Research Article

Oxidative stress and antioxidant systems in Guava (*Psidium guajava* L.) fruits during ripening

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ABSTRACT

Two varieties of guava *viz.*, L-49 and Hisar Safeda differing in their shelf lives were analyzed for various components of oxidative stress and of enzymatic and non-enzymatic antioxidative system at different stages of fruit ripening. Indices of oxidative stress *viz*., lipoxygenase activity, malondialdehyde value and H_2O_2 content increased throughout during ripening in both the varieties. The extent of oxidative stress was more pronounced in Hisar Safeda (shelf life 3-4 days) than in L-49 (shelf life 7-8 days). Except for superoxide dismutase, activities of all other antioxidative enzymes *viz*., catalase, peroxidase, ascorbate peroxidase and glutathione reductase increased up to color turning stage and decreased thereafter. Superoxide dismutase activity, however, increased upto ripe stage followed by a decline. Contents of ascorbic acid and glutathione (total, oxidized and reduced) were found to be the maximum at turning and mature stage, respectively. It is inferred that ripening of guava fruit is accompanied by a progressive increase in oxidative/ peroxidative stress which induces antioxidant system but not until later stages of ripening. Over-accumulation of ROS due to dysfunctioning of ROS scavenging system at later stages of fruit ripening appears to be responsible for loss of tissue structure as observed in ripened and over-ripened fruits. **[Physiol. Mol. Biol. Plants 2009; 15(4) : 327-334]** *E-mail : sarla_malhotra@hau.ernet.in*

Key words : *Ascorbate, Glutathione, Guava, Fruit ripening, Reactive oxygen species, ROS scavenging enzymes, Psidium guajava,*

Abbreviations : *APX, ascorbate peroxidase; CAT, catalase; DHAR, dehydroascorbate reductase; GR, glutathione reductase; IMG, immature green; LOX, lipoxygenase; MDA, malondialdehyde; MDHAR, monodehydoascorbate reductase; MG, immature green; OR, over ripe; POX, peroxidase; ROS, reactive oxygen species; R-ripe; SOD, superoxide dismutase; T, turning*

INTRODUCTION

In plant tissues including ripening fruit, reactive oxygen species (ROS) such as superoxide radical ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), hydroxyl radical (OH^-) etc. are inevitably generated *via* a number of metabolic pathways. Electron transport systems in plastids and mitochondria generate $O_2^{\bullet -}$ during α-oxidation in glyoxysomes while H_2O_2 is produced during photorespiration in leaf peroxisomes. Over-accumulation of these ROS leads to oxidative damage in cellular molecules such as nucleic acids, proteins and lipids. However, plant cells have been provided with antioxidative defence systems which scavenge ROS and thus provide protection against their deleterious effects. These include antioxidant enzymes such as superoxide dismutase (SOD), peroxidase (POX), catalase (CAT), ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR) and glutathione reductase (GR), water soluble antioxidant compounds like ascorbate, glutathione and flavonoids and lipid soluble antioxidants such as carotenoids and tocopherols. In spite of such an efficient defense system, plant cells suffer from oxidative damage under biotic and abiotic stress conditions (Apel and Hirt, 2004). Over-accumulation of ROS occurs either because of their over-production (del Rio *et al*., 1998) or due to dysfunctioning of antioxidative defense system becoming unable to scavenge them fully (Kanazawa *et al*., 2000; Palma *et al*., 2006).

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Fruit ripening, which results in the transformation of unripe fruit into an edible ripe product, is a metabolically active process consisting of not only simple degradative reactions but also of complex biosynthetic reactions. It is a stressful process and is considered to be a functionally modified protracted form of senescence. Therefore, mechanism of membrane deterioration and loss of tissue structure during ripening may also probably be similar to that characterized in true senescing systems involving the accumulation of ROS. Fruits like saskatoon (Rogiers *et al*., 1998), tomato (Mondal *et al*., 2004) and orange (Huang *et al*., 2007) fruits have been reported to accumulate ROS during ripening. Guava (*Psidium guajava*) is a crop of economic importance and is considered to be a poor man's fruit in India. However, factors controlling its growth and development have not attracted considerable research interests. Our laboratory has been engaged in understanding the biochemistry of fruit ripening in guava (Jain *et al*. 2001, 2003). Recently, Mondal *et al*. (2008) have documented guava to be a climacteric fruit with a burst in ethylene production coinciding with eating ripeness. However, there is no information what-so-ever, on the oxidative stress and antioxidative systems in this fruit during ripening. This study was, therefore, undertaken to investigate the role of ROS producing and scavenging systems in ripening guava.

