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Rapid burst of ethylene evolution by premature seed: A warning sign for the onset of spongy tissue disorder in Alphonso mango fruit?

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Moisture stress induced in premature seeds due to the breakdown of funiculus in Alphonso mango led to the burst of ethylene evolution, which in turn caused a sudden increase of polyphenol oxidase activity in the pulp, resulting in the development of a black spot near the seed base. Reduced levels of very long chain fatty acids in 70% mature seeds with black spots were associated with a sudden increase of cytokinins followed by a rapid rise of starch-metabolizing enzymes culminating in the onset of pre-germination events. Concurrently, an overproduction of *p*-OH benzoic acid inhibited amylase and polygalacturonase enzymes and led to partial degradation of the stored starch and pectin in the pulp. A parallel drop in climacteric ethylene production by the pulp led to incomplete ripening coupled with changes in composition, texture and aroma of the pulp, characteristic of spongy tissue. The results have provided strong experimental evidence to support the fact that increased competition for resources among developing fruits for the synthesis of seed fat plays a critical role in spongy tissue formation in Alphonso mango. The major highlight of the study is that rapid ethylene evolution by premature seed is an early warning sign for the initiation of spongy tissue formation in Alphonso mango.

Keywords. Alphonso mango; ethylene burst; physiological disorder; premature seed; spongy tissue; VLCFAs

1. Introduction

It is now a well-established fact that initiation of seed-germination-associated events in the pre-harvest phase of fruit development followed by the flow of water from the mesocarp into seed results in spongy tissue (ST) formation in the Alphonso mango fruit (Shivashankar 2014). Premature germination of developing seed is prompted by the reduced synthesis of very long chain fatty acids (VLCFAs) in the seed during the pre-harvest fruit growth phase, leading to increased cytokinin production, thus setting in motion the commencement of spongy tissue formation (Shivashankar *et al.* 2015a). We had earlier reported that the development of spongy tissue begins with the appearance of a tiny black spot on the seed coat when the fruit is about 70% mature, which subsequently grows in size as the fruit matures. Even though a good deal of work has been carried out to determine the changes occurring in the pulp of spongy tissue affected fruits (Katrodia and Rane 1989; Selvaraj *et al.* 2000) and the sequence of biochemical reactions culminating in ST formation in the pulp of Alphonso mango fruits (Shivashankar *et al.* 2007), the factor/s that lead to breakdown of funiculus and the events occurring in the seed immediately following disconnection of funiculus have remained obscure till date. Furthermore, the significance of black spot in the development of ST in Alphonso mango and the series of reactions that follow until the appearance of ST symptoms have not been understood thus far. Hence, the present work was conducted on developing fruits of Alphonso mango beginning from 60% maturity until full maturation, to trace the progression of events in the seed and to determine the link between seed events and changes in pulp until the appearance of symptoms of spongy tissue. In order to accomplish the above, biochemical changes between fruits, with and without black spot on the stone, were followed and are reported here.

2. Materials and methods

2.1 *Tree growth conditions*

'Alphonso' mango fruits were harvested during the 2015–2016 season from 25-year-old trees receiving the recommended supply of fertilizers and plant protection measures and were maintained under uniform growth conditions in the experimental orchard of the institute.

2.2 *Selection of fruits*

Fruits from panicles bearing two or more fruits were selected and harvested, beginning from 60% maturity up to full maturity. The presence of a black spot on the seed coat, which was first noticed in 70% mature fruits, was treated as a marker of spongy tissue as fruits with symptoms of sponginess at full mature ripe stage were invariably found to have a black spot on the stone. On the contrary, fruits free from black spot were considered as healthy.

2.3 *Biochemical analysis*

2.3.1 *Preparation of acetone powder:* Acetone powder of experimental samples were prepared by homogenising 50 g of fresh fruit tissue in 200 ml chilled (−20°C) acetone. The homogenate was rapidly filtered through Whatman No.1 filter paper in a Buchner funnel. The residue was air-dried at 4°C until free of acetone, and the resulting white powder was stored at −20°C.

2.3.2 *Assay of antioxidant enzymes, dehydrogenases, free radicals, MDA and EC:* The activities of antioxidant enzymes, polyphenol oxidase (PPO), superoxide dismutase (SOD), peroxidase (POX), catalase (CAT) and total protein content were assayed as reported previously (Shivashankar *et al.* 2012). Activities of malate dehydrogenase (MDH) and succinate dehydrogenase (SDH), levels of free radicals, malondialdehyde (MDA) and electrical conductivity (EC) were determined as described previously (Shivashankar *et al.* 2007).

2.3.3 *Total phenolic compounds and phenolic acids:* Total phenolic compounds were extracted in 80% (v/v) methanol and estimated following Singleton and Rossi (1965) using gallic acid as standard. The content of total phenolic compounds was expressed as mg gallic acid equivalents g^{-1} fresh weight (FW) of sample tissue. Extraction of phenolic acids followed by high performance liquid chromatography (HPLC) analyses, gas chromatography-flame ionization detector (GC–FID) analysis and gas chromatography–mass spectrometry (GC–MS) were performed as described previously (Shivashankar *et al.* 2015a, b).

2.3.4 *Assay of starch metabolizing enzymes:* Amylase was determined as described previously (Shivashankar *et al.* 2007). Glucose 6-phosphate dehydrogenase (G6PDH) was assayed according to Tian *et al.* (1998). Two sets of tubes containing 100 µl each of crude extract and total dehydrogenase assay buffer, 50 mM Tris–HCl, pH 8.1, plus 0.1 µmol $MgCl_2$ and 0.01 µmol NADP were prepared. Into one set, 0.02 µmol glucose 6-phosphate was added, while 0.02 µmol 6-phosphogluconate was added to the second set. The reduction of NADP in both sets was measured as the rate of change of absorbance at 340 nm for the initial 6 min. Glucose 6-phosphate dehydrogenase activity was calculated as the difference of activities between the above two sets.

