Antioxidant systems in ripening tomato fruits

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Abstract

Two cultivars of tomato (*Lycopersicon esculentum* Mill.), Selection-7 (shelf life 7-8 d) and ARTH-3 (shelf life 14-15 d) were analyzed for oxidative stress and the antioxidant enzyme system at different stages of fruit ripening. The results presented here suggest that during the early stages of fruit ripening, efficient antioxidant system protects the tomato fruits against the damaging effect of progressive oxidative stress. At later stages, however, oxidative damage occurs due to decreased activities of the ROS scavenging enzymes.

Additional key words: fruit softening, *Lycopersicon esculentum*, reactive oxygen species, scavenging enzymes,.

Introduction

Reactive oxygen species (ROS) such as superoxide radical ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\bullet -}$) are inevitably generated in plant cells as a consequence of normal metabolism. The antioxidant defence system including enzymes like superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT), dehydroascorbate reductase (DHAR), glutathione reductase (GR) and monodehydroascorbate reductase (MDHAR) and water soluble compounds such as ascorbic acid, glutathione and flavonoids and lipid soluble compounds like carotenoids and tocopherols, *etc*., help in scavenging of ROS (Foyer *et al*. 1994). In spite of the presence of an efficient antioxidant system, oxidative damages still occur in plant cells either due to uncontrolled production or inefficient scavenging of ROS. Senescence of green plant tissues is generally accompanied by increased contents of malondialdehyde (Hodges *et al.* 1999, Ye *et al.* 2000), higher production of ROS (Del Rio *et al.* 1998) and gradual loss in the ability of scavenging enzymes to neutralize the free radicals (Pastori and Del Rio 1997, Kanazawa *et al.* 2000). Since the overall process of fruit ripening is considered as functionally modified protracted form of senescence, the mechanism of membrane deterioration during ripening may also probably be similar to that characterized in senescing systems. Tomato fruits exhibit climacteric type of ripening physiology. Ethylene evolution and respiration increase substantially as the fruits mature. Ripening is also accompanied by substantial decrease in the concentration of starch and concomitant increase in total, reducing and non-reducing sugars (Singh *et al.* 2000). The extent to which oxidative stress accompanies and perhaps contributes to the changes, was the focus of the present study.

Materials and methods

The experiments were carried out with detached tomato (*Lycopersicon esculentum* Mill.) fruits of Selection-7 (a cultivar with short shelf life of 7 - 8 d) and ARTH-3 (a cultivar with long shelf life of 14 - 15 d). The plants of these cultivars were field grown by following the recommended agronomic practices at the Experimental Farms of the Department of Vegetable Crops, CCS Haryana Agricultural University, Hisar, India. Freshly

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Abbreviations: APX - ascorbate peroxidase; CAT - catalase; DHAR - dehydroascorbate reductase; GR - glutathione reductase; IMG - immature green; LOX - lipoxygenase; MDA - malondialdehyde; MDHAR - monodehydroascorbate reductase; MG - mature green; POX - peroxidase; ROS - reactive oxygen species; RR - red ripe; SOD - superoxide dismutase; TR - turning red.
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harvested fruits, free of any visible defects were surface sterilized with 0.2 % (m/v) mercuric chloride, rinsed in tap water and then in distilled water and air dried. After removal of the blossom to expose locule tissue, the fruits were identified as immature green (IMG, half the size of mature fruit), mature green (MG, full sized green fruit with ripe seeds surrounded by jelly like tissue), turning red (TR, pink colour developing around the apical end of the fruit and spread over the entire surface) and red ripe (RR, firm and red fruit without chlorophyll) based on visible observations of firmness, liquifaction and lycopene appearance (pigmentation).

