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Is it Possible to Combine High Content in Phenolics with Low Browning in Fruits and Vegetables? A case in Eggplant

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Abstract. Demand among consumers for fruits and vegetables with improved contents in bioactive compounds is increasing. In particular, a lot of attention is being paid to phenolic compounds, as they have been reported to present many beneficial effects for human health. However, oxidation of phenolic compounds present in the tissues of fruits and vegetables by polyphenol oxidases (PPOs) can result in browning, which affects negatively the quality of the produce. Eggplant (*Solanum melongena*) presents a high content in phenolics, in particular chlorogenic acid (CGA), which confers nutraceutical properties to this crop. In order to obtain information relevant for the development of varieties with high content in CGA and low browning, we have studied the diversity for CGA, PPO activity, and fruit flesh browning and their relationships in a collection of 18 Spanish accessions of eggplant. Also, using an interspecific mapping population between *S. melongena* and *S. incanum* we have mapped the genes involved in the synthesis pathway of CGA as well as the eggplant PPO genes. The results confirmed that eggplant presents high levels of CGA, and that a wide diversity exists for the three traits studied. Low levels of correlation have been found between CGA and PPO activity on one side and browning on the other, indicate that PPO is not a limiting factor in browning in the germplasm collection studied. The six genes of the pathway for the synthesis of CGA from phenylalanine have been mapped to five different linkage groups. Only two of the genes are linked indicating that selection of materials with the alleles favourable of different parents will be easily achieved. However, the five PPO genes mapped (PPO1 to PPO5) cluster together in the same linkage group, which will difficult obtaining recombinants. Mapping of these genes is of interest for marker assisted selection for high content in CGA and reduced browning. Overall, the results indicate that selection of eggplant varieties with high content in CGA and low browning is feasible. The information obtained is also useful for the genetic improvement of other fruits and vegetables in order to develop new cultivars with increased added value resulting from high content in phenolics and low browning.

Keywords: breeding, browning, chlorogenic acid, diversity, genetic map, polyphenol oxidases, *Solanum melongena*

INTRODUCTION

During the last years there has been an increased demand for fruits and vegetables having a higher content in nutraceutical compunds (Herath *et al*., 2006; Kris-Etherton *et al*., 2002). In this respect, breeding programmes for improving the content of bioactive compounds in fruits and vegetables are becoming more important for public and private breeders (Diamanti *et al*., 2011). Phenolic compounds present in fruits and vegetables are raising an increased interest due to their multiple beneficial effects for human health, which in many cases is derived from their high antioxidant acitivty (Crozier *et al*., 2009; Dai and Mumper, 2010). Furthermore, many of the phneolic compounds have a great thermal stability and therefore are not lost after cooking (Friedman and Jürgens, 2000; Lo Scalzo *et al*., 2010).

However, increasing the content in phenolics in fruits and vegetables may lead to browning and a consequent loss of quality (Toivonen *et al*., 2008). When the internal tissues of a fruit or vegetable with significant amounts of phenolic compounds are exposed to the air and cell damage occurs (due to cutting or biting), the phenolic substrates, which are mostly found in vacuoles, become accessible to polyphenol oxidases (PPO) (Mayer, 2006; Toivonen *et al*., 2008). The PPOs catalyze the oxidation of phenolic compounds to quinones, which subsequently react with air oxygen and other cellular compounds to give brown-coloured compounds which cause browning. As a consequence, breeding programmes aimed at improving the content in phenolics in fruits and vegetables should also take into account browning.

One of the vegetables with a highest content in phenolics is eggplant (*Solanum melongena* L.) (Gajewski *et al*., 2009; Hanson *et al*., 2006; Mennella *et al*., 2012; Prohens *et al*., 2007; Stommel and Whitaker, 2003). Because of this, eggplant is considered as a model vegetable crop for the improvement of nutraceutical quality (Plazas *et al*., 2013). The main phenolic compound of eggplant is chlorogenic acid (CGA), which is an hydroxicinnamic acid with multiple beneficial properties for human health (Mennella *et al*., 2012; Stommel and Whitaker, 2003). CGA has displayed anti-oxidant, anti-carcinogenic, anti-inflammatory, anti-obesity, cardiprotective, neuroprotective, and analgesic effects (Plazas *et al*., 2013). As a result, CGA plays a major role in the nutraceutical properties of eggplant (Akanatapichat *et al*., 2010; Cao *et al*., 1996; Das *et al*., 2011; Kwon *et al*., 2008). This has resulted in the development of breeding programmes specifically aimed at increasenf the CGA content of eggplant (Plazas *et al*., 2013).