MATERIALS AND METHODS

Plant material

Field grown guava fruits of cultivars L-49 (shelf life 7-8 days) and Hisar Safeda (shelf life 3-4 days) were harvested from ten year old trees grown in the orchards of CCS Haryana Agricultural University, Hisar, India. They were identified as immature green (IG), mature green (MG) and turning (T) fruits on the basis of visual observations of size, firmness, liquefaction and pigmentation. To get ripe (R) and over-ripe (OR) stage, fruits harvested at turning stage were wrapped in a newspaper and kept for 2 and 4 days (Hisar Safeda) and 3 and 5 days (L-49), respectively at room temperature. Freshly harvested, ripe and over-ripe fruits, free of any visible defects were surface sterilized with 0.2 % (*w/v*) mercuric chloride, rinsed in tap water and then in distilled water and air dried for further analysis. All the estimations were carried out in three replicates with two extractions each. The value reported for each parameter are, therefore, the means of six estimations.

Analytical methods

Rate of lipid peroxidation was measured by the formation of malondialdehyde (MDA) as described by Heath and Pecker (1968). One g tissue was homogenized in 5 ml of 0.1 % (*w/v*) trichloroacetic acid (TCA) and centrifuged at 8,000 x g for 15 min. 1 ml of the supernatant was thoroughly mixed with 4 ml of 20 % (*w/v*) TCA containing 0.5 % (*w/v*) 2-thiobarbituric acid (TBA) and the mixture was incubated at 95 °C for 30 min. The samples were cooled in an ice bath, centrifuged at 8,000 x g for 15 min and the absorbance of the supernatant was recorded at 532 nm. Non specific absorbance of the sample at 660 nm was subtracted from the absorbance at 532 nm (Hodges *et al*., 1999). The concentration of MDA was calculated using the molar extinction coefficient of 155 mM^{-1} cm^{-1}.

H_2O_2 was extracted by homogenizing 4 g tissue in 5 ml of ice cold 0.01 M phosphate buffer (pH 7.0) and centrifuging the homogenate at 8,000 x g for 10 min (Sinha, 1972). 50 ml of the sample was added to 1.95 ml of 0.01 M phosphate buffer (pH 7.0). To the mixture, was added 2 ml of 5 % potassium dichromate and glacial acetic acid (1:3, *v/v*). The optical density was read at 570 nm against the reagent blank without sample extract. The quantity of H_2O_2 was determined by comparing with the standard (10 to 160 mmol).

Ascorbic acid was estimated according to the method of Roe (1964) which is based on the reduction of 2, 6-dichlorophenol indophenol by ascorbic acid. Method of Smith (1985) was employed for determining the level of oxidized, reduced and total glutathione.

Enzyme extraction and assays

Extraction conditions were standardized with respect to type, molarity and pH of the buffer, concentration(s) of stabilizing agent(s) and other constituents of the extraction medium to achieve maximum extraction of the enzyme. Extraction medium for SOD, CAT, APX, GR and LOX consisted of 0.1 M phosphate buffer (pH 7.5) containing 5 % (*w/v*) polyvinylpolypyrrolidone (PVPP), 1 mM EDTA, and 10 mM β-mercaptoethanol. For POX, however, the extraction buffer consisted of 0.01 M phosphate buffer (pH 7.0) containing 4 % (*w/v*) PVPP. The homogenate was prepared by grinding 4 g (fresh weight) of guava fruits in 5 ml of ice cold extraction medium in pre-cooled mortar and pestle. The homogenate thus prepared was centrifuged at 10,000 x g for 15 min at 4 °C.

