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Oxidative Stress and Antioxidative System in Ripening Ber (*Ziziphus mauritiana* Lam.) Fruits

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Summary

The present investigations were undertaken to ascertain the extent of lipid peroxidation, oxidative stress and antioxidative system during ripening of two varieties of ber fruits (*Ziziphus mauritiana* Lam.) differing in their shelf lives, *viz.* Umran (8–9 days) and Kaithali (4–5 days). Based on visual observations, fruits were categorized as immature green (IG), mature green (MG), turning colour (TC), ripe (R) and overripe (OR). Lipoxygenase (LOX) activity, malondialdehyde (MDA) value and H_2O_2 content increased significantly during ripening in both varieties, obtaining 3-, 3- and 2.5-fold increase in Umran and 4-, 3.5- and 3-fold increase in Kaithali, respectively. Kaithali being short-lived variety, exhibited higher values of these parameters at almost all the stages of fruit ripening. Activity of superoxide dismutase (SOD), glutathione reductase (GR) and ascorbate peroxidase (APX) increased during initial stages but thereafter decreased, whereas that of peroxidase (POX) decreased continuously during ripening. On the other hand, catalase (CAT) activity increased continuously as ripening progressed. In general, antioxidative enzymes exhibited comparatively higher activity in Umran. Total ascorbic acid content increased from IG to TC stage, while total glutathione increased up to MG stage and thereafter decreased in both varieties. Kaithali had higher content of ascorbate than Umran. β-Carotene and α-tocopherol also decreased as ripening progressed. However, the decrease was more pronounced in Umran as compared to Kaithali.

Key words: *Ziziphus mauritiana*, antioxidative enzymes, antioxidative metabolites, oxidative stress

Introduction

Fruits, being a rich source of essential nutrients, are generally important in our diet for their high content of fibres, vitamins and minerals. However, water content in fruits represents very high percentage of fresh mass, consequently exhibiting relatively high metabolic activity, which continues post harvest and makes most fruits highly perishable commodities. It is this perishability and inherent short shelf life that present the greatest problem for the successful transportation and marketing of fresh fruits. Efforts have been made to increase the shelf life of fruits using antisense RNA technology to inactivate the expression of ripening-related proteins such as ethylene biosynthetic enzymes, *viz.* 1-aminocyclopropane-1-carboxylate (ACC) synthase and ACC oxidase (*1*), and cell wall degrading enzymes like polygalacturonase and

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Abbreviations: 2,6-DCPIP: 2,6-dichlorophenolindophenol; EDTA: ethylenediaminetetraacetic acid; fm: fresh mass; GSH: reduced glutathione; TCA: trichloroacetic acid; Tris-HCl: Tris-(hydroxymethyl)aminomethane hydrochloride

pectin methylesterase (*2*). In spite of considerable efforts and huge resource commitments worldwide, the enhancement of fruit shelf life has not met with more than limited success. If a big breakthrough in this field is to be achieved, the problem has to be handled from different angles and, therefore, there is a continuous search for alternative targets. One of the fertile areas in this direction appears to be that of scavenging of reactive oxygen species (ROS) such as $O_2^{\cdot-}$ (superoxide anion), H_2O_2 (hydrogen peroxide), $^{\cdot}OH$ (hydroxyl radical) and $^1O_2$ (singlet oxygen), which are essentially generated during normal metabolism as by-products of inevitable leakage of electrons to molecular oxygen from the electron transport activities in chloroplast, mitochondria and plasma membrane (*3*). Accumulation of ROS is linked to deteriorative changes such as DNA mutation, protein denaturation, enzyme inactivation and lipid peroxidation (*4*).

