Influence of seed fatty acids on seed viability and corky tissue development in sapota (*Manilkara achras*) fruits cv. "Cricket ball"

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Abstract- Corky tissue (CT) is a major physiological disorder adversely affecting fruit quality in sapota (*Manilkara achras*) cv.Cricket ball in India. Corky tissue affected fruits are characterized by a dry acidic flesh and hard lump in the pulp, without showing external symptoms. Loss of seed viability during fruit growth has been found to cause the formation of the disorder. However, the exact process which triggers CT development remains unclear. It has been frequently observed that seed fat content in seeds from CT affected fruits remains significantly lower in comparison with that of seed in healthy fruit. Since seed fats play a major role in determining seed viability, the purpose of the present study was to determine the possible association between seed fats and seed viability in the development of corky tissue. The study found that the seed moisture content, total dehydrogenase activity (TDH), fat content and percent seed germination in seeds of CT affected fruits were significantly lower compared to healthy seed. There was an excessive production of free radicals in seeds of CT affected fruit while the activities of antioxidative enzymes like peroxidase (POD), catalase (CAT) and superoxide dismutase (SOD) were markedly lower. Consequently, the extent of lipid peroxidation was significantly higher in seed from CT affected fruit compared to seeds in healthy fruit. There was a significant rise in the level of iron content in seed from CT affected fruit which possibly accounted for the disproportionate production of free radical and substantially higher levels of lipid peroxidation. There was a marked decline in the level of VLCFAs and the contents of linoleic acid and oleic acid associated with an increase of EC and a fall in the pH of seed indicating a loss of seed viability. Analysis of results suggested that the reduced fat content of seed accompanied by changes in the composition of fatty acids coupled with an enhanced production of free radicals resulted in increased lipid peroxidation and membrane damage leading to a loss of seed viability and culminating in the development of corky tissue in sapota fruit cv. Cricket ball.

Index Terms- Corky tissue, sapota, *Manilkara achras*, physiological disorder, seed viability, germination, seed fatty acids, VLCFAs, free radical, lipid peroxidation

I. INTRODUCTION

Corky Tissue (CT) of sapota fruit, *Manilkara achras* (Mill.) Fosberg, cv.Cricket ball is a physiological disorder distinguished by the presence of a semi-dehydrated hard lump/s embedded inside the soft pulp tissue of ripe fruits, slightly sour to taste and located near the peel. The rate of incidence of the disorder shows wide variations within and among trees, locations and seasons even within the same orchard. Fruits harvested in summer season display higher incidence of CT compared to winter crop and thus present a major challenge for the production of consistently high quality fruits. A unique characteristic of the disorder is that the CT affected fruits do not show external symptoms and are identified only after cutting the fruits open.

Past work based on measurements of seed dehydrogenase activity had shown that the incidence of corky tissue in 'Cricket ball' sapota occurred due to a reduction in seed viability (Shivashankar *et al*., 2013). Subsequent work showed that changes in seed viability influenced the metabolism of fruit pulp resulting in CT formation and the associated deterioration of fruit quality in affected fruits (Shivashankar *et al*., 2014)

Among the numerous varieties of sapota being grown commercially, cv."Cricket ball" is worst affected by the CT disorder. A preliminary examination of sapota varieties for fat content showed that "Cricket ball" contained significantly higher level of fats in its seed compared to other varieties. However, the CT affected sapota seed contained less fats compared to the seed from healthy fruit (Shivashankar *et al*., 2007). Changes in fat metabolism producing differences in the composition of fatty acids of seed is reported to cause a loss of viability in some plants (Lin and Pearce, 1990) as fatty acid composition is the most important factor which determines its susceptibility to oxidation. As the biosynthesis of fats in plant cells occurs from a supply of carbohydrates at the expense of high energy input, we speculated that the lower level of fat content in CT seed could arise from a competition for assimilates among fruits on the same panicle which might in turn, influence seed viability and cause the disorder. In order to test this hypothesis, the present work was initiated with the objective of determining the possible association between seed fats and seed viability and its influence on the development of corky tissue in sapota cv. Cricket ball.