Several works have revealed that there is a wide diversity for CGA content, PPO activity and browning in eggplant (Mishra *et al*., 2012; Prohens *et al*., 2007; Stommel and Whitaker, 2003). However, no studies have been made on the relationships between these three traits in a wide collection of germplasm. These studies would provide information of relevance for the breeding of eggplant cultivars with high content in CGA and low browning. Also, the genes involved in the synthesis of CGA (Comino *et al*., 2007; Joët *et al*., 2010; Mahesh *et al*., 2007; Menin *et al*., 2010; Niggeweg *et al*., 2004) and PPO genes (Shetty et al., 2011) are known, which opens the way to marker assisted selection. In this respect, mapping of these genes in eggplant would facilitate molecular breeding for high content in CGA and reduced browning in eggplant (Plazas *et al*., 2013).

We present the results of: a) a study of the diversity and relationships of CGA, PPO activity, and fruit flesh browning in a germplasm collection of eggplant, and b) the mapping of genes involved in CGA synthesis and of genes codifying for PPOs. The results are of interest for the breeding of eggplants with improved content in phenolics of nutraceutical value and low or moderate fruit flesh browning. It also provides information of general interest for the development of fruit and vegetable varieties with high content in phenolics with low browning.

MATERIALS AND METHODS

Diversity for CGA, PPO, and browning

A collection of 18 eggplant accessions from the region of Valencia (Spain) were used for the study of diversity and relationships of CGA, PPO, and browning. Accessions were cultivated in the open field during the summer season at the Agricultural Experimental Farm of Carcaixent (Valencia, Spain). The accessions present a wide morphological and genetic diversity for fruit characteristics and belong to different cultivar groups. Fifteen fruits per accession, which were divided in five samples of three fruits, were used for studying the diversity for CGA, PPO and browning.

CGA was measured by high performance liquid chromatography using lyophilized powdered tissue. CGA was extracted according to Naranjo *et al*. (2007). CGA was extracted with methanol and measurement was made at 320 nm (Fig. 1). PPO activity was measured according to Bellés *et al*. (2006) using lyophilized powdered tissue and using CGA as substrate. The PPO enzymatic reaction was followed colorimetrically at 420 nm for the first 1.5 min. One unit of PPO activity was defined as the increase in 0.1 absorbance units/min. For fruit flesh browning estimation, CIELAB L*, a*, and b* colour coordinates were measured with a chromameter in the flesh of fruits at 0 min and 10 min after being cut. The degree of browning was estimated as the difference between 0 min and 10 min in the Euclidean distance to pure white (L*=100, a*=0, b*=0) (Prohens *et al*., 2007).

The mean, minimum, maximum, maximum/minimum ratio, average standard deviation (obtained from ANOVA), and coefficient of variation were calculated for the three traits. Pearson linear correlations were also calculated using accession means.