Plants, however, possess an impressive array of defense mechanisms against oxidative stress including the enzymatic and nonenzymatic antioxidant systems. The antioxidant enzymes include superoxide dismutase (SOD), peroxidase (POX), catalase (CAT), ascorbate peroxidase (APX) and glutathione reductase (GR) (*5*), while the nonenzymatic antioxidants include water-soluble (ascorbate, glutathione, phenolic compounds and flavonoids) and lipid-soluble (α-tocopherol, β-carotene, lycopene) metabolites (*6*). Superoxide dismutases (SODs), metalloenzymes, are believed to play a crucial role in antioxidant defense (*7*) because they catalyze the dismutation of $O_2^{\cdot-}$ to H_2O_2, while the removal of H_2O_2 is taken care of by CAT and/or POX and the enzymes of ascorbate-glutathione pathways (*8*). In spite of such an efficient scavenging system, activated O_2 species (AOS) accumulation has been found to be stimulated by various environmental, mechanical and physical stresses (*9*) and biotic factors such as invasion of various pathogens (*10*). In addition, AOS production has also been reported to be stimulated during plant senescence (*11*) and during ripening and storage of fruits (*12,13*), the events which are characterized by the breakdown of cell wall components, membrane disruption and cellular decompartmentation (*14*). Although ber fruit is a crop of worldwide economic importance and it prevents chronic diseases such as diabetes and inhibits oxidation of low-density lipoproteins (*15*), it is highly perishable, and undergoes cell wall degradation and fruit softening during ripening and storage (*4*). Changes in the extent of various indicators of oxidative stress and counteracting antioxidant system during ripening of ber are presented here.

Materials and Methods

Plant material and chemicals

Ber fruits of two varieties differing in shelf lives, *viz.* Umran (8–9 days) and Kaithali (4–5 days) (*16*) were harvested from ten-year-old trees grown at horticulture farm, CCS HAU, Hisar (Haryana, India). The fruits were harvested from multiple trees and treated as one replicate. Based on visual observations of colour, texture and liquefaction, fruits were categorized as immature green (IG), mature green (MG), turning colour (TC), ripe (R) and overripe (OR). All chemicals used during present investigations were of analytical grade and obtained from Sigma-Aldrich Chemical Company, St. Louis, MD, USA (glutathione (GSH) reductase, 4-vinyl pyridine), Sisco Research Laboratories Pvt. Ltd., Mumbai, India (riboflavin, L-methionine, nitroblue tetrazolium, guaiacol, 2,6-dichlorophenolindophenol – DCPIP) and HiMedia Laboratories Ltd., Mumbai, India (Tris-HCl, β-carotene, α-tocopherol).

Preparation of various extracts

Preliminary experiments were conducted to optimize the extraction conditions with respect to pH, molarity and type of buffer, concentration of stabilizing agent(s) and other constituents of extraction medium. Unless stated otherwise, all extraction procedures were carried out at 0–4 °C.

Enzyme extraction

The standardized extraction medium for SOD and POX consisted of 0.1 M Tris-HCl buffer (pH=7.5) containing 3 % (by mass per volume) polyvinylpyrrolidone, 1 mM EDTA and 1 mM $CaCl_2$. The extraction medium for CAT, APX, GR and LOX consisted of 0.1 M potassium phosphate buffer (pH=7.5) in place of Tris-HCl buffer, the rest of the extractants were the same. The enzymes were extracted by macerating 25 g of tissue with 90 mL of ice cold extraction medium in a pre-chilled pestle and mortar using acid-washed sand as abrasive. The homogenate was filtered through four layers of cheesecloth and the filtrate was centrifuged at 15 000 rpm for 20 min in a refrigerated centrifuge at 4 °C. The supernatant was carefully decanted and used as crude enzyme preparation. The extract prepared in phosphate buffer was also used for H_2O_2 estimation.

TCA extraction

Ascorbic acid, glutathione and malondialdehyde were extracted in trichloroacetic acid (TCA). A mass of 15 g of ber tissue was ground with 75 mL of 0.1 % TCA. The extract was filtered through four layers of cheesecloth and centrifuged at 12 000 rpm for 15 min at 4 °C.

Extraction of fat-soluble antioxidants

For β-carotene and α-tocopherol extraction (*17*), 25 g of ber tissue were ground with petroleum ether:acetone (1:1), immersed with 50 mL ether:acetone solvent and covered with aluminium foil to avoid volatile loss. The extract was kept overnight, decanted and filtered through filter paper. Residue/meshed tissue was washed twice or three times using about 30 mL of extraction mixture each time and the filtrates were pooled. To the pooled extract, 100 mL of water were added and the water layer was separated through separatory funnel to eliminate water-soluble debris, if any. The water solvent treatment was repeated twice and organic layer was collected. A pinch of sodium sulphate was added to remove any trace of water/moisture left. The final volume of organic solvent phase containing fat-soluble antioxidants was made to 100 mL and used for estimation of β-carotene and α-tocopherol.