II. MATERIAL AND METHODS

II.1. Material

"Cricket ball" sapota fruits were collected during the 2013–14 season from 10-year-old trees receiving the recommended supply of fertilizers and plant protection measures and maintained under uniform growth conditions in the experimental orchard of IIHR, Bangalore.

II.2. **Moisture content**

Moisture contents of seed tissue samples were determined gravimetrically immediately after cutting the fruit open.

II.3. **Seed germination**

Seeds were sown on moist filter paper in closed petri dishes and placed in an incubator in darkness at 28°C. The number of germinated seeds was recorded daily up to 120 days. Seeds were considered as germinated when the radicle length was more than 2 mm (ISTA, 1993). Seed germination tests were performed on 10 seeds each replicated ten times.

II.4. **Seed viability**

Seed viability was measured by assay of total dehydrogenase (TDH) activity using triphenyl tetrazolium chloride (TTC) test (ISTA, 1985). After removal of the seed coat; the embryo was soaked overnight in water. To 100mg of embryo, 3mL of 1% TTC reagent was added and incubated for 24 hours at a constant temperature of 37°C in a water bath. The tissue was homogenized with 10mL of methanol, centrifuged at 3000 rpm for 10 min and the absorbance of the clear supernatant was read in a spectrophotometer at 485 nm. A standard curve prepared from known concentrations of triphenyl formazan was used to express dehydrogenase activity in terms of µg formazan produced per gram of fresh tissue

II.5. **Fat content**

Total fat content in tissue samples was extracted using Soxhlet extractor as described by Osborne and Voogt (1978) and estimated gravimetrically. 1g of dry tissue powder packed in a thimble was placed in a Soxhlet extractor and extracted under reflux on a steam bath for 3 hrs using petroleum ether as solvent (bp.40°-60°C). The solvent containing the dissolved fats was quantitatively transferred to a clean and dry pre-weighed flask (W_1), evaporated to dryness on a boiling water bath and weighed again (W_2). The difference in weights was used to calculate the percent of fat in sample using the formula, (W_2-W_1) / Weight of sample×100

II.6**. Extraction and methylation of fatty acids**

Healthy and spongy affected fruit tissues were homogenized in a mixture of chloroform-methanol (2:1 v/v) and filtered through Whatman no.1 filter paper. The chloroform phase containing the lipids was separated, dried in a rotary vacuum evaporator at 40°C and stored at −20°C until further used (Folch *et al.*, 1957).The extracted lipids were methylated by dissolving in methanol and refluxed for 10 min at 70°C, followed by addition of 14% BF_3 in methanol. The mixture was further refluxed for 30 min at 70°C according to the modified method of Morrison and Smith (1964). Methyl esters of fatty acids (FAME) were subsequently extracted in heptane and dried on anhydrous sodium sulfate and filtered through 0.2 µm nylon membrane.

II.7. **GC-FID analysis**

GC-FID analysis of fatty acid methyl esters was carried out using a Varian-3800 Gas chromatograph system equipped with flame ionization detector (FID) on a fused silica capillary column (VF-5 Factor Four, Lake Forest, CA, USA), 30 m × 0.25 mm i.d and 0.25µm film thickness. The temperature program for the column was as follows: Initial oven temperature was set to 100°C for 4 min, increased by 3°C per min up to 220°C and held for 4 min. The temperature was further increased to 260°C at the rate of 5°C per min and held for 10 min. Injector and detector temperatures were maintained at 250°C and 260°C respectively. Helium at a flow rate of 1 mL/min was used as the carrier gas. Flow rates of H_2 and air were maintained at 20 mL/min and 250 mL/min respectively. Injection was initially done in split-less mode followed by split mode (1:30) after 1.5 minutes.