LOX activity was measured at 30 °C by monitoring the formation of conjugated dienes from linoleic acid at 234 nm (Surrey, 1964). SOD activity was determined by quantifying the ability of the enzyme extracts to inhibit light induced conversion of nitroblue tetrazolium (NBT) to formazan (Beauchamp and Fridovich, 1971). One enzyme unit was defined as the amount of enzyme which could cause 50 per cent inhibition of the photochemical reaction (McCord and Fridovich, 1969). CAT and POX activities were assayed at 37 °C as described by Sinha (1972) and Dias and Costa (1983), respectively. Method of Nakano and Asada (1981) was employed to assay APX. GR activity was determined at 30 °C by adding 100 ml of enzyme extract to 1 ml of 0.2 M phosphate buffer (pH 7.0) containing 1 mM EDTA, 0.75 ml distilled water, 0.1 ml of 20 mM oxidized glutathione (GSSG) and 0.1 ml of 2 mM NADPH (Halliwell and Foyer, 1978). Oxidation of NADPH by GR was monitored at 340 nm and the rate (nmol min^{-1}) was calculated using the extinction coefficient of 6.12 mM^{-1} cm^{-1}.

RESULTS AND DISCUSSION

Indices of lipid peroxidation

Oxidative stress can be best assessed by the extent of lipid peroxidation catalyzed by lipoxygenase which plays a central role in membrane deterioration by peroxidizing free polyunsaturated fatty acids. LOX initiates oxidation of the *cis, cis*-1, 4-pentadiene moieties present in linoleic and linolenic acid. The linoleyl-hydroperoxides thus formed decompose into oxy-free radicals, ethane and thiobarbituric acid reactive substances (TBARS), all of which are sensitive markers of lipid peroxidation. Membrane lipids and free fatty acids (FFA) are highly susceptible to oxidation and therefore, increasing oxidative stress is thus indicated by the accumulation of lipid peroxidation products.

LOX activity (Fig. 1) was the lowest at the IG stage (374 and 512 moldiene g^{-1} f. wt. in L-49 and Hisar Safeda, respectively) and increased significantly (about 2.5 to 3 fold) reaching the maximum values of 1022 and 1232 mol, respectively at OR stage. Malondialdehyde (MDA) (Fig. 2) content followed a pattern similar to that exhibited by LOX. It was minimum at IG stage with 12 and 14 mols g^{-1} f. wt. in L-49 and Hisar Safeda, respectively which increased significantly during ripening and was maximum at OR stage (50 and 60 mols g^{-1} f. wt. in L-49 and Hisar Safeda, respectively). Fig. 3 depicts the results on H_2O_2 content, yet another reactive oxygen species and toxic for cell macromolecules. It increased progressively from 350 and 376 to 714 and 774 mol g^{-1} f. wt. from IG to OR stage in L-49 and Hisar safeda, respectively. Consistently higher values for LOX, MDA and H_2O_2 in Hisar Safeda suggest that membrane deterioration and hence loss of membrane integrity and tissue structure is much faster in soft variety than in the firm one. Membrane deterioration due to increased LOX activity has been reported to be responsible for loss of tissue structure in saskatoon, avocado, pear and tomato fruit during ripening (Kausch and Handa, 1997; Rogiers *et al*., 1998; Mondal *et al*., 2004; Liu *et al*., 2008). Lurie and Ben Arie (1983) demonstrated increased lipid peroxidation in ripening apple and attributed this to increase in LOX activity.

To determine the role of ROS scavenging systems in combating the oxidative stress, ROS scavenging enzymes and metabolites were characterized in fruits of different developmental stages. Fig. 4 reveals the SOD activity to increase from 62.5 and 65.5 in L-49 and Hisar Safeda at IG stage to 76 and 81.5 units g^{-1} f. wt. at ripe stage, respectively followed by a decrease at OR stage to 65 and 73.5 units g^{-1} f. wt. Hisar Safeda had slightly higher activity of SOD than L-49 throughout the ripening period. These observations are in agreement with those reported earlier in tomato fruits (Ahn *et al*., 2002; Thakur and Pandey, 1999). However, contrary to this, Mondal *et al*. (2004) and Reddy and Srivastava (2003) found highest SOD activity during MG stage in tomato and mango, respectively. SOD has been reported to decrease during senescence in many plant systems resulting in an increased accumulation of $O^{\bullet-}_2$ radicals and thus oxidative stress (Dhindsa *et al*., 1981). Respiration is a primary source of superoxide radical because it is produced by leakage of electrons to O_2 during their transport in plastids and mitochondria (Purvis *et al*., 1995). Due to a substantial increase in respiration of guava, the climacteric fruit, oxygen free radical production probably increases during the later stages of development. The concomitant decline in SOD activity at OR stage would thus contribute to accumulation of O^{-}_2, thus affectively increasing the oxidative stress and deterioration in over-ripened fruits.