Enzyme assays

The activity of LOX was assayed spectrophotometrically at 234 nm (*18*) and expressed as nmol of conjugated dienes produced per min per g of fresh mass (fm). SOD was assayed by measuring its ability to inhibit the photoreduction of nitroblue tetrazolium (NBT) (*19*). The enzyme activity was expressed as μmol of SOD required to inhibit NBT photoreduction per min per g of fm. POX was assayed by determining the rate of guaiacol oxidation in the presence of H_2O_2 at 470 nm (*6*) and the enzyme activity was expressed as nmol of guaiacol oxidized per min per g of fm. CAT activity was measured and expressed as μmol of H_2O_2 consumed per min per g of fm (*20*). APX activity was expressed as nmol of ascorbate oxidized per min per g of fm (*21*), while GR activity was expressed as nmol of NADPH oxidized per min per g of fm (*22*).

Estimation of metabolites

The level of lipid peroxidation was measured in terms of MDA by using thiobarbituric acid (TBA) reaction (*23*). The concentration of MDA was calculated using the molar absorption coefficient of 155/(mM·cm) and expressed as nmol of MDA per g of fm. The amount of H_2O_2 was expressed as μmol of H_2O_2 per g of fm (*20*). Ascorbic acid content was estimated based on the reduction of DCPIP (*24*). The results were expressed as μmol of ascorbic acid per g of fm. Glutathione content was expressed as μmol of glutathione per g of fm (*25*). β-Carotene was estimated spectrophotometrically and the results were expressed as ng of β-carotene per g of fm (*17*). α-Tocopherol content was expressed as μmol of α-tocopherol per g of fm (*26*).

Statistical analysis

The statistical analysis was done using complete randomized design (CRD). Multiple fruits from multiple trees were treated as one replicate and each replicate was repeated three times. The experiments were planned as two factorial experiments. The least significant difference (LSD) of stages, varieties and interactions was calculated at $p \leq 0.05$.

Results and Discussion

Indices of oxidative stress

Changes in LOX activity during ripening of Umran and Kaithali are presented in Table 1. The activity which was the lowest at IG stage (9.8 and 11.5 nmol of conjugated dienes produced per g of fm per min in Umran and Kaithali, respectively) increased throughout ripening and obtained the highest value of 27.9 (Umran) and 46.8 (Kaithali) nmol of conjugated dienes produced per g of fm per min at OR stage with about 3- to 4-fold increase. It has been reported that membrane deterioration and loss of tissue structure in saskatoon fruits during ripening are due to increased activity of LOX (*12*). Similar observations of increased lipid peroxidation during fruit ripening have been reported in tomato fruits (*27*). The extent of lipid peroxidation as judged by the formation of MDA in the two varieties of ber is presented in Table 1. Parallel to LOX, MDA value also increased continuously throughout ripening from a minimum of 1.32 (Umran) and 1.70 (Kaithali) at IG stage to a maximum of 4.54 (Umran) and 5.97 (Kaithali) nmol of MDA per g of fm at OR stage. Similarly, the increase in MDA value has been reported in tomato (*27*) and guava (*28*). Hydrogen peroxide is yet another toxic ROS which is generated in a number of ways during normal metabolic processes. Similar to LOX and MDA, H_2O_2 content (Table 1) was minimum at IG stage (731.7 and 632.87 μmol per g of fm in Umran and Kaithali, respectively), increased continuously throughout the ripening period and reached maximum at OR stage (1844.3 and 2057.3 μmol per g of fm in Umran and Kaithali, respectively). The results are in agreement with those reported for saskatoon fruits (*12*). However, the results are contrary to those in tomato fruits (*27*), where H_2O_2 content was found to be maximum at IG stage and decreased gradually as the fruit