Glyceraldehyde 3-phosphate dehydrogenase (GA_3PDH) activity was determined by measuring the increase in absorbance caused by the reduction of NAD in a 3 ml reaction mixture containing 2.5 ml of 100 mM Taps buffer (pH 8.6), 2 µmol NaH_2PO_4, 0.1 µmol NAD, 0.6 µmol cysteine and 0.15 µmol glyceraldehyde 3-phosphate. Enzyme extract (0.1 ml) was added, the contents mixed, and the increase in absorbance at 340 nm was recorded for 5 min. One unit was defined as the amount of enzyme needed to cause an initial rate of reduction of one µmol of NAD min^{-1} at 25°C (Burrell *et al.* 1994). Phosphoenol pyruvate carboxylase (PEPCase) activity was determined spectrophotometrically by coupling the reaction to NADH oxidation mediated by malate dehydrogenase. The reaction mixture contained 1.7 ml of 50 mM HEPES, pH 7.5, 0.5 µmol $MgCl_2$, 0.1 µmol NADH, 0.075 µmol $NaHCO_3$, 0.25 units of malate dehydrogenase, 0.015 µmol Phosphoenol pyruvate (PEP), and 0.1 ml of enzyme extract. The control was without PEP. The contents of tubes were quickly mixed by inversion and the decrease in absorbance was recorded at 340 nm for 5 min. One unit of enzyme was defined as the formation of 1 µmol of oxaloacetate from phosphoenol pyruvate min^{-1} at pH 7.5 (Sadasivam and Gowri 1981). For the assay of Phosphoglucoisomerase (PGI) activity, the reaction mixture containing 0.5 ml of 75 mM glycylglycine, 0.1 µmol D-fructose 6-phosphate, 0.05 µmol NAD, 1 µmol $MgCl_2$, 0.05 units of G6PDH, 0.1 ml of enzyme extract and 2 ml of water was rapidly mixed by inversion. The increase in absorbance caused by the reduction of NAD was recorded at 340 nm for 5 min. One unit of enzyme was the amount required to convert 1 µmole of D-fructose 6-phosphate to D-glucose 6-phosphate min^{-1} at pH 8.5 at 25°C (Simcox *et al.* 1977). The assay buffer for Phosphoglucokinase (PGK) contained 2.4 ml of 100 mM HEPES buffer, 0.01 µmol of β-NADH, 0.05 µmol EDTA, and 0.1 µmol $MgSO_4$, pH 7.6 at 25°C. Then, 1.3 µmol of 3-phosphoglyceric acid and 0.1 µmol of ATP were added as substrates and 0.165 units of GAPDH was added and mixed by inversion. To this 0.1 ml of the enzyme extract was added. The decrease in absorbance was measured at 340 nm for 5 min. One unit was defined as the amount of enzyme required to reduce 1 µmole of 3-phosphoglycerate to D-glyceraldehyde-3-phosphate min^{-1} at 25°C (Burrell *et al.* 1994). Pyruvate kinase (PK) was determined following Burrell *et al.* (1994). The assay mixture containing 2.3 ml of 50 mM 3-(*N*-morpholino) propanesulfonic acid buffer (pH 7.5), 1.5 µmol $MgCl_2$, 10 µmol KCl, 0.02 µmol ADP, 0.016 µmol PEP, 0.015 µmol NADH and 0.015 units of LDH was mixed at 25°C. The reaction was initiated by adding 0.1 ml of enzyme extract and the decrease in absorbance was read at 340 nm for 5 min. One unit of enzyme was the amount needed to produce one µmole of pyruvate from phospho (enol) pyruvate min^{-1} at pH 7.5 in the presence of ADP.

2.3.5 *Determination of glycolytic and TCA cycle intermediates:* Fructose-6-phosphate was measured following the change in absorbance at 340 nm as described by Du *et al.* (1998) with some modifications. The assay mixture (3 ml) contained 2.6 ml of 100 mM Tris–HCl, pH 8.5, 1.0 µmol $MgCl_2$, 0.025 µmol NADP and 0.1 ml extract. To the mixture, 1 unit of PGI was added and the change in absorbance was read at 340 nm for 5 min. The content of F-6-P was expressed as nmol g^{-1} FW. The assay mixture for Glucose-6-phosphate contained 2.6 ml of 100 mM Tris–HCl, pH 8.5, 1.0 µmol $MgCl_2$, 0.025 µmol NADP, 0.05 units of G6PDH and 0.1 ml of sample extract. The change in absorbance was read at 340 nm for 10 min at 25°C and expressed as nmol g^{-1} FW. Glucose-1-phosphate was measured in a final volume of 3 ml reaction mixture containing 2.6 ml of 100 mM Tris–HCl, pH 8.5, 1.0 µmol $MgCl_2$, 0.025 µmol NADP, 0.05 units of G6PDH and 0.1 ml of sample extract. The change in absorbance was read at 340 nm for 10 min at 37°C and expressed as nmol g^{-1} FW (Du *et al.* 1998). Fructose-1, 6-bis phosphate was measured following Wirtz *et al.* (1980) with modifications. To the assay mixture containing 2.6 ml of 100 mM Tris–HCl, pH 8.1, 0.5 µmol $MgCl_2$, 0.02 µmol NADH and 0.1 ml extract, 0.025 units of GAPDH was added to give a final volume of 3.0 ml and the absorbance was measured at 340 nm for 5 min. The F-1, 6-P_2 concentration was expressed as nmol g^{-1} FW. Dihydroxyacetone phosphate (DHAP) was measured by a method modified from Wirtz *et al.* (1980); in an assay mixture of 3 ml containing 2.6 ml of 100 mM Tris–HCl, pH 8.1, 0.5 µmol $MgCl_2$, 0.02 µmol NADH, and 0.1 ml of extract, 0.012 units TPI was added. The change in absorbance was recorded at 340 nm for 5 min and expressed as nmol g^{-1} FW. Glyceraldehyde-3-phosphate (GAP) was measured by a method modified from Wirtz *et al.* (1980) in a 1 ml assay mixture containing 100 mM Tris–HCl, pH 8.1, 0.5 µmol $MgCl_2$, 0.04 µmol NADH and an aliquot of extract. To the mixture, 0.2 U ml^{-1} of aldolase was added and the change in absorbance at 340 nm was measured for 5 min. The concentration of GAP was expressed as nmol g^{-1} FW. Pyruvate was assayed as described by Chen *et al.* (2002) with some modifications. To the reaction mixture containing 200 mM Tris–HCl, 0.2 µmol EDTA-NaOH, pH 7.0, 0.015 µmol NADH and an aliquot of extract, 0.03 units Lactate dehydrogenase (LDH) was added. Pyruvate content was determined as the difference in absorbance at 340 nm before and after the addition of LDH and expressed as nmol g^{-1} FW. Phosphoenol pyruvate (PEP)was assayed as described by Chen *et al.* (2002) with some modifications. To a reaction mixture containing 200 mM Tris–HCl, pH 7.6, 0.18 µmol EDTA-NaOH, pH 7.0, 0.02 µmol NADH and 0.1 ml of extract, 0.01 units of LDH was added, followed by 0.22 µmol ADP, 1.0 µmol $MgSO_4$, 2.5 µmol KCl, 0.03 units LDH and 0.012 units PK to give a final volume of 1 ml. PEP content (nmol g^{-1} FW) was determined as the difference in absorbance at 340 nm before and after addition of LDH and PK. Adenosine diphosphate (ADP) was assayed as described by Stitt *et al.* (1989) with modifications. ADP was measured in a volume of 1 ml containing 100 µl of extract and 100 mM Tris–HCl, pH 8.1, 1.5 µmol $MgCl_2$, 2.0 µmol KCl, 0.12 µmol NADH, 0.02 µmol PEP and 0.03 units LDH. After 2 min of incubation, 0.05 units PK and 0.02 units myokinase were added. The absorbance was measured at 340 nm and expressed as nmol g^{-1} FW. Adenosine triphosphate (ATP) was assayed as described by Stitt *et al.* (1989) with some modifications. The assay medium in a final volume of 1 ml contained 100 µl of extract, 100 mM Tris–HCl, pH 8.1, 1 µmol $MgCl_2$, 0.015 µmol NADP, 0.2 µmol glucose, 0.011 units G6PDH and 0.02 units phosphoglucoisomerase (PGI). After 2 minutes of incubation, 0.005 units hexokinase was added. The absorbance was measured at 340 nm and expressed as nmol g^{-1} FW. Nicotinamide adenine dinucleotide (NAD) was determined as described by Tezuka *et al.* (1994). The sample was homogenised in 0.1 M HCl at 95°C, cooled in an ice bath and the pH was adjusted to 6.5 with 0.1 N NaOH. 0.5 ml of 0.2 M glycylglycine (pH 6.5) was added to the coenzyme fraction and centrifuged at 10000 g for 20 min at 4 ° C. The supernatant was immediately used for the assay of NAD. 0.1 ml of the extract was added to the reaction mixture containing 50 µmol glycylglycine (pH 6.5), 4.0 µmol nicotinamide, 0.1 µmol phenazine methosulfate (PMS), 0.05 µmol thiazolyl blue (MTT) and 0.018 µmol 3,3′-diaminobenzidine (ADB). After placing the cuvette containing the reaction mixture in a UV-visible spectrophotometer for measurement at 570 nm, 40 ml of 80% ethanol was added to start the reaction. The results were expressed as nmol g^{-1} FW. Measurement of Nicotinamide adenine dinucleotide (NADH) was performed as described by Tezuka *et al.* (1994). The seed sample was homogenised with 0.1 M NaOH at 95°C, cooled in an ice bath, and the pH was adjusted to 7.5 with 0.1 N HCl. 0.5 ml of 0.2 M glycylglycine (pH 7.5) was added to the coenzyme fraction and centrifuged at 10000*g* for 20 min at 4°C for 10 min, and the resulting supernatant was immediately used to assay NADH. For measurements, 0.1 ml of the extract was added to the reaction mixture containing 50 µmol glycylglycine (pH 7.5), 4.0 µmol nicotinamide, 0.1 µmol phenazine methosulfate (PMS), 0.05 µmol thiazolyl blue (MTT) and 0.018 µmol ADB. 40 ml of 80% ethanol was added to start the reaction. The absorbance was read at 570 nm and expressed as nmol g^{-1} FW.