II.8. **Gas chromatography-mass spectrometry (GC-MS)**

GC-MS analysis was performed on Varian-3800 gas chromatograph coupled with Varian 4000 GC-MS-MS ion trap mass selective detector. Fatty acids were separated on VF-5MS fused silica capillary column (Varian, USA) (30 m × 0.25 mm id with 0.25 µm film thickness) by applying the same temperature program as described above for GC-FID analysis. The carrier gas was helium at a flow rate of 1ml/min; injector temperature, 260°C; ion source-temperature, 220°C; trap temperature, 200°C and transfer line temperature, 260°C. Mass detector conditions were: EI-mode at 70 eV with full scan range, 50–450 amu. Fatty acids were identified by comparing the relative retention times of FAME peaks with those of reference standards (Sigma-Aldrich, USA) and also by comparing the spectra with those available in Wiley and NIST-2007 spectral libraries (Liu, 1994). The total quantity of FAME was estimated as the sum of all GC-FID peak areas in the chromatogram and individual compounds were quantified by comparing the known individual FAME procured as standard. All the analyses were performed on three samples run separately.

II.9. **Free radical production**

Superoxide anion ($O_2^{\bullet-}$) levels were estimated following Doke (1983). The levels of hydroxyl radicals (•OH) were determined as described by Von Tiedemann (1997), using 2-deoxyribose as the scavenger molecule. Hydrogen peroxide (H_2O_2) content was measured according to Schopfer *et al.* (2001) and expressed as ng H_2O_2 generated /g FW of tissue.

II.10. **Lipid peroxidation**

Lipid peroxidation was monitored by measuring the conversion of lipids to malondialdehyde (MDA), using the thiobarbituric acid reactive substances (TBARS) assay, as described by Draper and Hadley (1990). TBARS reagent (1 mL) was added to a 0.5 mL aliquot of tissue homogenate and heated for 20 min at 100 ° C. The antioxidant, butylated hydroxy-toluene, was added before heating the samples. After cooling on ice, samples were centrifuged at 840g for 15 min and absorbance of the supernatant was read at 532 nm. Blanks for each sample were prepared and assessed in the same way to correct for the contribution of A_{532} to the sample. TBARS results were expressed as MDA equivalents using 1,1,3,3- tetraethoxypropane as standard.

II.11. **Electrolyte leakage and pH**

One g of pulp tissue was suspended in 10 mL of distilled water and electrolyte leakage was measured as conductance using a conductivity bridge (ELICO model CM-180) and expressed as dS/ m. pH was recorded using a combination electrode.

II.12. **Enzyme assays**

Peroxidase and catalase enzymes were extracted by homogenizing 1g fresh tissue in a chilled mortar and pestle in an ice bath in 100 mM sodium phosphate buffer (pH 7.0). The homogenate was centrifuged at 18 000*g* for 15 min at 4°C and the supernatant was used as enzyme source.

II.12.1**.Peroxidase (POD)**

POD (EC 1.11.1.7) was assayed in a reaction mixture (5 mL) containing 50 mM sodium phosphate buffer (pH 7.0), 20 mM guiacol and 100 µL enzyme extract. The reaction was initiated by the addition of 0.042% (v/v) H_2O_2 and the increase in absorbance at 420 nm was monitored for 3 min at 30 sec intervals with a Beckman Model DU 64 UV-Visible spectrophotometer. The activity was expressed as (nmol. min^{-1} mg^{-1} protein) (Saroop *et al.*, 2002)

II.12.2. **Catalase (CAT)**

CAT (EC 1.11.1.6) was assayed in a reaction mixture (3 mL) containing 100mMTris-HCl buffer (pH 7.0), 0.1 mL exudates and 200 mM H_2O_2. CAT activity was determined using the molar extinction coefficient of H_2O_2 at 240nm (= 43.6 M^{-1} cm^{-1}) and expressed as nmoles mg $protein^{-1}$ min^{-1} (Luck, 1965)