Catalase is one of the primary enzymatic defenses against oxidative stress induced by senescence (Zimmermann *et al*., 2006) Its activity (Fig. 5) increased from IG to turning stage followed by a continuous decline upto OR stage. In contrast to SOD (H_2O_2 producing enzyme), catalase (H_2O_2 degrading enzyme) activity throughout was higher in L-49 as compared to

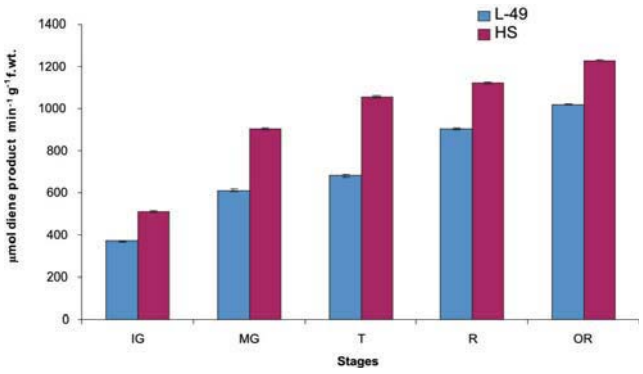

Fig. 1. Lipoxygenase activity in guava fruit at different stages of ripening

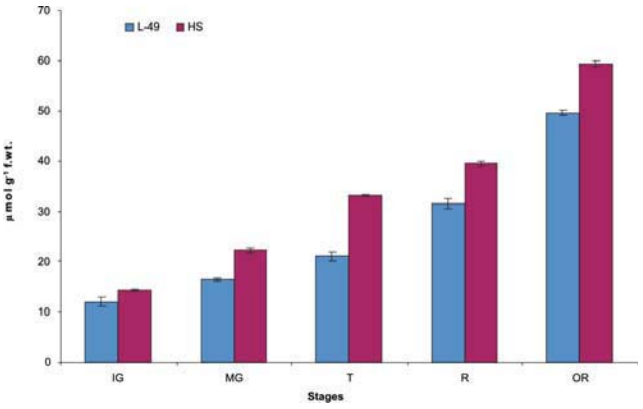

Fig. 2. MDA content in guava fruit at different stages of ripening

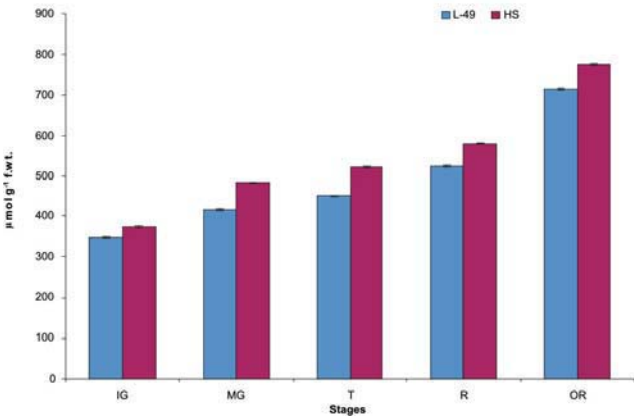

Fig. 3. H_2O_2 content in guava fruit at different stages of ripening

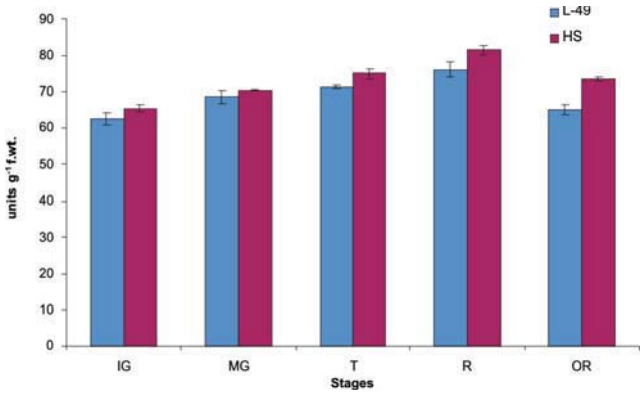

Fig. 4. SOD activity in guava fruit at different stages of ripening

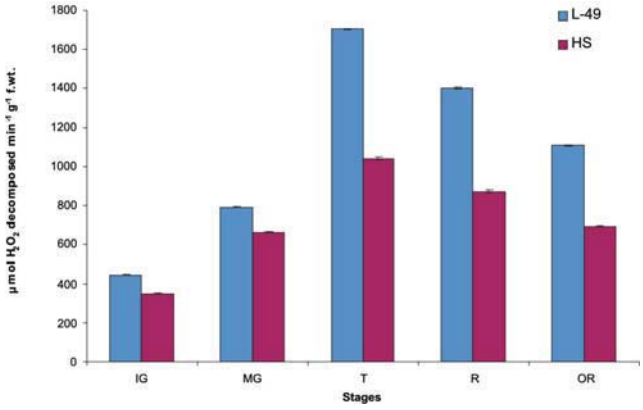

Fig. 5. Catalase activity in guava fruit at different stages of ripening

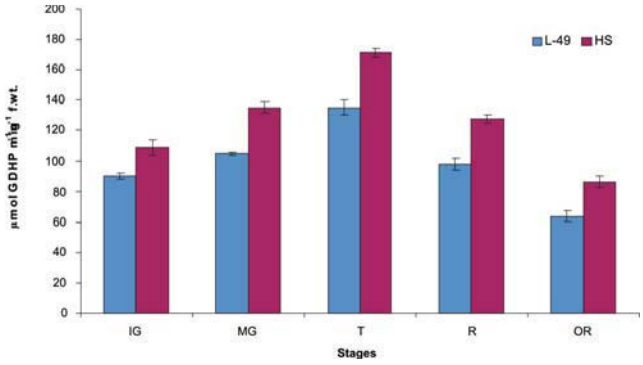

Fig. 6. Peroxidase activity in guava fruit at different stages of ripening

Hisar Safeda. This probably would explain higher content of H_2O_2 in Hisar Safeda than in L-49 throughout ripening. Present results correspond to those reported for mango (Masia, 1998). However, catalase activity increased continuously during ripening of tomato (Andrews *et al*., 2004; Mondal *et al*., 2004) fruit, whereas, in saskatoon (Rogiers *et al.* 1998) and orange (Huang et al., 2007), it has been reported to decrease continuously.