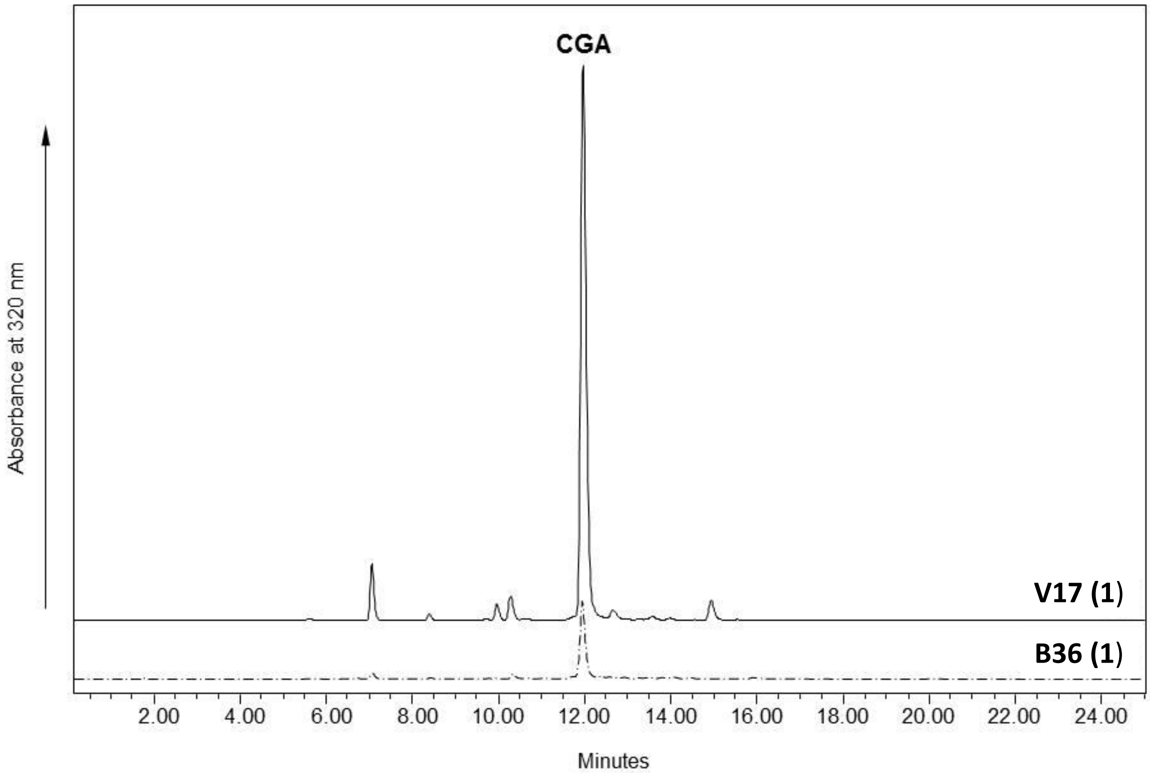

Fig. 1. Representative C_{18} column HPLC chromatograms from methanolic extracts of two samples of eggplant with contrasting contents in chlorogenic acid (CGA) content. High and low CGA content samples are represented in continuous and dashed lines, respectively. Absorbance was measured at 320 nm.

Genetic mapping of CGA and PPO genes

The first backcross (BC1) generation resulting from the cross between an interspecific *S. incanum* × *S. melongena* hybrid and its *S. melongena*, as well as the two parents and the interspecific hybrid were used. This population had been used to obtain an interspecific genetic map of eggplant (Vilanova *et al*., 2010) and consists of 12 linkage groups in which more than 200 SSR, COS and AFLP markers have been positioned.

Sequencing of introns of the six genes (phenylalanine ammonia-lyase, PAL; 4-coumaroyl:CoA ligase, 4CL; hydroxycinnamoy-CoA shikimate/quinate hydroxycinnamoyl transferase, HCT; cinnamic acid 4-hydroxilase, C4H; p-coumarate 3-hydroxylase, C3'H; and, hydroxycinnamoyl CoA quinate hydroxicinnamoyl transferase, HQT) involved in the synthesis of CGA from phenylalanine (Plazas *et al*., 2013) was performed in order to look for single nucleotide polymorphisms (SNPs) between parents. For the six eggplant PPO genes (PPO1 to PPO6) (Shetty *et al*., 2011), as the genes have no introns (Thypiapong *et al*., 2007; Tran *et al*., 2012) the exon sequence was used to look for polymorphism. SNP polymorphisms were transformed into cleaved amplified polymorphic sequences (CAPs) and used for genotyping individual plants and for mapping the CGA synthesis and PPO genes in the BC1 population. When no CAP marker sequence was available, detecting of polymorphism was performed with high resolution melting (HRM).

RESULTS AND DISCUSSIONS

Diversity for CGA, PPO, and browning

The results obtained show that eggplant presents a high content of CGA when compared with other vegetable crops (Plazas *et al*., 2013). In this respect, the average value has been of 3.55 g/kg, with a maximum value of 6.27 g/kg. An important variation was found in the collection of accessions for the three traits studied, with differences of 2.54-fold for CGA, 2.86-fold for PPO activity, and 3.36-fold for fruit flesh browning (Tab. 1). These results are in agreement with former studies in which the diversity of CGA content, PPO activity, and browning have been studied in eggplant (Mennella *et al*., 2012; Mishra *et al*., 2013; Prohens *et al*., 2007; Stommel and Whitaker, 2003). In addition, the high diversity values found represent a confirmation that Spain is a secondary center of diversity for eggplant (Hurtado *et al*., 2012) and that a wide variation can be found for these traits in a single geographical region.