Rate of lipid peroxidation was measured by the formation of malondialdehyde (MDA) as described by Heath and Packer (1968). The concentration of MDA was calculated using the coefficient of absorbance 155 $mmol^{-1}$ cm^{-1}. H_2O_2 was extracted by homogenizing 4 g tissue in 5 cm^3 of ice cold 0.01 M phosphate buffer (pH 7.0) and centrifuging the homogenate at 8 000 *g* for 10 min (Sinha 1972). 0.05 cm^3 of the sample was added to 1.95 cm^3 of 0.01 M phosphate buffer (pH 7.0). Two cm^3 of 5 % potassium dichromate and glacial acetic acid (1:3, v/v) was added to the mixture. The absorbance 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 μmol). For extracting ascorbic acid, 1 g tissue was macerated with 5 cm^3 of 5 % metaphosphoric acid, filtered and made the volume with 5 % mataphosphoric acid. An aliquot (5 cm^3) was titrated with 0.025 % 2,6-dichlorophenol-indophenol (DCPIP) until a pink colour reached which persisted for 15 s. The quantity of ascorbic acid was calculated by comparing cm^3 of DCPIP reagent used for unknown with that used for known amount of standard 0.1 % ascorbic acid.

Enzyme 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 enzymes. Extraction medium for SOD, CAT, APX, GR and LOX consisted of 0.1 M phosphate buffer (pH 7.5) containing 5 % (m/v) polyvinylpyrrolidone (PVP), 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 % (m/v) PVP. The homogenate was prepared by grinding 4 g (fresh mass) of tomato fruits in 5 cm^3 of ice cold extraction medium in pre-cooled mortar and pestle. The homogenate thus prepared was centrifuged at 10 000 *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). 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 0.1 cm^3 of enzyme extract to 1 cm^3 of 0.2 M phosphate buffer (pH 7.0) containing 1 mM EDTA, 0.75 cm^3 distilled water, 0.1 cm^3 of 20 mM oxidized glutathione (GSSG) and 0.1 cm^3 of 2 mM NADPH. Oxidation of NADPH by GR was monitored at 340 nm and the rate (nmol min^{-1}) was calculated using the coefficient of absorbance 6.2 $mmol^{-1}$ cm^{-1}.

Results and discussion

LOX activity (Fig. 1*A*) and MDA content (Fig. 1*B*) increased in tomato fruits during ripening. LOX activity increased from 18 and 35 units to 80 and 130 units in ARTH-3 and Sel-7 from IMG to RR stage, respectively. MDA content during ripening followed a pattern similar to that of LOX activity. The MDA content increased substantially from 0.4 to 1.6 and from 0.6 to 2.3 nmol g^{-1} from IMG to RR stage in ARTH-3 and Sel-7, respectively. At RR stage, both LOX activity and MDA content were 1.5 fold higher in Sel-7 than in ARTH-3, suggesting that membrane deterioration in Sel-7 was much faster than in ARTH-3 leading to its shorter shelf life. Lurie and Ben-Arie (1983) demonstrated increased lipid peroxidation in ripening apples and attributed this to increased LOX activity. Similar observations of lipid peroxidation leading to membrane deterioration have been correlated with higher LOX activity during ripening of saskatoon (Rogiers *et al.* 1998), avocado, pear and tomato (Brennan and Frenkel 1977, Kausch and Handa 1997) fruits as the enzyme specifically initiates oxidation of *cis*,*cis*-1,4 pentadiene moieties of free polyunsaturated fatty acids such as linoleic and linolenic acids. The hydroperoxides thus formed decompose into oxy-free radicals, ethane and thiobarbituric acid reactive substances, all of which are sensitive markers of lipid peroxidation (Rogiers *et al*. 1998). However, free fatty acids are to be cleaved first by lipases for subsequent reactions of LOX. Accordingly, an increase in LOX activity as observed here may not be regarded as the direct cause of oxidative stress. Similarly, the increase in MDA content need not be directly connected with increased LOX activity. In that case, this increase could be explained better by non-enzymatic oxidation reactions carried out by ROS. It is also confirmed from a decrease

in antioxidant enzyme activities during ripening. Contrary to MDA and LOX activity, H_2O_2 content (Fig. 1*C*) was maximum in IMG tomatoes and exhibited only a marginal decrease during ripening. This may be attributed to decrease in SOD (an H_2O_2 producing enzyme) activity (Fig. 2*A*) and increase in catalase (an H_2O_2 consuming enzyme) activity (Fig. 2*D*) during ripening. ARTH-3 had lower content of H_2O_2 than Sel-7 throughout the ripening period. H_2O_2 is a strong oxidant that can initiate localized oxidative damage leading to disruption of metabolic functions and losses of cellular integrity at sites where it accumulates (Foyer *et al.* 1997).