II.12.3**. Superoxide dismutase (SOD)**

SOD enzyme (EC. 1.15.1.1) was extracted by homogenization of 1g fresh tissue in 50 mM sodium phosphate buffer pH 7.8 and centrifuged at 10,000 × *g* for 10 min at 4°C. The clear supernatant containing the enzyme was used for assays. SOD was assayed based on its ability to inhibit the photochemical reduction of nitro blue tetrazolium (NBT) following Beauchamp and Fridovich (1971). The reaction mixture in a final volume of 3.0 ml, contained 50 mM sodium phosphate buffer pH 7.8, 100 µM EDTA, 130 mM methionine, 0.75 mM NBT, 20 µM riboflavin and enzyme extract. The reaction was started by illuminating the tubes under four 40 W fluorescent lamps for 8 min at the end of which time absorbance was recorded at 560 nm against a blank maintained in dark. One unit of SOD activity was defined as the amount of enzyme required to inhibit the photoreduction of NBT by 50% of that caused by the superoxides generated from the reaction between photoreduced riboflavin and oxygen under the assay conditions. SOD enzyme activity was expressed in units mg $^{-1}$ protein.

II.13. **Iron content**

One g of oven-dried seed was digested with 10 ml of 9:4 (v/v) mix of 16 M nitric acid: 11.6 M perchloric acid. Iron concentration was estimated using an AAnalyst-200 atomic absorption spectrometer (Perkin-Elmer, Waltham, MA, USA; Jones *et al.*, 1991).

II.14. **Statistical analysis**

Experimental data were subjected to ANOVA adapting the Fisher's analysis of variance technique (Panse and Sukhatme, 1978) and mean values were tested for significance using student's t-test. The results were expressed as mean ± standard error (SE).

III. RESULTS

Results presented in Table 1 showed that the total fat content of seed decreased from 15.76% in H seed to 11.12% in CT seed. Moisture content also declined from 43% in H seed to 40% in CT seed. The rate of germination in CT seed was lower at 48% compared to 94% in H seed while TDH activity was also lower (12.8) in CT seed compared to 27.9 in H seed (Figure 1).

The rate of production of free radicals was significantly higher in CT seed as compared to H seed. The generation of hydroxyl radical in CT seed was 5.8 times higher than that in H seed while the superoxide production was 4.3 times higher (Figure 2) .The activity of POD showed a reduction to an extent of 35.3% in CT seed compared to H seed. Similar trend was noticed in the activities of both CAT and SOD which showed a decline of 12.5% and 17.4% respectively in CT seed in comparison with H seed (Figure 3)

There was a 62.6% increase in the rate of generation of MDA in CT seed compared to H seed. The pH of CT seed was lower (6.40) as against 6.51 in H seed (Figure 3). The electrical conductivity was significantly higher in CT seed compared to H seed. The iron content showed a four-fold increase in CT seed as compared to H seed (Table 1).

The H seed was found to contain a higher level of fatty acids (305.13) compared to CT seed (259.74) (Table 2). Of the 18 fatty acids detected in seed, four fatty acids viz, palmitic, linoleic, oleic and stearic acids constituted 94.8% of the total fatty acid content of the H seed and 93.3% in CT seed. Palmitic acid , the single largest fatty acid present in seed, constituting 47.5% of the total fatty acids in H seed increased to 50.9% in CT seed, although the total content fell by 8.93% . Linoleic acid constituted the second major fatty acid component of seed with 62.3% in H seed. The short chain fatty acids, caprylic acid and lauric acids were present in only H seed at a low level while capric acid was marginally higher in H seed. Tridecanoic acid was present in CT seed at 0.70 but not in H seed. Myristic acid registered a rapid increase of 315.3% from 0.72 to 2.99 in CT seed compared to H seed, while pentadecanoic acid showed a marginal rise in CT seed. Heptadecanoic acid increased by 43.4% in CT seed while eicosanoic, arachidic and behenic acids decreased by 4.8%, 19.9% and 67.9% respectively. Oleic acid content reduced by 25.8% in CT seed compared to H seed. The total content of VLCFAs, eicosanoic, arachidic, and behenic acids decreased from 9.76 in H seed to 7.00 in CT seed. The content of linoleic acid (18:2) reduced by 40.5% in CT seed as against a drop of 1.26% in the case of linolenic acid (18:3). Fe content increased four-fold in CT seed compared to H seed.