Table 1. ANOVA results for LOX activity, MDA value and H_2O_2 content

Variety or stage	LOX activity nmol/(min·g)	MDA value nmol/g	H_2O_2 content μmol/g	Interaction	LOX activity* nmol/(min·g)	MDA value** nmol/g	H_2O_2 content** μmol/g
Umran	18.480	2.816	1284.190	U×IG	9.800[a]	1.323	731.700
Kaithali	24.480	3.859	1278.720	U×MG	15.233[b]	1.787[a]	817.300[a]
SEM±LSD	0.3±0.8	0.02±0.06	2.4±NS	U×TC	17.733[d]	2.660	1431.100
				U×R	21.733	3.770	1596.530
				U×OR	27.900	4.540	1844.300
IG	10.650	1.512	682.280	K×IG	11.500[a]	1.700[a]	632.870
MG	15.483	2.073	817.870	K×MG	15.733[b,c]	2.360	818.430[a]
TC	17.500	3.348	1250.130	K×TC	17.267[c,d]	4.037	1069.170
R	26.400	4.498	1706.180	K×R	31.067	5.227	1815.830
OR	37.367	5.257	1950.800	K×OR	46.833	5.973	2057.300
SEM±LSD	0.4±1	0.03±0.1	4±11	SEM±LSD	0.6±1.7	0.05±0.14	5.4±15.8

*a, b, c and d were on par with LSD
**both interactions 'a' were on par with LSD

ripened. The parallel increase in LOX activity and MDA value during ripening indicates membrane disruption, which in turn leads to increased production of ROS and H_2O_2 content, thus causing softening of the fruit. Higher values for these parameters in Kaithali, a short-lived variety, further suggest their role in membrane deterioration and softening of fruit. On the F-test analysis basis, significantly lower values of LOX and MDA parameters were observed for Umran variety, whereas LOX, MDA and H_2O_2 parameters regarding different stages of ripening show that IG stage exhibited respective minimum value (Table 1). Further, for interaction effect (variety×stage), the most effective combination observed with respect to LOX and MDA was U×IG, while for H_2O_2, minimum value observed was K×IG combination. The combinations U×IG and K×IG were found to be statistically on par with each other. The varieties, stages and interactions were statistically significant for stages except at varietal level difference for H_2O_2, which was not significant (Table 1).

Antioxidative enzymes

The activity of SOD increased from 2.4 and 1.41 at IG stage to 5.3 and 4.22 μmol of NBT inhibited by photoreduction per g of fm per min at TC stage, respectively, in Umran and Kaithali (Table 2). Thereafter, it decreased to 1.917 (Umran) and 1.35 (Kaithali) μmol of NBT inhibited by photoreduction per g of fm per min at OR stage. In general, Umran variety exhibited higher SOD activity at all the stages of fruit ripening, thus, dismutating $O_2^{\cdot-}$ anion significantly. Conflicting results have been reported regarding SOD activity during ripening. Maximum SOD activity at TC stage observed during the present investigations is in accordance with Thakur and Pandey (*29*), where SOD activity was reported to be maximum at turning pink stage of tomato. However, SOD activity has been reported to be maximum at MG stage and to decrease thereafter during ripening of tomato (*27*) and guava (*28*). The decrease in SOD activity during ripening of fruit has also been observed in saskatoon fruit (*12*) and orange (*30*). Peroxidase activity (Table 2) was maximum at IG stage in both varieties and decreased continuously during ripening, reaching the minimum value at the OR stage. In Umran, the activity decreased from 151.733 at IG to 54.067 nmol of guaiacol oxidized per g of fm per min at OR stage, while in Kaithali the corresponding values were 126.3 and 34.133. These results are in fair agreement with those observed in tomato (*27*) and guava (*28*), where a continuous decrease in POX activity has been reported during ripening. On the contrary, an increase in POX activity during ripening of tomato fruits has also been observed (*29*). The activity profile of CAT (Table 2) showed that there was a progressive increase in the activity during ripening of ber fruits. The lowest activity of 440.667 and 763.233 μmol of H_2O_2 consumed per g of fm per min in Umran and Kaithali at IG stage attained the maximum value of 1634.6 and 1320.733 μmol of H_2O_2 consumed per g of fm per min, respectively, at OR stage. Surprisingly, the activity was found to be higher in Kaithali, a short shelf life variety, than in Umran at IG, MG and TC stages although during the later stages, Umran exhibited higher CAT activity. A similar continuous increase in CAT activity had been reported earlier during ripening of tomato (*27*,*29*). However, the results different from these, *i.e.* an initial increase and then decrease in the CAT activity, have been reported in saskatoon fruit (*12*). Ascorbate peroxidase activity (Table 2) decreased from 432.0 and 398.767 nmol of ascorbate oxidized per g of fm per min at IG stage to 356.567 and 274.9 nmol of ascorbate oxidized per g of fm per min at TC stage in Umran and Kaithali, respectively. The activity increased drastically at R stage and again decreased at OR stage in both varieties. Umran had higher activity than Kaithali at all the stages of fruit ripening except at OR stage where Kaithali had slightly higher activity compared to Umran. Conflicting results regarding APX activity have been reported in literature. APX activity has