Activity profile of peroxidase (Fig. 6) and ascorbate peroxidase (Fig. 7) revealed patterns similar to that followed by catalase. These results are supported by the observations on tomato (Andrews *et al*., 2004) and papaya (Silva *et al*., 1990) in which these activities were low at greenish stage, increased during ripening and then gradually fell as the fruit turned to over ripe stage. A continuous decrease in POX and APX has been reported during ripening of tomato (Mondal *et al*., 2004) and orange (Huang *et al*., 2007) fruits, during flower transition in *Arabidopsis thaliana* (Ye *et al*., 2000) and during maturation of phyllodes in *Acacia mangium* (Yu and Ong, 2000). In contrast to catalase and ascorbate peroxidase, activity of POX was found to be higher in HS throughout ripening. This suggests that POX may not be playing an important role in scavenging H_2O_2 during fruit ripening (Moller *et al.,* 2007). These observations are further supported by the findings of Andrews *et al*. (2000) where POX has also been reported to catalyze cell wall softening reactions during fruit ripening.

Glutathione reductase (Fig. 8), which was higher in L-49 than in Hisar safeda, exhibited maximum activity at MG stage, declined continuously and reached a minimum at OR stage. L-49 exhibited significantly higher GR activity than Hisar Safeda at all the ripening stages. The results are in agreement with those reported earlier (Mondal *et al*. 2004; Andrews *et al*., 2004) during tomato fruit ripening and are different from those observed during ripening of saskatoon (Rogiers *et al*., 1998) and orange (Huang *et al*., 2007) which exhibited continuous increase in glutathione reductase activity with a remarkably sharp increase at the later stages of ripening. However, during ageing of pea plant (Olsson, 1995) and water melon seeds (Hsu and Sung, 1997), glutathione reductase has been reported to decrease.

