, ≥99.5% purity), ferulic acid (Aldrich, 99% purity), L-glutamic acid (Aldrich, 98.5% purity) at three different concentrations (0.2, 0.5 and 1 %) and randomly packed in ordinary atmosphere bags (polyethylene terephthalate, PET), covered by a double barrier film, and refrigerated for 7 days (4.0 ± 0.5 °C, 95 % RH).
Three bags were collected daily and PPO activity was measured in fruits. In the last sampling day only, the browning index was also measured. For this purpose, eggplant cubes were homogenizing into an iced bath for 5 min, minimizing light and oxygen exposition by wrapping the samples in aluminum film.

2.2. Polyphenol oxidase (PPO) determination

Ten grams of eggplant homogenate were added of 25 mL cold acetone (-20 °C) continuously stirred for 10 min. The homogenate was filtered through Whatman No. 42 paper under vacuum on Buchner funnel and the acetone powder collected and suspended in 15 mL 0.1 M citrate phosphate buffer at pH 6.5 and kept overnight at 4 °C, before being again filtered under vacuum. Clear solution was ultrafiltered in a stirred cell with 50 kDa membrane (Millipore, Milan, Italy) and utilised as enzymatic extract assayed spectrophotometrically (Cary IE-100 UV-VIS, Varian, USA) at 505 nm.
The PPO activity was assayed using catechol as phenolic substrate, N,N-Dimethylformamide (DMF), citrate phosphate buffer and MBTH (3-metil-2-benzotiazolinone idrazone) as chromophore agent, according to a modified version of the method proposed by Spagna et al. (2005). The reaction was stopped with H_2SO_4 1M. Blank was prepared by inverting the order between the enzymatic mixture and H_2SO_4.
One unit of PPO activity was defined as the amount of enzyme which produces 1 µmol of adduct per min at 25 °C under the conditions above described.
The results were expressed as relative enzymatic activity, which represents the residual PPO activity reached after adding different natural inhibitors to the packages.

2.3. Browning index

Browning index was measured from the homogenized product into an iced bath, after centrifugation at 10,000 x *g* per 20 min at 2 °C, filtration through Whatman No. 2 under vacuum on Buchner funnel and following spectrophotometric reading at 420 nm (Jeong et al., 2008).
High absorbance values correspond to a greater tissue browning. Results were expressed as percentage reduction respect to control in each measurement.

2.4. Reagents

All chemicals used were of analytical grade and supplied by Sigma-Aldrich Chemicals Co. (Milan, Italy).

2.5. Statistical analysis

Data were statistically analysed by analysis of variance (ANOVA) using CoStat version 6.003 (CoHort Software). Differences among means were evaluated for significance using the Tukey-HSO test at $p \leq 0.05$. Different letters above bars represent statistical differences.

3. Results and Discussion

The PPO activity was inhibited by addition of anti-browning agents (**Figure 1**). In order to better evidence the enzymatic changes in comparison to the untreated control, the results were graphically represented as relative activity, by fixing equal to one the initial enzyme activity.

For each sampling among the different treatments performed, columns with same letter are not significantly different according to Tukey-Kramer test ($p \geq 0.05$).

The greatest increase in PPO activity was measured at t=1 as a consequence of stress due to cut and following contact between degenerative enzymes and their substrates.

The inhibitors started to be effective from t=2, in which ferulic, glutamic and L-ascorbic acids, in decreasing order respectively, resulted the most effective. Probably some additional enzymatic reactions (such as phenylalanine ammonia lyase and peroxidase) or non-enzymatic reactions, occur inside packaging (Barbagallo et al., 2009).

The L-ascorbic acid treatment (**Figure 1a**) reduced PPO activity by 10-23 % (0.2 % concentration) and 15-21 % (0.5 %) whereas at the highest concentration (1 %) PPO activity decreased by 17-25 %. From t=3 onwards, the highest ascorbic acid concentration induced proportional decreasing percentage reduction of PPO activity.

The action of ascorbic acid in browning prevention consists of a chemical reduction of *o*-quinones to di-phenols, leading to the formation of uncoloured compounds, as well as a direct inhibition effect on enzyme. Ascorbic acid acts also as scavenger by removing the molecular oxygen from the reactions catalyzed by PPO and moreover it has a chelating effect on Cu present on prosthetic group of the enzyme. Nevertheless, it is generally a temporary effectiveness since ascorbic acid is irreversibly oxidised during the process (McEvily et al., 1992).

The treatment with benzoic acid (**Figure 1b**) reduced the PPO activity by 2-8 % at t=1 according to the concentration used, then the incidence of the oxidative process compensated the inhibitor action which progressively increased to 3-15 % until t=5. At the end of storage period (t=7), no differences were noticed in PPO inhibition between tests performed at 0.5 and 1 % inhibitor concentration.