Tab. 1

Mean minimum, maximum, maximum/minimum ratio, average standard deviation for accession, and coefficient of variation for the chlorogenic acid content, polyphenol oxidase activity, and fruit flesh browning in a collection of 18 eggplant accessions.

Parameter	Chlorogenic acid content (g/kg dw)	Polyphenol oxidase activity (units)	Fruit flesh browning (units)
Mean	3.55	1.552	5.15
Minimum	2.47	0.870	2.47
Maximum	6.27	2.490	8.31
Maximum/Minimum	2.54	2.86	3.36
Average standard deviation	1.73	0.773	1.88
Coefficient of variation (%)	39.2	54.4	46.1

The coefficient of correlation between CGA and fruit flesh browning has been significant ($P<0.05$) although the value has been low ($r=0.253$; Fig. 2). The coefficient of determination (r^2) for this correlation has been $r^2=0.064$, indicating that only 6.4% of the variation found in fruit flesh browning is explained by variation in CGA. The coefficient of correlation between PPO activity and fruit flesh browning has been non-significant ($P>0.05$; $r=0.185$) (Fig. 3).

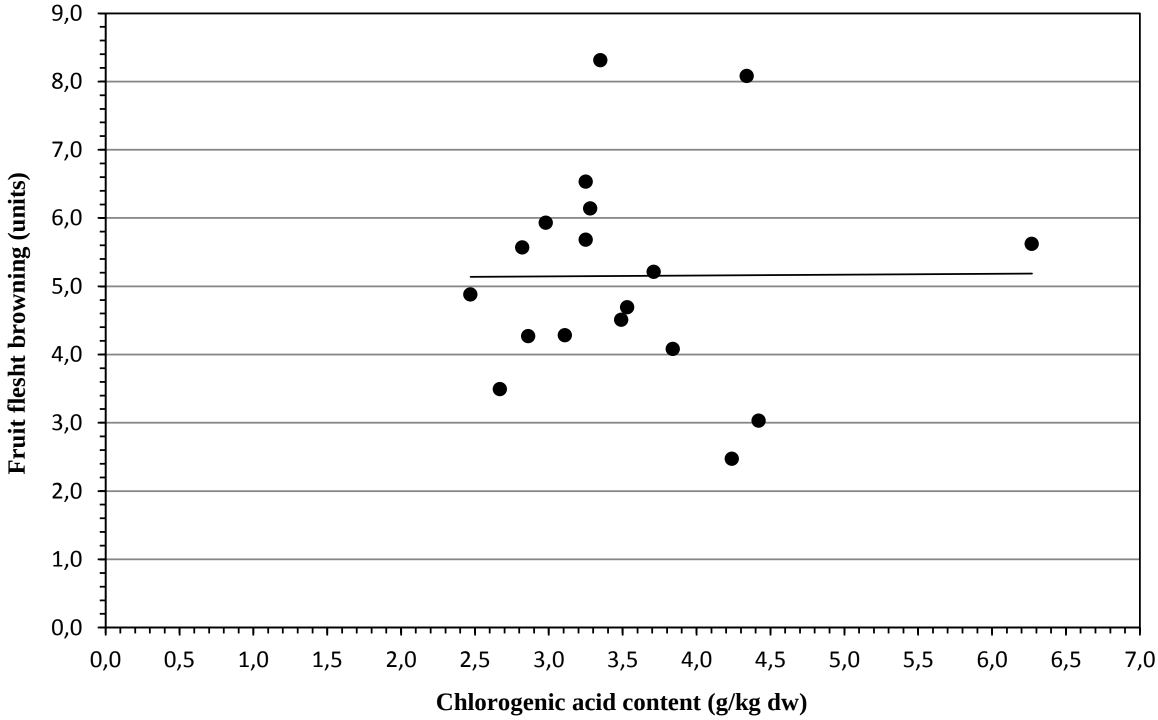

Fig. 2. Relationships between chlorogenic acid content and fruit flesh browning in a collection of 18 eggplant accessions. The linear regression line is displayed.