Table 2. ANOVA results for SOD, POX, CAT, APX and GR activity

Variety or stage	SOD activity μmol/min·g	POX activity nmol/min·g	CAT activity μmol/min·g	APX activity nmol/min·g	GR activity nmol/min·g	Interaction	SOD* activity μmol/min·g	POX** activity nmol/min·g	CAT*** activity μmol/min·g	APX**** activity nmol/min·g	GR**** activity nmol/min·g
Umran	3.183	107.820	1067.127	488.360	1381.367	U×IG	2.400^{b}	151.733	440.667	432.000	1409.167^{a}
Kaithali	2.316	76.987	1094.833	384.113	984.020	U×MG	3.100^{a}	135.167^{a}	820.033	$379.400^{a,b}$	1768.467
SEM±LSD	0.02±0.07	1.6±4.7	3.9±11.5	4±12	4±12	U×TC	5.300	113.333	1019.800^{a}	356.567^{b}	1426.367^{a}
						U×R	3.200^{a}	$84.800^{b,c}$	1420.533	914.600	1306.000
						U×OR	1.917	54.067^{d}	1634.600	359.233^{b}	996.833^{b}
IG	1.905	139.017	601.950	415.383	1203.700	K×IG	1.410^{c}	126.300^{a}	763.233	398.767	998.233^{b}
MG	2.617	114.600	909.800	351.600	1639.783	K×MG	2.133	94.033^{b}	999.567^{a}	323.800	1511.100
TC	4.760	95.617	1093.817	315.733	1175.883	K×TC	4.220	77.900^{c}	1167.833	274.900	925.400
R	2.833	68.683	1321.667	717.300	1064.767	K×R	2.467^{b}	52.567^{d}	1222.800	520.000	823.533
OR	1.633	44.100	1477.667	381.167	829.333	K×OR	1.350^{c}	34.133	1320.733	403.100^{a}	661.833
SEM±LSD	0.04±0.1	2.5±7.4	6±18	6.5±19	6.4±19	SEM±LSD	0.05±0.15	3.5±10	8.7±25.7	9±27	9±27

*a, b and c were on par with LSD
**a, b, c and d were on par with LSD
***both interactions 'a' were on par with LSD
****a and b were on par with LSD

been reported to decrease in pea (*31*). The highest APX activity at MG stage followed by a continuous decrease during ripening has been reported in tomato (*27*) and guava (*28*). Decline in APX activity at OR stage resulted in the inefficiency of the antioxidant system to scavenge ROS, causing oxidative stress. The decrease in APX activity at OR stage may be either due to substrate limitation or the enzyme inactivation. The down-regulation of APX may be suggested to be responsible for the accumulation of endogenous H_2O_2, membrane deterioration and loss of tissue structure during fruit ripening. Such a decrease in APX activity that initiates senescence process in gladiolus tepals has also been reported earlier (*32*). For GR, both varieties obtained highest enzyme activity at MG stage (1768.467 and 1511.1 nmol of NADPH oxidized per g of fm per min in Umran and Kaithali, respectively), which decreased gradually to the respective values of 996.833 and 661.833 nmol at OR stage (Table 2). Umran showed comparatively higher enzyme activity than Kaithali throughout the ripening period. The results are in agreement with those reported for ripening tomato (*27*) and guava (*28*) fruits. However, the present results are contrary to those of Rogiers *et al.* (*12*), who observed a continuous increase in GR during ripening of saskatoon fruits. Data revealed that significantly higher activities were observed in Umran variety for SOD, POX, APX and GR and in Kaithali for CAT enzyme (Table 2). Furthermore, the enzymatic activities were influenced significantly by stages of fruit ripening. The varieties, stages and interactions were statistically significant (Table 2).