Ascorbic acid and reduced glutathione are important ROS scavenging metabolites. Results presented in Fig. 9 reveal that fruits at turning stage had maximum content of ascorbic acid (83 and 99 mg/100g f. wt. in L-49 and Hisar Safeda, respectively). Throughout ripening, Hisar Safeda, the variety with short shelf life had higher ascorbic acid content than L-49. This may probably be due to higher APX activity in L-49 which utilizes ascorbic acid as the substrate. However, the increase in ascorbic acid during turning stage, when APX is also increasing could not be explained. These results are supported by the observations on tomato fruit during ripening (Mondal *et al*., 2004) which exhibited increase in ascorbic acid content up to turning stage followed by a decline at ripe stage. Here also the soft variety had higher ascorbic acid content than the one with higher shelf life. The ascorbic acid content has been reported to increase during ripening of jujube (Bal and Josan, 1980) and cavandish banana (Mustaffa *et al.*, 1998) while a continuous decline has been observed in sapota (Lakshminarayan and Subramanyam, 1966) and cherry laurel (El-Bulk *et al*., 1997; Kadioglu and Yavree, 1998). However, in guava fruit (Dhillon *et al*., 1987) ascorbic acid content has been reported to show a sigmoidal increase during ripening.

Data on total (Fig 10), reduced (Fig 11) and oxidized (Fig 12) glutathione reveal that fruits of both the varieties had maximum content of all the three components at the MG stage. Reduced glutathione was more than the oxidized one at all the stages of ripening in both the cultivars. Hisar Safeda had lower content of reduced glutathione than L-49 while ratio of reduced/oxidized glutathione was always higher in Hisar Safeda except at OR stage when the ratio was higher in L-49. Moreover, GSSG increased by 3 per cent in L-49 and by 6 per cent in Hisar Safeda from IG stage to ripe stage indicating an increase in cellular oxidative status during ripening. Glutathione system has been reported to be involved directly in maintaining a low redox potential and thus a highly reduced intracellular environment (Tanaka *et al*. 1994). Tomato and saskatoon fruit have also been reported to respond to the increase in oxidative stress by increasing reduced and oxidized glutathione during development (Rogiers *et al*. 1998; Andrews et al., 2004). In present investigations, guava fruits also responded similarly to increased oxidative stress during ripening. Both GSH and GSSG concentrations increased about 2.4 and 2.8 fold as fruits developed from IG to MG stage.

From the results on changes in ROS species taken in conjunction with the results on ROS scavenging enzymes in the two cultivars of guava fruit during ripening, it could be suggested that although ripening of guava fruit entails a multitude of physicochemical changes (many of which are catabolic in nature), it is accompanied by a progressive increase in oxidative/peroxidative stress.

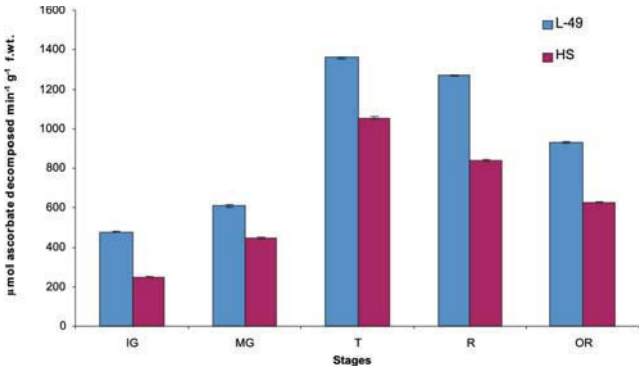

Fig. 7. Ascorbate peroxidase activity in guava fruit at different stages of ripening

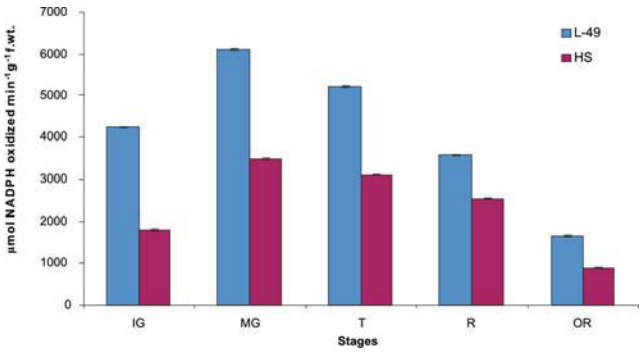

Fig. 8. Glutathione reductase activity in guava fruit at different stages of ripening