2.3.6 *Fat content:* Total fat was extracted using Soxhlet extractor (Osborne and Voogt 1978) and estimated gravimetrically. One gram of dry tissue packed in a thimble was inserted in a Soxhlet extractor and extracted under reflux on a steam bath for 3 h using petroleum ether as solvent (bp 40°–60°C). The extract containing the fats was transferred to a dry pre-weighed flask (W1), evaporated on a boiling water bath, and weighed (W2). The difference in weight was employed to compute the percent of fat in sample using the formula, (W2 − W1)/Weight of sample × 100.

2.3.7 *GC–FID analysis of fatty acids:* Healthy and spongy affected fruit tissues were homogenized in a mixture of chloroform–methanol (2:1 v/v) and filtered on Whatman no.1 filter paper. The chloroform phase was separated out, dried in a rotary vacuum evaporator at 40°C and stored at −20°C (Folch *et al.* 1957). Methylation of fats was done by dissolving in methanol and refluxing for 10 min at 70°C, followed by adding 14% BF_3 in methanol and further refluxing for 30 min at 70°C as described by Morrison and Smith (1964). Methyl esters of fatty acids (FAME) were extracted in heptane, dried on sodium sulfate and filtered through 0.2 µm nylon membrane. GC–FID analysis of fatty acid methyl esters was done using a Varian-3800 gas chromatograph system coupled 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 0.25 µm film thickness. The temperature program for the column was maintained as follows: Initial oven temperature was 100°C for 4 min, raised by 3°C per min up to 220°C, held for 4 min, temperature increased at 5°C per min up to 260°C and held for 10 min. Injector and detector temperatures were maintained at 250°C and 260°C, respectively. Helium was used as carrier gas at a flow rate of 1 ml/min. Flow rates of H_2 and air were maintained at 20 ml/min and 250 ml/min, respectively. The mode of injection was split-less, initially, followed by split mode (1:30) after 1.5 min.

2.3.8 *Gas chromatography–mass spectrometry (GC–MS):* GCMS analysis was carried out on Varian-3800 gas chromatograph coupled with Varian 4000 GC–MS–MS ion trap mass selective detector. Fatty acids were separated on a VF-5MS fused silica capillary column (Varian, USA) (30 m × 0.25 mm i.d. with 0.25 µm film thickness) by following the temperature program described above. Helium at a flow rate of 1 ml/min was used as carrier gas and the injector temperature was 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. Identification of fatty acids was done based on retention times of FAME peaks as related to reference standards (Sigma-Aldrich, USA) and by comparison of the spectra with the data of Wiley and NIST-2007 spectral libraries (Liu 1994). The total quantum of FAME was computed as the sum of areas of GC–FID peaks in the chromatogram. Individual fatty acids were estimated by comparison with known FAME standards. Three samples were estimated separately for the analysis.

2.3.9 *Ethylene:* Ethylene evolution was measured by placing the pulp and seed of healthy and ST-affected fruits in a 1-liter glass jar hermetically sealed with rubber stopper at 24°C for one hour. One µl of headspace gas was withdrawn from the chamber and injected into a gas chromatograph

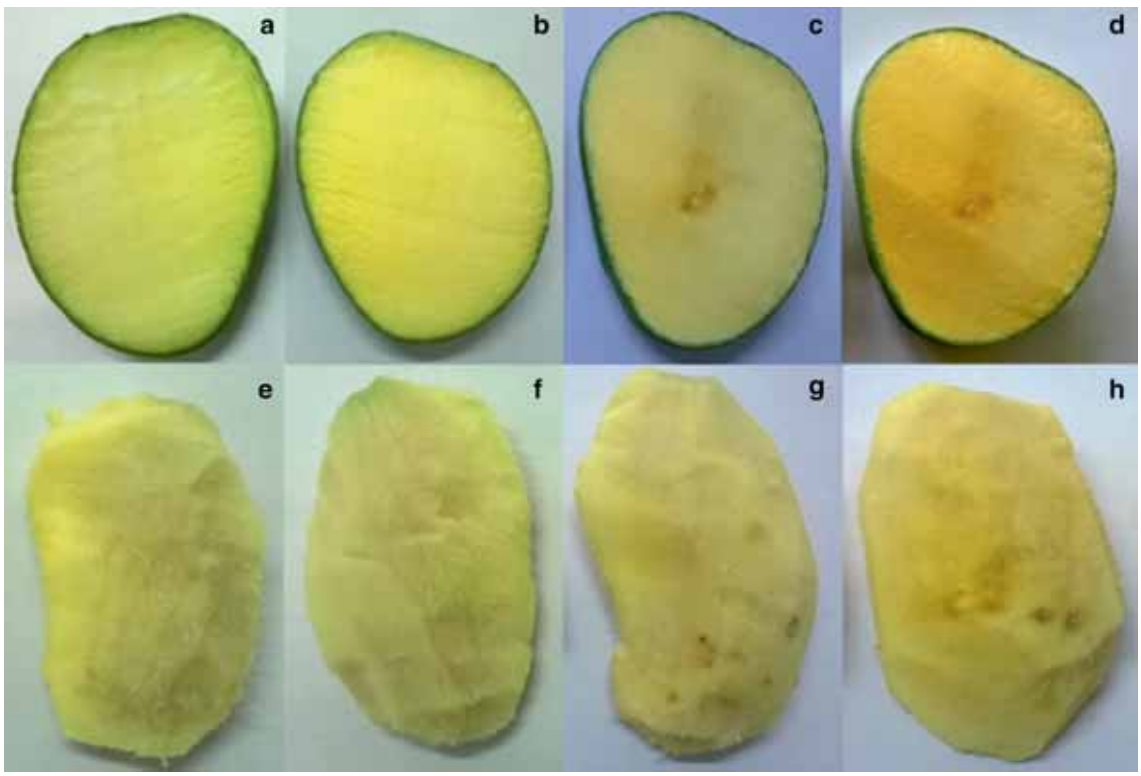

Figure 1. Healthy pulp (**a**, **b**), seed (**e**, **f**), black spotted (ST) pulp (**c**, **d**) and seed (**g**, **h**) of Alphonso mango at 70% maturity. The black spot on the stone was first apparent in 70% mature fruit, which subsequently turned spongy.

(model GC-4CM, Shimadzu, Kyoto, Japan) fitted with activated alumina column and flame ionization detector. Helium gas at a flow rate of 1.2 ml min^{-1} was used as carrier gas. The oven temperature was programmed to increase from 80°C to 120°C at the rate of 10°C min^{-1}. The amount of ethylene liberated was expressed as nmol g^{-1} h^{-1}.