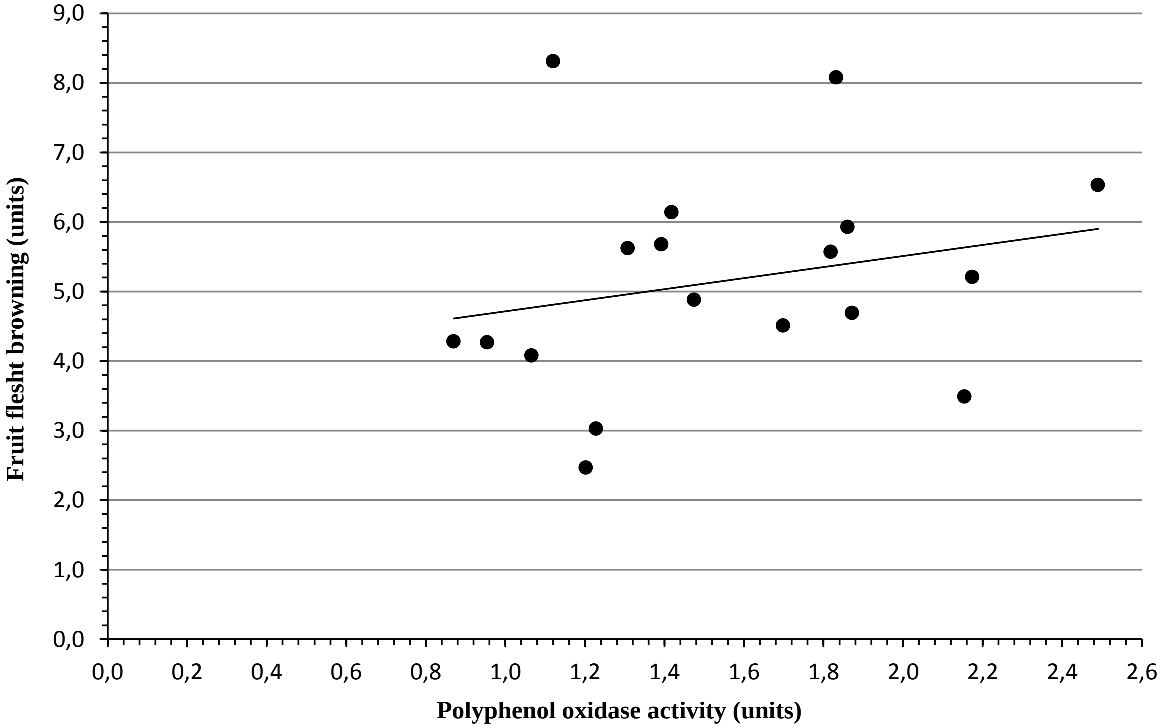

Fig. 3. Relationships between polyphenol oxidase (PPO) activity and fruit flesh browning in a collection of 18 eggplant accessions. The linear regression line is displayed.

The results from the correlation studies indicate that additional factors other than CGA content or PPO activity are influencing fruit flesh browning in the eggplant collection studied. In this respect, cell and cell organelle size, morphology and distribution, presence of phenolic compounds other than CGA and of other non-phenolic compounds able to produce browning, amount of CGA and PPO release from cell compartments, diffusion of CGA and PPO, accessibility of O_2 to CGA, intracellular pH, or differences in the concentration of intracellular antioxidants may have considerable impact in fruit flesh browning (Barbagallo *et al*., 2012; Concellón *et al*., 2012; Mishra *et al*., 2012; Ghidelli *et al*., 2013; Toivonen *et al*., 2008). In other crops, like potato, low correlations between PPO activity and browning have been reported in some populations (Culley *et al*., 2002)

The results obtained suggest that it is possible to select eggplant accessions with high content in CGA and low browning. The lack of correlation between PPO activity and browning suggests that even the lowest levels of PPO activity found by us in the eggplant collection studied are more than sufficient to produce high levels of browning. Other studies have shown that PPO inhibition reduces browning of the eggplant fruit flesh (Barbagallo *et al*., 2012; Ghidelli *et al*., 2013; Hu *et al*., 2010; Massolo *et al*., 2011; Mishra *et al*., 2012), which may indicate that selection of PPO genes with no or very low activity may be of interest for reducing fruit flesh browning in eggplant.