Benzoic acid is an aromatic carboxylic acid present in several plant tissues and in blueberry fruits in particular, also used as an additive (from E210 to E219), with antimicrobial function in non-alcoholic beverages (Sapers et al., 1989). The fact that benzoic acid showed the same inhibitor action at 0.5 % and at 1 % concentration, should represent an advantage for eventual dipping at low dosage, since benzoates and parabens may provoke allergy to consumers.

Citric acid (**Figure 1c**) reduced PPO activity by 13-21 % at t=1, then inhibitor action reduced up to t=3 at lowest dosage used, whereas at 1 % PPO inhibition showed again high values (21-27 %).

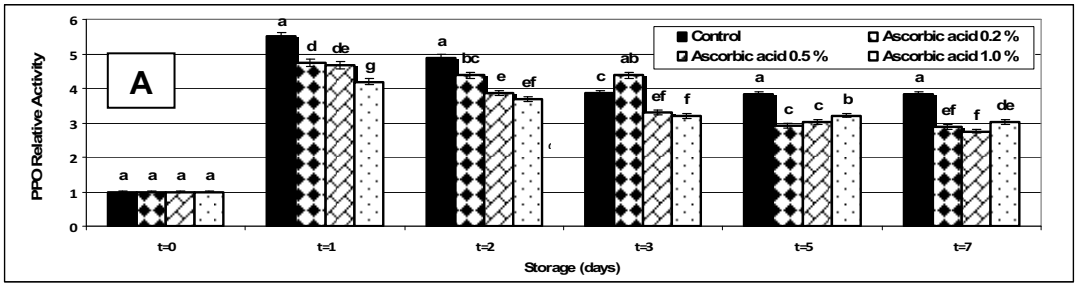
Citric acid acts as anti-browning agent by both pH reduction and direct action on PPO enzyme due to chelating action on Cu present on active site of the enzyme (Richardson and Hyslop, 1985; Raju and Bawa, 2006). In fact, the ionizing groups of protein structure of the enzymes are influenced by pH. These groups must be in appropriate ionic form to maintain the active site form, to bind the substrate or catalyze the enzymatic reactions. However, all changes of ionization form of enzymes are generally reversible. Also the substrate stability is influenced by pH variations, undergoing chemical degradation and acting as enzymatic inhibitor (Tipton and Dixon, 1983).

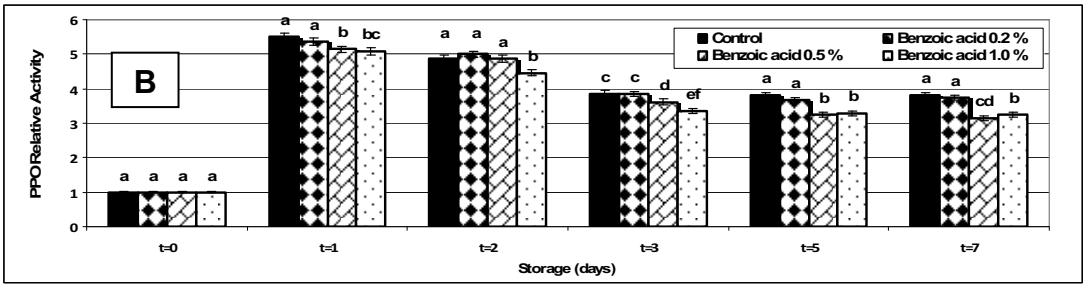
Treatment with ferulic acid (**Figure 1d**) was effective reducing the enzymatic activity up to t=7 with increasing concentrations (9-13 % and 25-36 % PPO activity reduction, at 0.2 e 0.5 % inhibitor concentration, respectively), whereas at 1 % PPO activity reduced by 36-37 % already from t=1 without any decrease at following collections.