2.4 *Statistical analysis*

Experimental data were subjected to ANOVA adapting Fisher's analysis of variance technique (Panse and Sukhatme 1978) and mean values were tested for significance using Student's *t*-test at $P < 0.05$. The results were expressed as mean ± standard error (SE). All measurements were performed using three biological replicates

3. Results

3.1 *Development of black spot on stone*

An examination of developing fruits of various maturity levels revealed the appearance of a tiny black spot on the seed coat of some fruits at 70% maturity which subsequently enlarged in size with maturity (figure 1) while others did not show the black spot. The fruits with black spot were treated as ST-affected while the rest were considered as healthy (H).

3.2 *Initiation of germination events*

Analysis of fatty acid profiles in 70% mature fruits (table 1) showed that the content of free fatty acids in the seed was significantly lower by 37% in ST compared to H. The contents of the two VLCFAs, lignoceric acid and cerotic acid were lower by 45% and 42%, respectively, in ST seed. While the amount of a majority of fatty acids showed a reduction in seed of ST compared to H, margaric acid and nonadecylic acid increased by 21% and 204% respectively while behenic acid increased sharply by 617% in ST. Data presented in figure 2 showed that there was a significant increase in the activities of the starch metabolizing enzymes, *viz.,* G6PDH and amylase in ST seed compared to H seed. The activities of the TCA cycle enzymes, MDH, SDH and PEPCase were significantly higher in ST-affected Alphonso mango compared to H (figure 3). The activities of glycolytic pathway enzymes, GAPDH, PGI, PGK, and PK were higher in the seed of ST-affected Alphonso mango compared to H (figure 4). These changes were associated with a rapid rise in the concentrations of metabolites such as Fructose-6-phosphate, Glucose-6-phosphate, Glucose-1-phosphate, DHAP, GAP, ADP, ATP, NAD, NADH, Fructose-1,6-bis phosphate, Phosphoenol pyruvate and pyruvate in ST seed compared to H seed (table 2). Concurrent with the above changes, there was a sudden rise in the levels of cytokinins and gibberellins associated with a fall in abscisic acid level in ST seed (figure 5). The total content of phenolic acids in ST seed decreased by 10.1% compared to H seed, while, on the

Table 1. Fatty acid composition of seed and pulp of 70% mature healthy (H) and spongy tissue (ST) affected Alphonso mango

	Seed			Pulp		
Fatty acid	H	ST	P value	H	ST	P value
Caprylic acid	ND	ND		0.08 ± 0.004	0.05 ± 0.003	**
Capric acid	ND	ND		1.90 ± 0.091	0.43 ± 0.032	***
Tridecylic acid	0.85 ± 0.099	0.01 ± 0.001	**	1.76 ± 0.110	1.15 ± 0.072	**
Myristic acid	0.39 ± 0.035	0.12 ± 0.015	**	0.16 ± 0.032	0.09 ± 0.015	ns
Pentadecylic acid	0.42 ± 0.055	0.18 ± 0.030	*	3.44 ± 0.193	0.90 ± 0.104	***
Palmitoleic acid	94.48 ± 1.17	59.79 ± 2.25	***	43.16 ± 1.218	23.90 ± 1.052	***
Palmitic acid	2.29 ± 0.078	1.67 ± 0.133	*	0.35 ± 0.052	0.20 ± 0.026	ns
Margaric acid	106.4 ± 1.79	128.9 ± 1.55	***	4.51 ± 0.326	0.33 ± 0.032	***
Linoleic acid	449.4 ± 4.15	258.8 ± 2.88	***	3.80 ± 0.176	0.89 ± 0.043	***
Oleic acid	1.60 ± 0.127	1.09 ± 0.030	*	5.52 ± 0.182	0.91 ± 0.113	***
α-Linolenic acid	450.8 ± 1.71	239.7 ± 1.45	***	1.69 ± 0.072	1.51 ± 0.110	ns
Stearic acid	33.36 ± 1.34	10.70 ± 0.22	***	2.37 ± 0.095	0.79 ± 0.043	***
Nonadecylic acid	0.41 ± 0.04	1.25 ± 0.06	***	0.09 ± 0.020	0.06 ± 0.012	ns
Gondoic acid	15.94 ± 0.62	10.41 ± 0.33	**	0.43 ± 0.017	0.24 ± 0.038	*
Arachidic acid	0.46 ± 0.04	0.21 ± 0.03	*	0.15 ± 0.023	0.12 ± 0.020	ns
Heneicosylic acid	0.22 ± 0.04	0.86 ± 0.10	**	0.05 ± 0.015	0.01 ± 0.003	*
Erucic acid	2.96 ± 0.14	1.98 ± 0.20	*	0.28 ± 0.017	0.16 ± 0.020	*
Behenic acid	1.09 ± 0.03	6.73 ± 0.23	***	0.49 ± 0.035	0.30 ± 0.029	*
Tricosylic acid	8.48 ± 0.14	6.44 ± 0.20	**	1.36 ± 0.043	0.94 ± 0.026	**
Lignoceric acid	9.31 ± 0.31	5.08 ± 0.21	***	0.19 ± 0.035	0.08 ± 0.015	*
Cerotic acid	1.37 ± 0.13	0.79 ± 0.04	*	0.14 ± 0.017	0.05 ± 0.003	**

Values are expressed as means (±SE) for three independent preparations (*P ≤ 0.05, **P ≤ 0.005, ***P ≤ 0.0001).

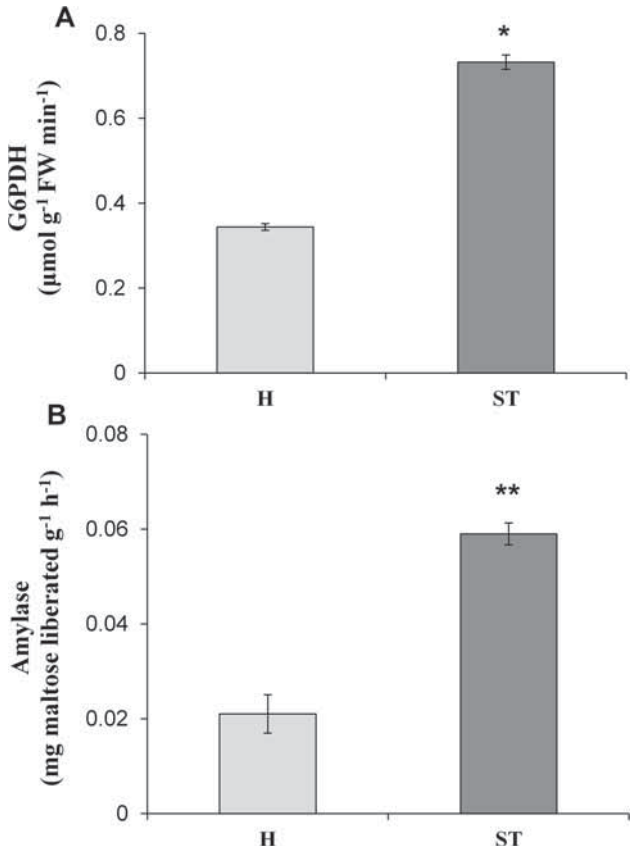

Figure 2. Activities of G6PDH and amylase in the seed of H and ST-affected 70% mature fruit of Alphonso mango showed that the rate of starch breakdown was significantly higher in ST seed. Data are expressed as the mean (±SE) of three replicate samples (*P ≤ 0.05, **P ≤ 0.005).

contrary, there was a two-fold rise in the levels of proto-catechuic acid, sinapic acid and chlorogenic acid levels in ST seed (table 3). A notable aspect of the study was the sudden and sharp rise in ethylene evolution (1438%) in ST seed, at 70% maturity, compared to H seed (figure 6).