IV. DISCUSSION

IV.1. Reduced seed germination in corky tissue affected fruits:

Seed from CT affected fruits showed a reduction in moisture content associated with a significant fall in the rate of germination. Moisture content of seed affords the medium for the hydrolytic breakdown of storage reserves and is essential for solubilization and transportation of metabolites in germinating seeds (Bewley and Black, 1994) (Figure 1). Therefore, it was apparent that the reduction in moisture content of seed resulted in a reduced germination rate. A number of reports have established that the rate of germination which represents a true measure of seed viability is strongly and positively correlated with TDH activity (Reuzeau and Cavalie, 1995; Bettey and Finch-Savage, 1996). The marked decline in TDH activity of CT seed in this study, therefore, confirmed the loss of seed viability compared to H seeds.

IV.2. Generation of free radicals, lipid peroxidation, MDA synthesis and membrane damage:

One of the major causes of seed deterioration is lipid peroxidation (Da Costa and Huang, 2007; McDonald, 1999) which is initiated by the action of free radicals on the unsaturated

fatty acids of membrane phospholipids. Data presented in Figure 2 showed that there were significantly higher levels of •OH and $O^{2\bullet-}$ radicals generated in CT seed compared to healthy seed. Hydroxyl radical is one of the most destructive free radicals in biological systems responsible for modification of macromolecules and cellular damage (Jiang Ming-Yi, 1999). CT seed recorded a remarkably high level of Fe^{2+}, which is reported to cause a rapid production of reactive oxygen species by oxidation to Fe^{3+} species in the Fenton reaction (Table 1). Iron, by its action of generating free radicals, especially hydroxyl radicals, is reported to trigger cell death by a process termed ferroptosis (Dixon and Stockwell, 2014). Thus, an excessive production of reactive oxygen species (ROS) could contribute to the destruction of cellular membranes if these are not controlled effectively in time (Lamb and Dixon, 1997). In order to counter the damaging and destructive effects of the free radicals on cell membranes, anti-oxidative enzymes such as, catalase (CAT), peroxidase (POX) and superoxide dismutase (SOD), which together constitute a mutually supportive defence system against ROS are also produced by plants. These antioxidant enzymes are known to provide the most sophisticated, effective and efficient protective mechanism against ROS (Monk *et al*., 1989; Halliwell and Gutteridge, 2002). SOD is involved in lowering the steady-state level of superoxide radicals while CAT and POX lower the level of H_2O_2 in higher plants. Data presented in Figure 3 showed that the activities of POD, CAT and SOD were markedly lower in CT seed compared to H seed. Hence, it was apparent that the greatly increased production of •OH and $O^{2\bullet-}$ radicals in CT seed coupled with a reduction in the activities of anti-oxidative enzymes implied destruction of membranes through increased peroxidation of lipids. Malondialdehyde (MDA) is the end product in the peroxidative decomposition of polyenic fatty acids and its accumulation in tissues is indicative of the extent of lipid peroxidation. Results presented in Figure 3 showed that the amount of MDA produced was significantly higher in CT compared to H seed. From this, it was evident that the production of high levels of free radicals together with lower activities of anti-oxidative enzymes contributed to increased rate of lipid peroxidation leading to a large accumulation of malondialdehyde in CT seed. Past work by other investigators showing a similar increase in lipid peroxidation during different forms of stress like, ozone injury, anoxia, drought stress and wounding (Kepler and Novacky, 1986) supported the present findings.