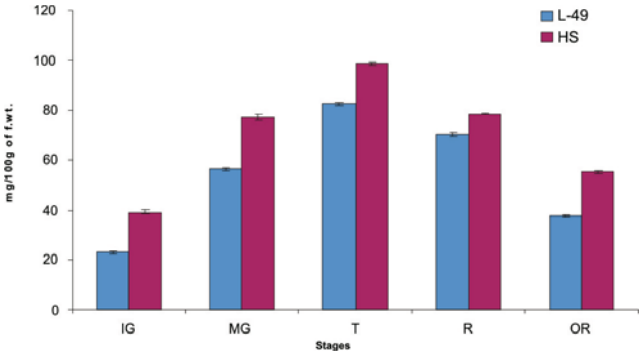

Fig. 9. Ascorbic acid content in guava fruit at different stages of ripening

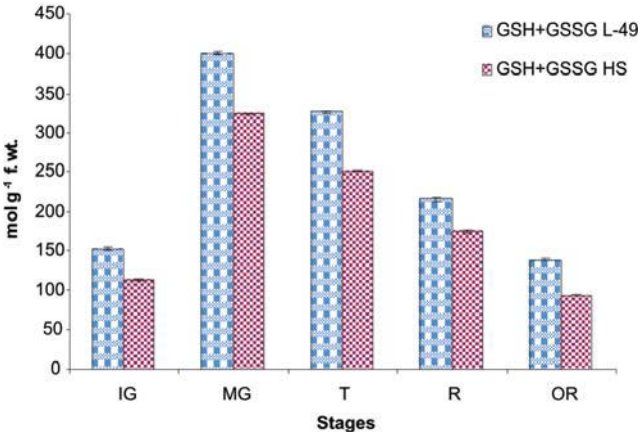

Fig. 10. Total glutathione content in guava fruit at different stages of ripening

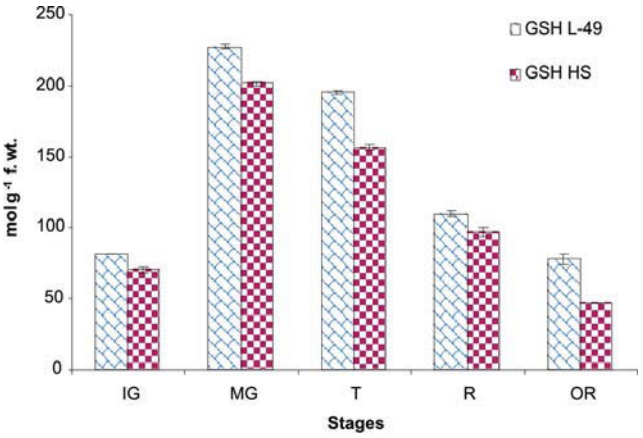

Fig. 11. GSH content in guava fruit at different stages of ripening

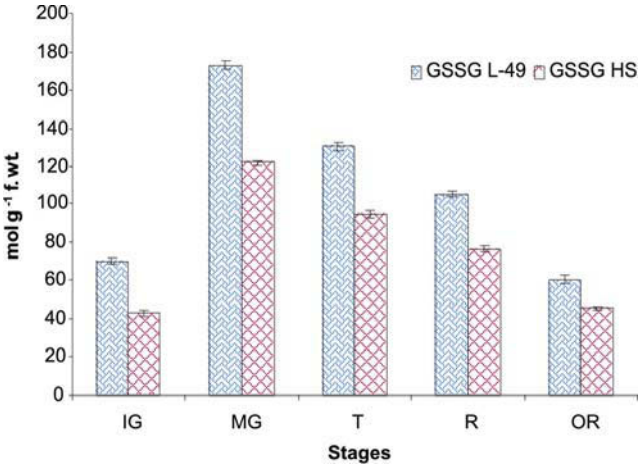

Fig. 12. GSSG content in guava fruit at different stages of ripening

Increment in lipoxygenase activity may cause loss of linolenate which is a major substrate of LOX leading to membrane damage. Lipid hydroperoxides from higher LOX activity, at later stages of ripening may contribute to an increase in cellular oxidative status which was evidenced by the increased oxidized glutathione (per cent of total) at fully ripe stage. Increased production of lipid hydroperoxides and other active oxygen species during ripening eventually induced higher activities of SOD, CAT, POX, APX and GR but not until the later stages of ripening. This might have led to the over accumulation of ROS. Although, it is too premature to speculate any mechanism, it appears that a decline in the free radical scavenging ability and the associated increase in oxidative stress may be mediating the biochemical/ physicochemical changes occurring during ripening of guava fruit as has been suggested for tomato (Mondal *et al*., 2004) and saskatoon (Rogiers *et al*., 1998) fruits. It can be documented that fruits with better ability to scavenge ROS may have higher shelf life. The results obtained can be exploited as an alternative strategy to enhance shelf life of fruits.

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