3.3 *Changes in pulp*

ST pulp at 70% maturity showed a marked reduction in the contents of total fatty acids (53%) and the two VLCFAs, lignoceric acid (57%) and cerotic acid (64%) compared to H pulp (table 1). The data presented in table 3 revealed that the levels of phenolic acids were significantly lower in ST pulp (56%) compared to healthy pulp. However, the concentration of *p*-OH benzoic acid registered a remarkable rise accounting for 44.3% of the total content of phenolic acids in ST pulp compared to 8.2% in H pulp. PPO activity in ST pulp increased by 257% (figure 7A) while the levels of total phenols was higher by 39.7% (figure 8A). There was a rapid and highly significant rise in the levels of free radicals in ST pulp (figure 9) associated with a marked

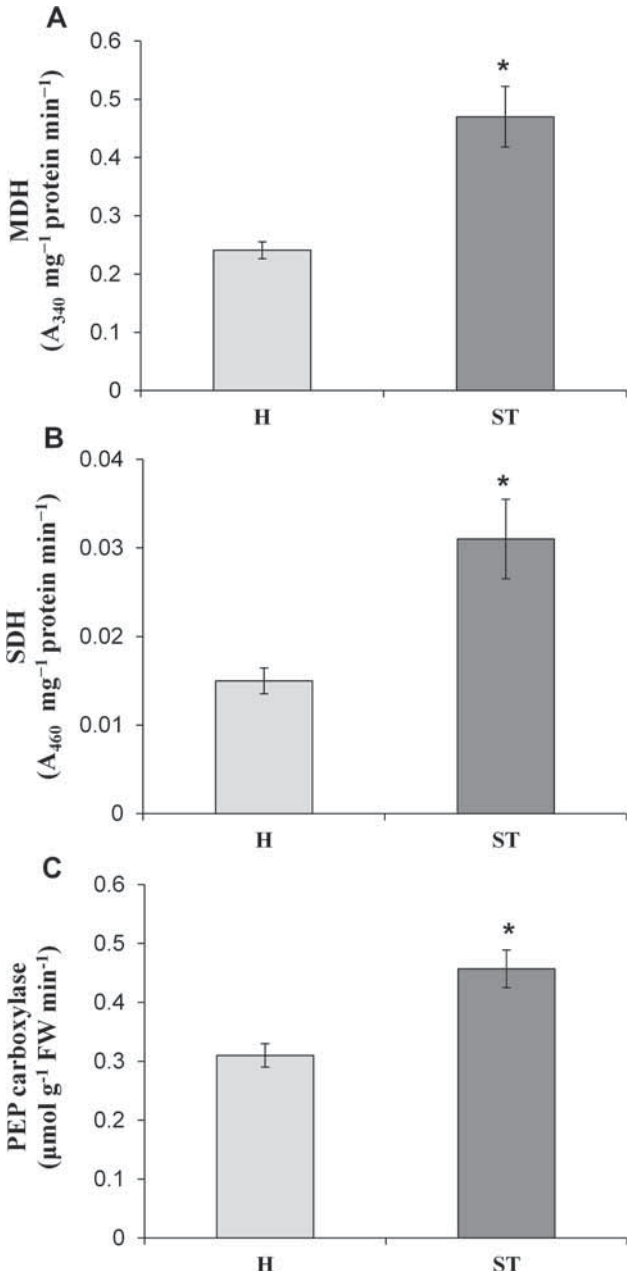

Figure 3. Activities of TCA cycle enzymes, MDH, SDH and PEPCase in the seed of H and ST-affected 70% mature fruit of Alphonso mango The data showed a higher rate of production of energy needed for biosynthetic processes in ST seed. Data are expressed as the mean (±SE) of three replicate samples (*P ≤ 0.05, **P ≤ 0.005).

reduction in the activity of POD (figure 7B). In contrast, CAT activity (figure 7D) increased rapidly in ST pulp. As a result, MDA concentration was higher by 76.7% and EC registered a significant rise (140.6%) in ST pulp (figure 8). Ethylene evolution by ST pulp at 70% fruit maturity was significantly higher (432.6%) compared to H pulp (figure 6).

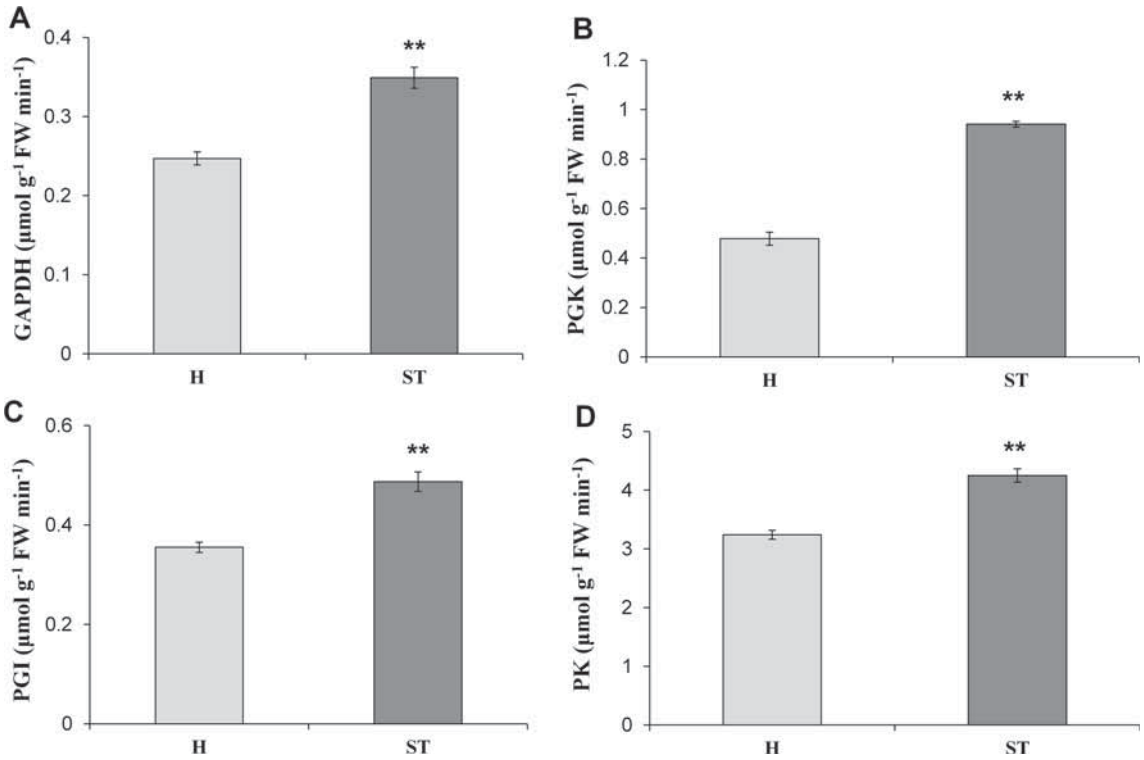

Figure 4. Activities of glycolysis pathway enzymes, GAPDH, PGK, PGI and PK in the seed of H and ST-affected 70% mature fruit of Alphonso mango. The data revealed that the rate of breakdown of sugars in seed was significantly higher in ST seed. Data are expressed as the mean (±SE) of three replicate samples (*P ≤ 0.05, **P ≤ 0.005).