IV.3. Changes in the composition of VLCFAs, loss of seed viability and CT formation:

A perusal of data presented in Table 2 showed that that there was a significant decline in the levels of very long chain fatty acids (VLCFAs) from 9.76 in H seed to 7.00 in CT seed Besides, the content of linoleic acid (18:2) and oleic acid reduced by 40.5% and 25% respectively in CT seed . VLCFAs have been found to be crucial for many vital processes such as cell expansion, cell proliferation, differentiation (Bach and Faure, 2010) and are essential for cell viability (Bach et al., 2008). The rapid loss of linoleic acid in seeds has been strongly correlated with ageing and viability loss in bean (*Phaseolus vulgaris*) (Lin and Pearce, 1990), and Norway maple *(Acer platanoides)* seeds (Pukacka, 1991). As stated earlier, lipid peroxidation is a major cause of seed deterioration in which free radicals attack the unsaturated fatty acids of membrane phospholipids and lead to membrane damage resulting in a decline of seed viability. Since polyunsaturated fatty acids are more susceptible to peroxidation compared to monounsaturated and saturated fatty acids, the preferential loss of polyunsaturated fatty acids like, linoleic acid and oleic acid is considered as an indication of lipid peroxidation. Considering the fact that linoleic acid and oleic acid contents registered a reduction of over 40% from 62.28 to 37.26 and 25% respectively, it was obvious that the extent of peroxidation was enormous generating a massive 62.6% increase in the rate of generation of MDA in CT seed. As a result, there was a rapid increase in electrical conductivity of CT seed signifying loss of membrane integrity (Bewley, 1986). Considering the striking reduction in the contents of linoleic acid, oleic acid and VLCFAs in CT seed compared to H seed, it was evident that viability was severely affected in CT seed due to which germination was reduced. Thus, the results of the study showed a loss of seed viability and cell death in CT seed resulting in development of CT symptom.

V. CONCLUSION

To sum up, the body of evidence presented in this paper supported the conclusion that a reduced rate of fat synthesis in the seed during fruit growth was accompanied by changes in the profile of fatty acids, especially long chain unsaturated fatty acids and VLCFAs. Concurrently, a large increase in the production of free radicals far exceeding the cellular antioxidant capacity contributed to a rapid increase of lipid peroxidation involving the long chain unsaturated fatty acids resulting in oxidative injury and consequently to destruction of cell membranes. The above sequence of events led to loss of seed viability culminating in the development of corky tissue in sapota fruits. Thus, the present study highlighted for the first time, the crucial role played by seed fatty acids in corky tissue formation in sapota fruit.

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Table 1: Biochemical composition of seeds of healthy and Corky tissue affected fruits

Parameters	Seed	
	H	CT
Moisture (%)	43±2.021	40±1.155
pH	6.51±0.292	6.4±0.231
Electrical conductivity (ds m^{-1})	0.128±0.006	0.183±0.008
Total fat (g $100g^{-1}$ FW)	15.76±0.778	11.12±0.198
Iron (ppm)	71±1.732	287±4.070

Table 2: Composition of fatty acids in seeds of healthy and Corky tissue affected fruits

Fatty acid	Content in seed (mg $100g^{-1}$ FW)	
	H	CT
Caprylic acid	0.43 ± 0.046	ND
Capric acid	0.02 ± 0.004	0.01 ± 0.003
Lauric acid	0.03 ± 0.003	ND
Tridecanoic acid	ND	0.70 ± 0.061
Myristic acid	0.72 ± 0.049	2.99 ± 0.243
Pentadecanoic acid	0.26 ± 0.046	0.28 ± 0.046
Palmitic acid	145.8 ± 4.042	132.8 ± 1.648
Heptadecanoic acid	1.80 ± 0.088	2.58 ± 0.165
Linoleic acid	62.28 ± 1.010	37.06 ± 0.709
Oleic acid	32.38 ± 0.867	24.03 ± 0.716
Linolenic acid	2.38 ± 0.090	2.35 ± 0.061
Stearic acid	49.18 ± 1.447	49.93 ± 0.979
Nonadecanoi acid	0.07 ± 0.004	ND
Eicosanoic acid	2.47 ± 0.121	2.35 ± 0.087
Arachidic acid	4.82 ± 0.164	3.86 ± 0.098
Behenic acid	2.47 ± 0.200	0.79 ± 0.046
Total	305.13	259.74