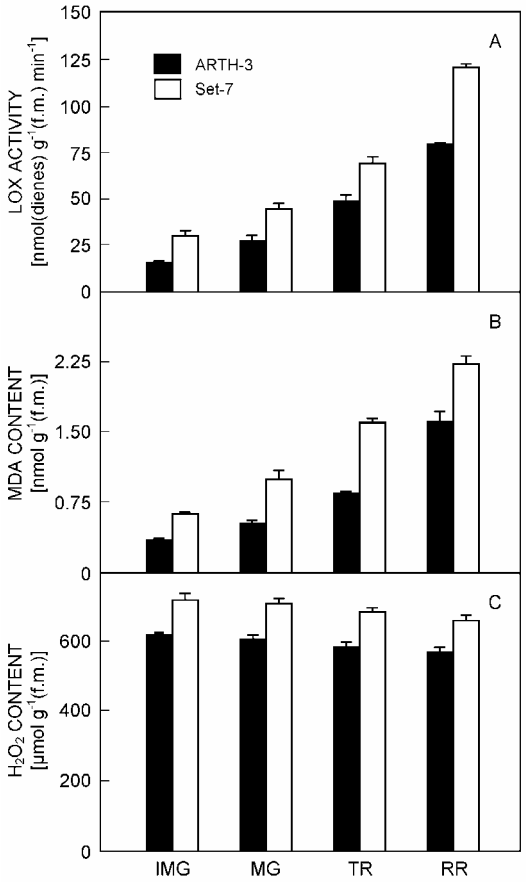

Fig. 1. LOX activity (*A*), MDA (*B*) and H_2O_2 (*C*) contents in fruits of two cultivars of tomato at different stages of ripening: IMG - immature green, MG - mature green, TR - turning red, RR - red ripe.

To determine whether the increasing oxidative stress accompanying tomato fruit ripening was associated with reduced ability to enzymatically catabolize ROS, changes in the activities of SOD, CAT, POX, GR and APX were determined as a function of fruit ripening. APX (Fig. 2*B*), GR (Fig. 2*C*), CAT (Fig. 2*D*) and POX (Fig. 2*E*) activities were higher in ARTH-3 than in Sel-7 at all stages of fruit ripening. These activities were the highest in MG fruits but declined as the fruit developed to the fully RR stage except CAT which exhibited a slight increase upto RR stage. POX activity (Fig. 2*E*) was quite high in ARTH-3 than in Sel-7 and showed only marginal decrease during ripening. These results thus correlated well with progressive increase in oxidative stress during fruit development. Respiration is a primary source of O_2^- (Purvis *et al.* 1995). Due to substantial increase in respiration of the climacteric fruits like tomato, oxy free radical production probably increases over later stages of development. Increases in lipid peroxidation products during later stages of fruit ripening could also be mediated through increased LOX activity. During plant senescence, LOX activity increases, while SOD, CAT and POX activities fall, resulting in a concomitant decline in the ability to scavenge free radicals (Dhindsa *et al.* 1981). Trends in the activities of LOX, SOD and CAT in ripening tomato fruits observed during the present studies were thus consistent with those evident in true senescing systems, even though the fruits were in a stage of active growth and had not begun to senesce. The increasing oxidative stress that probably results from lower activities of these enzymes is evidently needed to facilitate many of the metabolic changes associated with maturation and ripening of tomato fruits. Lower activities of these enzymes in Sel-7, the cultivar with short shelf life further supports the view that reduced ability to scavenge free radicals mediates biochemical changes leading to fast ripening/softening process in this cultivar.

Tomato fruits responded to the increase in oxidative stress during development by increasing ascorbic acid content in both the cultivars (Fig. 2*F*). ARTH-3 had lower content of ascorbate than Sel-7 at all stages of fruit ripening. It increased by about two folds as fruits developed from IMG to CT stage. However, at RR stage, the tomato fruits exhibited a marginal decline in the content of ascorbate. The increase in ascorbic acid upto CT stage probably coincides with the climacteric rise in respiration during ripening and since respiration is a major source of active ROS (Rich and Bonner 1978), the respiratory climacteric is probably a significant contributor to the increasing oxidative stress during this phase of development. From these results, it is evident that tomato fruits respond to the progressive increase in oxidative stress during earlier stages of maturation by increasing both, the activities of scavenging enzymes as well as the concentraion of ROS scavenging compounds. However, at later stages, the ROS scavenging system does not cope up with the production system, leading to the accumulation of ROS.