Table 2. Metabolite profile of seed and pulp of 70% mature healthy (H) and spongy tissue (ST) affected Alphonso mango

	H	ST	P value
Fructose-6-phosphate	31.58 ± 0.45	46.23 ± 0.37	***
Glucose-6-phosphate	54.39 ± 1.09	75.98 ± 0.73	***
Glucose-1-phosphate	42.36 ± 0.48	81.47 ± 0.60	***
Fructose-1,6-bis phosphate	47.85 ± 0.74	78.49 ± 1.43	***
DHAP	46.14 ± 0.65	66.54 ± 0.07	***
GAP	54.08 ± 0.54	124.36 ± 1.48	***
Pyruvate	55.79 ± 1.11	130.48 ± 1.25	***
Phosphoenol pyruvate	78.49 ± 1.37	184.57 ± 1.57	***
ADP	62.18 ± 1.27	112.58 ± 1.62	***
ATP	102.56 ± 1.38	168.49 ± 0.1.78	***
NAD	25.68 ± 1.32	47.28 ± 0.81	***
NADH	33.47 ± 1.39	54.71 ± 0.99	***

Values are expressed as means (±SE) for three independent preparations (*P ≤ 0.05, **P ≤ 0.005, ***P ≤ 0.0001).

4. Discussion

4.1 *Reduced synthesis of seed fats and VLCFAs indicates competition for resources*

During development of the fruit of mango cv. Alphonso on the tree, the appearance of a black spot on the seed coat was first apparent when the fruit was 70% mature. As reported earlier (Shivashankar *et al.* 2015a), this was found to be associated with the development of spongy tissue symptoms. The significantly lower content of total fat in fruits with a black spot on the stone compared to healthy fruits on the same panicle indicated that it was the result of competition among developing fruits for photosynthates giving rise to uneven distribution of resources to fruits on the same panicle. Analysis of 70% mature fruits of Alphonso mango showed that there was a significant reduction in the contents of free fatty acids and the two VLCFAs, lignoceric acid (45%) and cerotic acid (42%) in the seed of ST compared to H. Inter-fruit competition for photosynthates during early phase of development is known to lead to disconnection of vascular strands (funiculus) between peduncle and endocarp (stone), making the seed dependent on the mesocarp for its supplies (Wainwright and Burbage 1989). Accordingly, it was apparent that competition during early fruit development on the tree might have led to the breakdown of funiculus at the peduncle end.

4.2 *Evidence for initiation of premature germination-associated events*

Concurrent with the reduction of VLCFAs in the seed, there was a steep rise in the levels of cytokinins and gibberellins

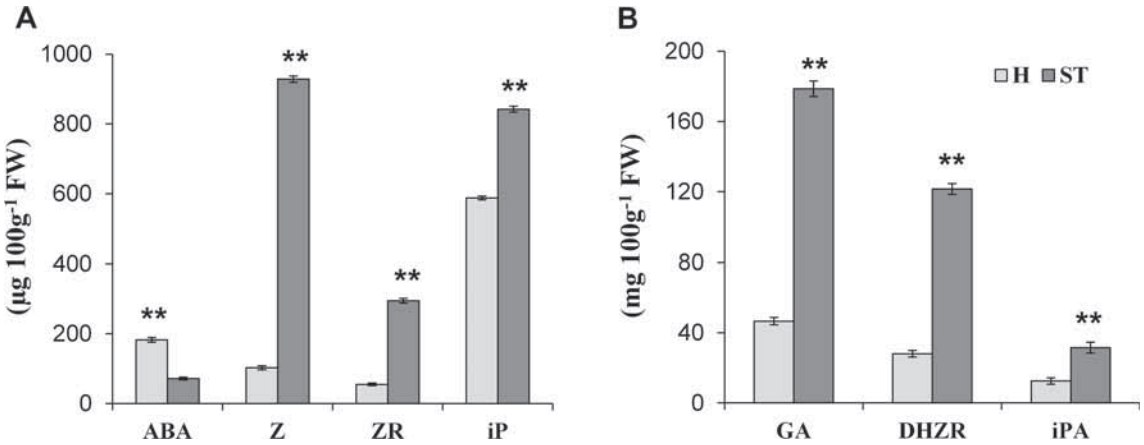

Figure 5. Changes in the levels of ABA, GA and cytokinins in the seed of H and ST-affected 70% mature fruit of Alphonso mango. The data clearly showed the onset of seed germination Data are expressed as the mean (±SE) of three replicate samples (*P ≤ 0.05, **P ≤ 0.005).

Table 3. Composition of phenolic acids in seed and pulp of 70% mature healthy (H) and spongy tissue (ST) affected Alphonso mango

	Pulp			Seed		
	H	ST	P value	H	ST	P value
Gallic acid	11.0 ± 0.19	2.4 ± 0.15	***	121.3 ± 0.75	108.2 ± 0.61	***
Protocatechuic acid	12.6 ± 0.63	6.3 ± 0.17	***	88.5 ± 0.94	189.6 ± 0.88	***
p-OH benzoic acid	8.7 ± 0.31	29.7 ± 0.46	***	135.6 ± 1.31	104.2 ± 0.78	***
Vanillic acid	2.4 ± 0.27	3.0 ± 0.21	ns	174.2 ± 1.32	147.5 ± 1.12	***
Syringic acid	11.1 ± 0.24	5.9 ± 0.27	***	62.1 ± 0.61	46.5 ± 0.79	***
p-coumaric acid	0.5 ± 0.12	0.7 ± 0.10	ns	276.2 ± 1.32	210.4 ± 0.91	***
Sinapic acid	7.9 ± 0.36	5.2 ± 0.18	**	58.5 ± 1.20	124.0 ± 0.82	***
Chlorogenic acid	3.4 ± 0.16	2.1 ± 0.25	*	124.3 ± 0.60	265.3 ± 1.24	***
Ferulic acid	8.4 ± 0.26	6.8 ± 0.42	*	494.2 ± 1.11	247.2 ± 1.55	***
o-coumaric acid	30.9 ± 1.20	0.6 ± 0.11	***	241.3 ± 1.07	187.5 ± 1.30	***
t-cinnamic acid	5.7 ± 0.15	0.3 ± 0.10	***	86.3 ± 0.62	54.7 ± 1.31	***
Salicylic acid	3.4 ± 0.21	3.8 ± 0.18	ns	38.69 ± 1.38	25.64 ± 0.90	**

Values are expressed as means (±SE) for three independent preparations (*P ≤ 0.05, **P ≤ 0.005, ***P ≤ 0.0001).

accompanied by a fall in abscisic acid (figure 5). This finding was similar to the report by Nobusawa *et al.* (2013) who had stated that a reduction in the content of seed VLCFAs triggered the synthesis of cytokinins. This, in turn, overcame ABA-suppressed seed germination as shown by Wang *et al.* (2011). Data presented in table 3 reveals that the levels of the three phenolic acids, protocatechuic acid, chlorogenic acid and sinapic acid, which are known to be stimulators of seed germination (Baodi Bi *et al.* 2017) increased significantly, while, on the contrary, there was a fall in the levels of gallic acid, *p*-OH benzoic acid, vanillic acid, syringic acid, *p*-coumaric acid, ferulic acid, *o*-coumaric acid, *t*-cinnamic acid and salicylic acid in ST seed. The concurrent increase of activities of starch metabolizing enzymes, *viz.,* G6PDH, amylase, TCA cycle enzymes and glycolytic pathway enzymes associated with a rapid rise in the concentrations of metabolites such as, Fructose-6-phosphate, Glucose-6-phosphate, Glucose-1-phosphate, Fructose-1, 6-bis phosphate, Phosphoenolpyruvate, DHAP, GAP, ADP, ATP, NAD, NADH and Pyruvate in ST seed compared to H seed (table 2) clearly demonstrates that the composition of phenolic acids in ST seed had changed so as to favour germination resulting in the breakdown of reserve carbohydrates in seed for production of energy needed for biosynthetic processes (Pritchard *et al.* 2002). As shown in figure 6, there was a sharp rise in ethylene evolution (1438%) of 70% mature ST seed compared to H seed. A number of studies have reported a sudden increase of ethylene production under stress (Apelbaum and Yang 1981), which was likely due to the interruption of supply of water and nutrients to the growing fruit following the breakdown of funiculus (Wainwright and Burbage 1989). Although, many studies have suggested that endogenous ethylene production is needed for seed germination (Machabée and