Antioxidant metabolites

Total ascorbate content (Table 3) increased from IG stage (430.4 and 446.587 μmol per g of fm) to TC stage (530.533 and 560 μmol per g of fm) for Umran and Kaithali, respectively, followed by a continuous decline; however, Kaithali had higher ascorbic acid content than Umran. The decline in ascorbic acid at later stages may be either due to its oxidation or utilization by APX. Similar results have been reported in apple (*33*) and tomato (*27*) where ascorbic acid was observed to exhibit an initial increase followed by a decline. Total glutathione (Table 3) was found to be maximum at MG stage in both ber varieties, *viz.* 11986.7 in Umran and 9922.370 μmol per g of fm in Kaithali, but thereafter decreased progressively up to OR stage. The maximum accumulation of glutathione at MG stage suggests its role as antioxidant due to overproduction of ROS at this stage. These results are in close agreement with those reported earlier in saskatoon (*12*) and guava (*28*) fruits during ripening. The data in Table 3 revealed that during ripening there was a continuous decrease in β-carotene from 108.633 and 96.6 ng per g of fm in Umran and Kaithali, respectively at IG stage to 5.6 and 15.2 ng per g of fm at OR stage, respectively. The present findings are contrary to the results reported previously. The carotenoid content has been reported to increase during ripening of *Capsicum* (*34*) and mandarin (*35*), respectively. The α-tocopherol content was 100.0 and 126.033 μmol per g of fm in Umran and Kaithali, respectively, at IG stage, which decreased gradually towards ripening and reached a minimum of 35.433 and 61.9 μmol per g of fm at OR stage (Table 3). The results are in fair agreement with those of Sattler *et al.* (*36*), who observed an antagonistic role of α-tocopherol with LOX activity and lipid peroxidation, *i.e.* as LOX activity increased, α-tocopherol content decreased. Similar results have also been reported in *Arabidopsis thaliana* (*37*). The data explicitly show that Kaithali variety had higher activities of antioxidant metabolites, namely total ascorbate, β-carotene and α-tocopherol, except total glutathione, which was higher in Umran. The varieties, stages and interactions were statistically significant (Table 3).

Conclusion

In the present investigations, throughout ripening, LOX activity, MDA value and H_2O_2 content increased considerably, suggesting that fruit ripening process is asso-

Table 3. ANOVA results for total ascorbate, total glutathione, β-carotene and α-tocopherol content

Variety or stage	Total Asc content	Total glutathione content	β-Carotene content	α-Tocopherol content	Interaction	Total Asc content*	Total glutathione content	β-Carotene content	α-Tocopherol content*
	μmol/g	μmol/g	ng/g	μmol/g		μmol/g	μmol/g	ng/g	μmol/g
Umran	453.527	9190.700	43.287	64.213	U×IG	430.400	9735.000	108.633	100.000
Kaithali	495.077	6930.090	53.367	93.213	U×MG	505.200	11986.700	65.600	75.200
SEM±LSD	1±3	3.6±10.7	0.2±0.5	0.1±0.3	U×TC	530.533	9881.600	30.600	62.500[a]
					U×R	440.000[a]	8061.400	6.000	47.933
					U×OR	361.500	6288.800	5.600	35.433
IG	438.493	9238.450	102.617	113.017	K×IG	446.587[a]	8741.900	96.600	126.033
MG	500.750	10954.530	69.533	94.050	K×MG	496.300	9922.370	73.467	112.900
TC	545.267	8233.300	42.383	74.617	K×TC	560.000	6585.000	54.167	86.733
R	476.650	6598.200	16.700	63.217	K×R	513.300	5135.000	27.400	78.500
OR	410.350	5277.480	10.400	48.667	K×OR	459.200	4266.170	15.200	61.900[a]
SEM±LSD	1.7±5.0	5.7±16.9	0.3±0.8	0.2±0.5	SEM±LSD	2.4±7.1	8.1±23.9	0.4±1.1	0.2±0.7

*both interactions 'a' were on par with LSD

ciated with the increasing oxidative stress. An increase in the activities of ROS-scavenging enzymes during initial stages of fruit ripening suggests that increased production of lipid hydroperoxides and other ROS during ripening eventually induces higher activities of these enzymes but not up to later stages. In general, lower activities of antioxidative enzymes associated with higher oxidative stress in Kaithali, a short shelf life variety, suggest the accumulation of ROS to be responsible for comparatively higher membrane deterioration and loss of tissue structure in Kaithali.

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