Values are the means ±SE of three replicates. ND= Not detected

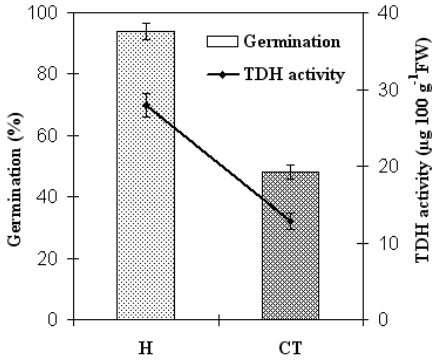

Figure 1: Relationship between germination and total dehydrogenase activity in seeds of healthy and CT affected fruits. Values are the means ±SE of eight replicates

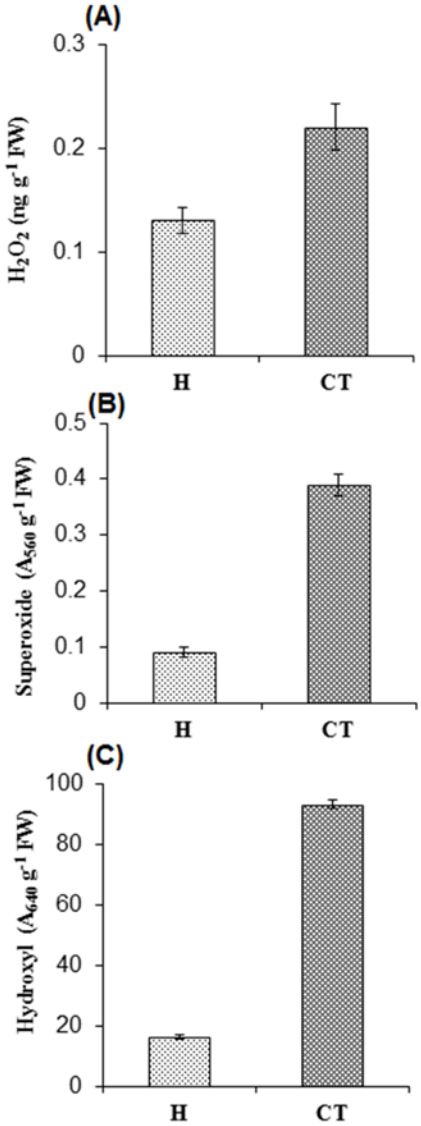

Figure 2: Free radical production in seeds of healthy and CT affected fruits Values are the means ±SE of eight replicates

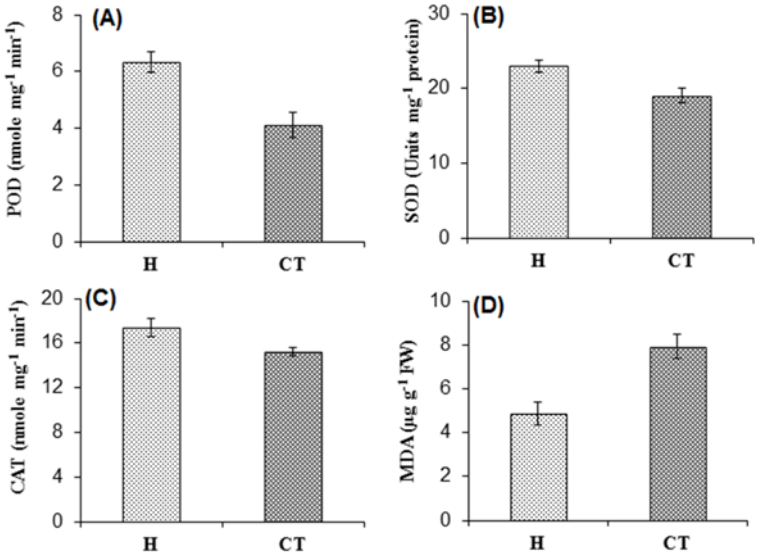

Figure 3: Changes in the activities of antioxidant enzymes and generation of MDA in seed of healthy and CT affected fruits. Values are the means ±SE of eight replicates