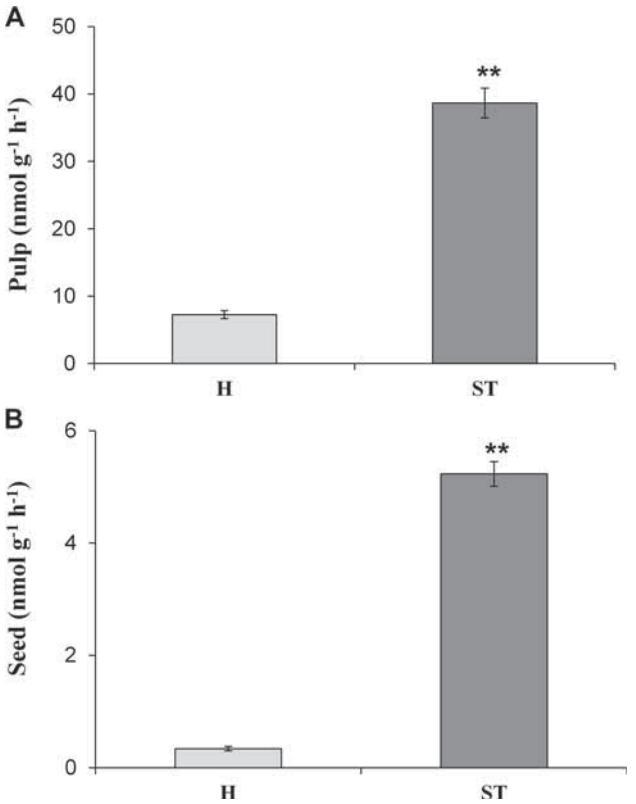

Figure 6. Ethylene evolution by pulp and seed tissues of H and ST-affected 70% mature fruit of Alphonso mango. The burst of ethylene by seed signified the stress response after the breakdown of the funiculus. Data are expressed as the mean (±SE) of three replicate samples (*P ≤ 0.05, **P ≤ 0.005).

Saini 1991; Petruzzelli *et al.* 1995; Calvo *et al.* 2004), there were speculations as to whether embryonic ethylene production is needed to trigger germination or *vice versa* (Abeles *et al.* 1992). In this context, the recent study by Hershkovitz *et al.* (2009) in avocado has confirmed that ethylene essentially increases embryo growth significantly before the seed is ready for germination. Thus, all the parameters presented above have provided ample evidence for the initiation of germination associated events in the premature seed of ST fruits. It has been reported that under conditions of stress for resources, recalcitrant seeds which are characterized by high sensitivity to desiccation and a lack of seed dormancy (Chandler and Robertson 1994; Farnsworth 2000) show pre-germination events, during development, on the mother plant itself. Based on the above, it was clearly apparent that vascular disconnection of Alphonso mango fruit from the tree at 70% fruit maturation had led to the development of stress for water and nutrients in some fruits on the panicle leading to a sudden surge of ethylene evolution by the seed, which in turn, led to embryo growth, followed by the onset of premature germination events in the developing seed.

4.3 *Formation of black spot on the seed coat*

The sudden surge of ethylene production by ST seed at 70% fruit maturity was associated with a large concurrent rise of ethylene evolution from the pulp near the seed base associated with an increase of PPO activity in ST pulp

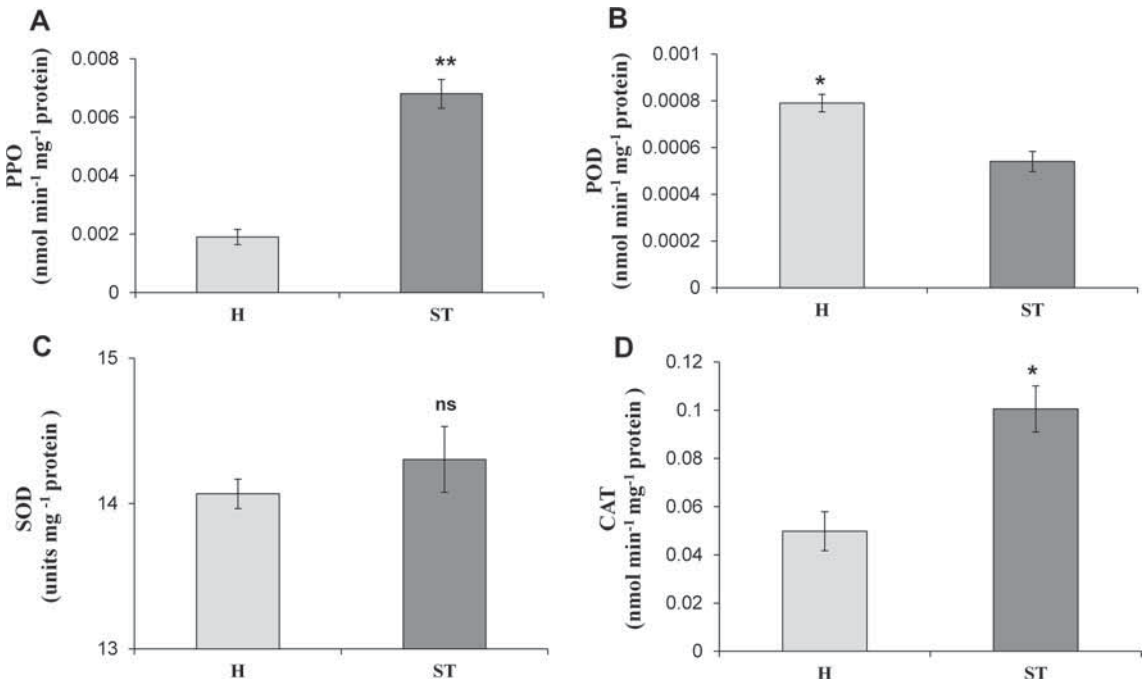

Figure 7. Activities of antioxidant enzymes in the seed of H and ST-affected 70% mature fruit of Alphonso mango. The data was indicative of the reduced antioxidant capacity of ST pulp. Data are expressed as the mean (±SE) of three replicate samples (*P ≤ 0.05, **P ≤ 0.005).