In summary, ripening of tomato fruits was accompanied by a progressive increase in oxidative/ peroxidative stress. The cultivar with short shelf life had higher oxidative stress than the cultivar with longer shelf life. Increase in production of lipid hydroperoxides and other reactive oxygen species during development

eventually induced higher activities of peroxidase, glutathione reductase, superoxide dismutase, catalase and ascorbate peroxidase, but not until the later stages of ripening. The activities of these enzymes were consistently low in the cultivar with short shelf life, suggesting that the reduced scavenging ability and associated increase in oxidative stress in the cultivar with short shelf life may be responsible for mediating many of the physicochemical changes that facilitate early ripening/softening of the fruits.

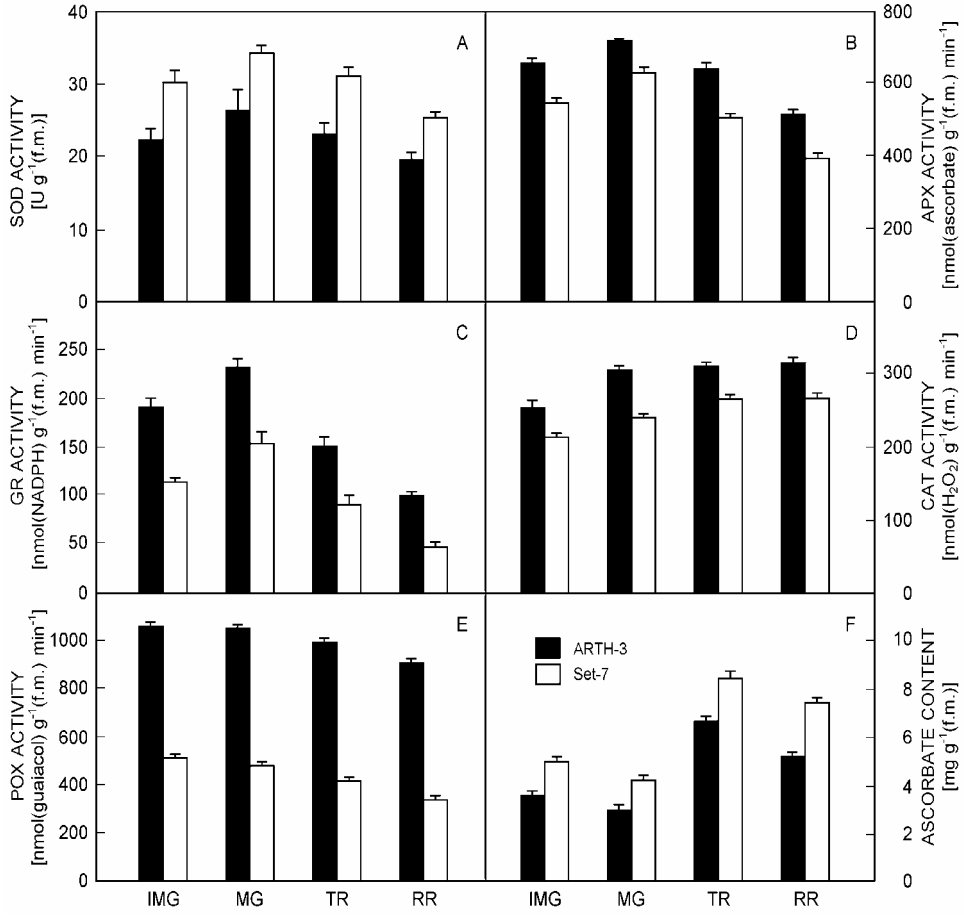

Fig. 2. SOD (*A*), APX (*B*), GR (*C*), CAT (*D*), POX (*E*) activities and ascorbate (*F*) content in fruits of two cultivars of tomato at different stages of ripening: IMG - immature green, MG - mature green, TR - turning red, RR - red ripe.

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