Genetic mapping of CGA and PPO genes

For the six genes involved in the CGA synthesis pathway (Comino *et al*., 2007; Joët *et al*., 2010; Mahesh *et al*., 2007; Menin *et al*., 2010; Niggeweg *et al*., 2004), orthologous genes could be found in tomato with a high identity to the eggplant unigenes. In all cases, polymorphism could be found between *S. melongena* and *S incanum*. For the PAL, 4CL, and C3'H genes CAPs markers could be developed, while for HCT, C4H, and HQT genes, SNP polymorphism was detected with HRM. The six genes could be situated in our interspecific genetic map (Tab. 2).

Tab. 2

Genes involved in the synthesis of chlorogenic acid content and their position in the genetic map of eggplant.

Gene	Linkage group	Distance (cM) from linkage group end
Phenylalanine ammonia-lyase (PAL)	9	15.0
4-Coumaroyl:CoA ligase (4CL)	3	90.3
Hydroxycinnamoy-CoA shikimate/quinate hydroxycinnamoyl transferase (HCT)	3	89.0
Cinnamic acid 4-hydroxilase (C4H)	6	107.1
p-Coumarate 3-hydroxylase (C3'H)	1	86.1
Hydroxycinnamoyl CoA quinate hydroxicinnamoyl transferase (HQT)	7	3.0
Polyphenol oxidases PPO1, PPO2, PPO3, PPO4	8	40.0
Polyphenol oxidase PPO5	8	35.5

The study of synteny with the tomato genome, revealed that these six genes mapped according to the syntenic position in tomato (Wu *et al*., 2009). The six genes are spread in five different linkage groups. In this way, the only genes situated in the same linkage group are 4CL and HCT, which are situated at a very small genetic distance. This shows that, with the exception of 4CL and HCT it may be relatively easy to combine the favourable alleles in a single material using marker assisted selection. For the 4CL and HCT genes, in case that the favourable allele of one of the genes is found in *S. melongena* and the favourable allele of the other gene is present in *S. incanum* it will be necessary to look for recombinants in segregating populations to find the desired genetic combination.

The PPO genes of tomato have no introns and are clustered together in chromosome 8 (Newman *et al*., 1993; Tran *et al*., 2012) and consequently, the levels of polymorphism are lower than those of intron sequences. Eggplant PPO genes PPO1 to PPO5 could be amplified and sequenced in both parents. However, PPO6 gene could only be amplified and sequenced in *S. melongena*, but not in *S. incanum*. This result suggests that the PPO6 gene of *S. incanum* presents substantial changes with respect to the homologous gene in *S. melongena*, or that is not present in *S. incanum*. HRM was used to detect the SNPs and for genotyping the mapping population.

The five PPO genes that could be mapped (PPO1 to PPO5) were situated in linkage group 8, in a position syntenic with the cluster of PPO genes in tomato (Wu *et al*., 2009). PPO1 to PPO4 genes are situated at the same distance from the linkage group end (40.0 cM), while PPO5 is a little bit separated, being situated at 35.5 cM from the linkage group end. It remains to be studied which PPO gene/s are expressed in the fruit. In any case, the fact that the PPO genes are found clustered in the same genomic region means that it will be difficult to obtain recombinants between *S. melongena* and *S. incanum* for the PPO genes. Further studies on diversity for PPO activity and identification of allelic variants that result in dramatically reduced PPO activity may result in eggplant varieties with low browning. In other crops, transgenic materials showing low or reduced PPO activity also present reduced browning (Murata *et al*., 2001).

CONCLUSION

We have found that considerable diversity exists for CGA, PPO activity and browning within a germplasm collection of eggplant from Spain. This indicates that these traits are amenable to selection. Lack of correlation between CGA and PPO on one side and fruit flesh browning on the other indicates that it is feasible to select eggplant materials with high content in CGA, and therefore with improved nutraceutical properties, and low browning. The mapping of the genes involved in the CGA synthesis pathway and of the genes codifying for PPOs will be useful for marker assisted selection in order to develop eggplant materials with improved CGA content and reduced browning. This information is of interest not only for eggplant breeding, but also for the genetic improvement of other fruits and vegetables with increased added value resulting from containing significant amounts of phenolics and reduced browning susceptibility.

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