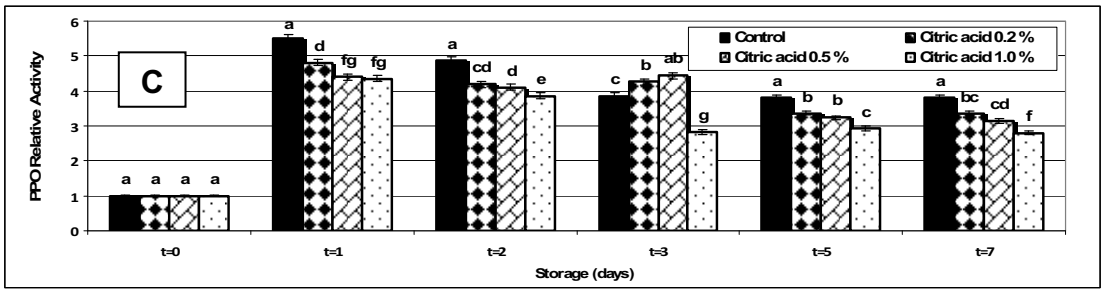
The anti-browning effect of ferulic acid is due to hydroxyl group (OH-) as electron donor to the intermediary quinone and to cross-linking the enzyme with a weak hydrogen bond. Ferulic acid could inhibit PPO activity as competitive inhibitor. In fact, aromatic carboxylic acids of cinnamic acids and analogues, as *p*-coumaric, ferulic and sinapic acids, represent PPO competitive inhibitors due to their structure similar to phenolic substrates (Kwak and Lim, 2005; Raju and Bawa, 2006).
L-Glutamic acid dipping (**Figure 1e**) reduced PPO activity by 12-19 %, 18-29 % and 30-33 % following the increase of inhibitor concentration and the storage days, but at lower dosage (0.2 %) the inhibitor action decreased up to t=5. At 1 % the maximum inhibition (33 %) was noticed at t=2. At intermediate concentration (0.5 %), the inhibitor action was 29 % at the end of storage period, similar result to that obtained with an higher concentration.
L-Glutamic acid is a di-carboxylic amino acid present in broccoli and cabbage, widely employed in food industry as a savour booster together with monosodium glutamate (E621). The side group of its chiral molecule gives an acid behaviour. It has a role in glutathione (GSH) formation, the most important antioxidant in human organism. However, high concentration of glutamate may provoke intolerances in some consumer, being the main neurotransmitter of central nervous system (Doğan et al., 2007). Then, an employment at intermediate concentrations in dipping treatment could be positive.
By considering the statistic significance ($p \leq 0.05$) for each sampling day among all performed treatments, at the end of storage period (t=7) and at the lowest applied concentration of anti-browning agents (0.2 %), no difference was noticed among treatments with citric, ferulic and L-glutamic acids.
At intermediate concentration (0.5 %), no significant difference ($p \geq 0.05$) was noticed between benzoic and citric acids application.
At highest concentration (1 %), the differences among tested inhibitors was again significant ($p \leq 0.05$). L-Ascorbic acid worked at its best condition at 0.5 % dosage. No significant differences were found between benzoic and ferulic acids at 0.5 or 1 %, while citric and L-glutamic acids showed best results at 1 %.
The greater reduction in PPO activity in terms of percentage was observed at the highest concentration of the inhibitors, with the following order: ferulic acid (-43 %), L-glutamic acid (-32 %), citric acid (-27 %), L-ascorbic acid (-21 %) and benzoic acid (-15%).
These positive effects were also translated in terms of browning index: ferulic acid (-39 %), glutamic acid (-25 %), citric acid (-19 %), ascorbic acid (-16 %) and benzoic acid (-13 %).

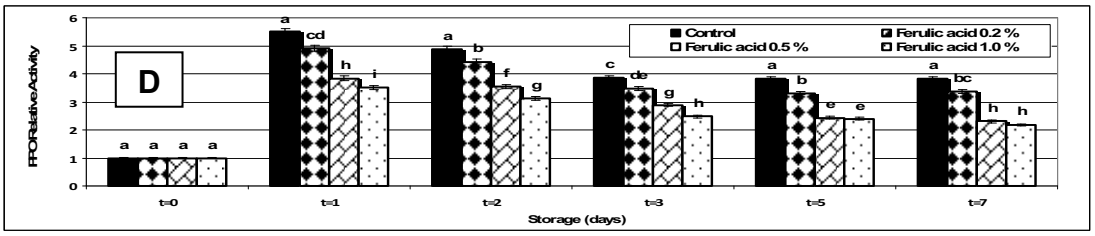
4. Conclusion

The minimally processed eggplants submitted to anti-browning dipping exhibited a significant decrease of PPO activity, especially at the highest concentrations (0.5 and 1 %) of the tested inhibitors.
These results were also confirmed by the browning index percentage, which was significantly reduced by those inhibitors. The anti-browning treatments may extend the shelf-life of minimally processed eggplant, thus suggesting an industrial application, e.g. the addition of one or more of these inhibitors into vegetable packages.









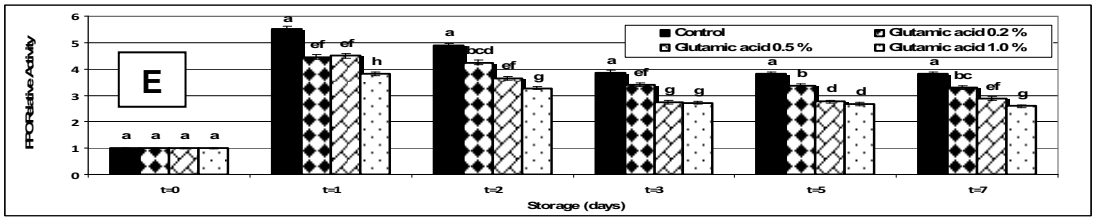


Figure 1: Changes of PPO relative activity during storage through anti-browning treatments with, L-ascorbic acid (A), benzoic acid (B), citric acid (C), ferulic acid (D), L-glutamic acid (E) respectively, at increasing concentrations (control, 0.2 %, 0.5 %, 1 %, respectively). For each storage day, columns with same letter are not significantly different according to Tukey-Kramer test ($p \geq 0.05$).

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