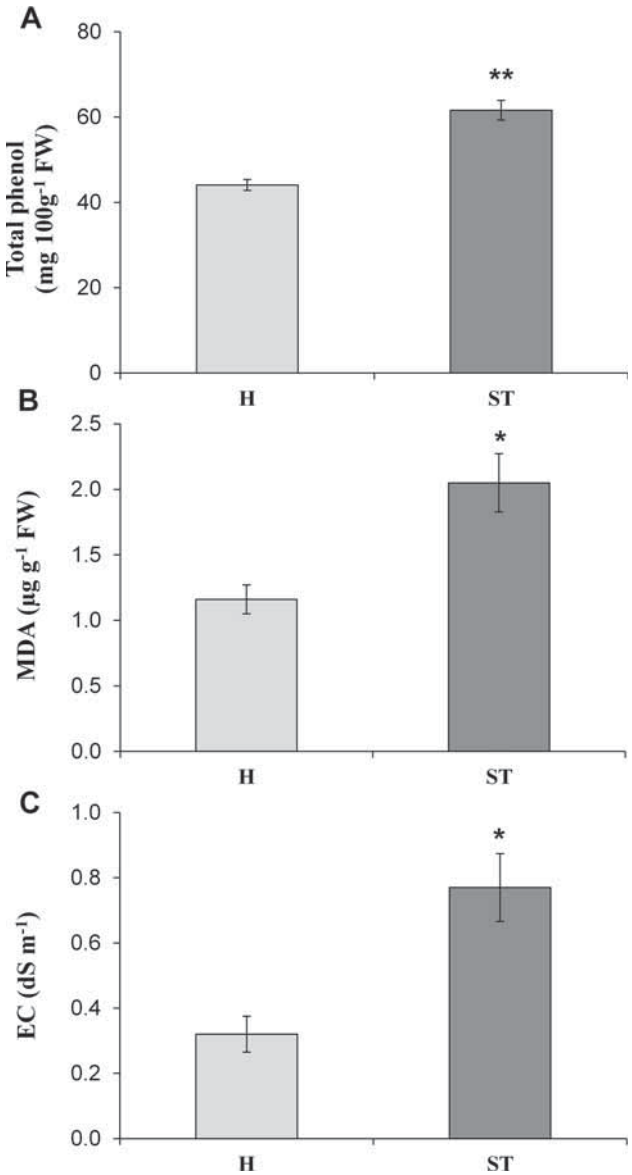

Figure 8. Total phenols, MDA and electrical conductivity in H and ST-affected 70% mature fruit of Alphonso mango. The data signified the increased rate of lipid peroxidation and membrane damage in ST. Data are expressed as the mean (±SE) of three replicate samples (*P ≤ 0.05, **P ≤ 0.005).

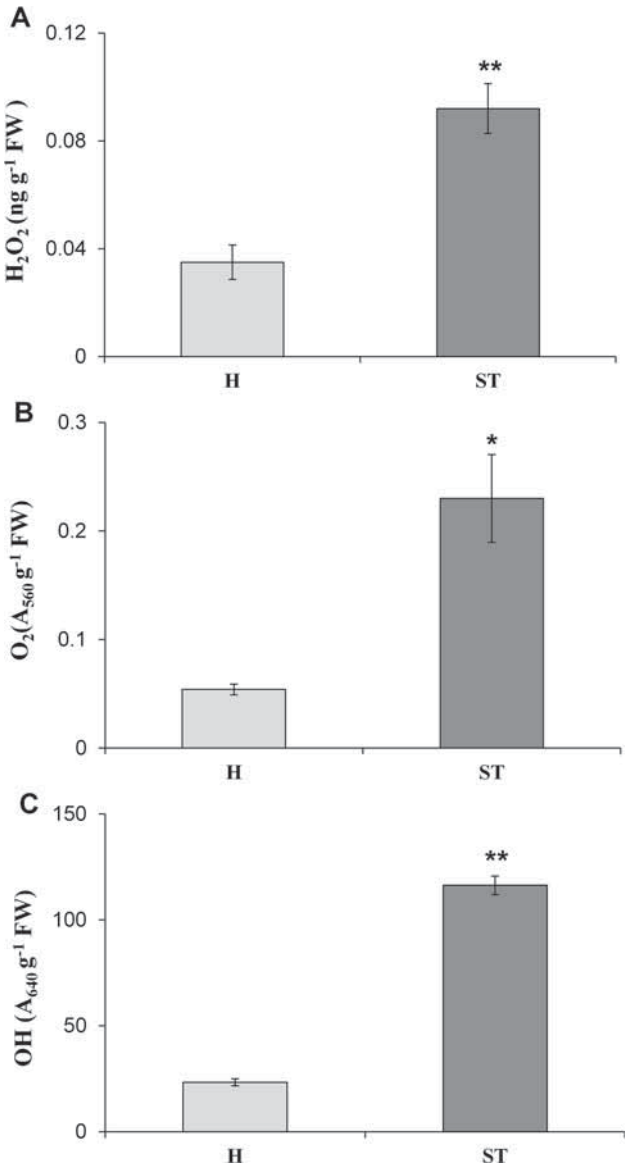

Figure 9. Levels of free radicals in H and ST-affected 70% mature fruit of Alphonso mango. The significantly higher levels of free radicals in ST might have led to the production of ethylene. Data are expressed as the mean (±SE) of three replicate samples (*P ≤ 0.05, **P ≤ 0.005).

compared to H pulp. Considering the report of Hershkovitz *et al.* (2009), in which treatment by ethylene induced PPO activity in the inner pulp of seeded avocado fruit, it was evident that the rise in ethylene evolution by seed of Alphonso resulted in higher PPO activity in the pulp of ST (figure 7A). With the content of total phenols increasing simultaneously in ST pulp in contrast to H pulp, enzymatic browning occurred as a result of the oxidation of phenolic compounds to quinones by PPO and their eventual polymerization to melanin pigments (Macheix *et al.* 1990; Whitaker 1995). Considering the report of Hershkovitz *et al.* (2009) who showed that the production of ethylene was higher near the seed base in avocado, it was evident that the black spot appeared on seed coat close to the embryo in Alphonso mango (figure 1).

4.4 *Inhibition of degradation of starch and pectin in pulp by p-OH benzoic acid*

Despite the fact that the concentration of phenolic acids decreased significantly in ST pulp, *p*-OH benzoic acid registered a sharp rise accounting for 44.3% of the total content of phenolic acids, as against 8.2% in H pulp (table 3). A higher level of *p*-OH benzoic acid is known to inhibit amylase activity (Wu *et al.* 2009). Indeed, several authors

have reported reduced amylase activity in the pulp tissue of ST-affected Alphonso mango (Katrodia and Rane 1989; Selvaraj *et al.* 2000) and Tommy Atkins (Lima *et al.* 2000) supporting our findings. Due to the reduction in amylase activity in the pulp, the stored starch was partially degraded during fruit ripening, leading to a lower level of sugars and higher starch content in ST pulp compared to H pulp (Shivashankar *et al.* 2007). The build-up of *p*-OH benzoic acid in ST pulp also inhibited polygalacturonase (PG) activity thereby reducing the rate of degradation of pectin (Srivastava *et al.* 2013). Consequently, the combined inhibition of both amylase and pectin degrading enzymes led to incomplete degradation of starch and pectin polymers resulting in changes in the composition and texture of ST-affected fruit compared to H fruit. Further, the higher level of *p*-OH benzoic acid is also known to enhance the production of free radicals (Shah and Verma 2011), which is also supported in the present study (figure 9). It is likely that the increased level of free radicals might have led to an abrupt rise in the level of ethylene in 70% mature fruit pulp of ST compared to H (figure 6), as reported by Mayak *et al.* (1983) and others (Beauchamp and Fridovitch 1970; Mapson and Wardale 1972; Riely *et al.* 1974) in various model systems. There was a reduction of POX activity coupled with increased CAT activity in ST pulp (figure 7D) leading to a lowering of the antioxidant capacity of ST pulp. This, coupled with increased free radical production, enhanced the lipid peroxidation and membrane damage through increased MDA synthesis (figure 8B) (Ahrabi *et al.* 2011), followed by changes in the characteristic aroma and off-flavour production in ST fruits of Alphonso mango (Selvaraj and Kumar 1989).

4.5 *Premature seed germination inhibits climacteric ethylene production and ripening*

The unusually high concentration of CO_2 produced by the combined effects of climacteric respiration in pulp, coupled with initiation of germination-associated events in ST-affected mango seed (Shivashankar 2014), inhibited ethylene production at the climacteric stage as shown by Nagamani *et al.* (2010). Similar results on the antagonistic effect of high concentrations of CO_2 on ethylene production has been reported in a number of plant responses such as fruit ripening, abscission and senescence (Lieberman *et al.* 1974; Li *et al.* 1983). CO_2 is known to influence ethylene production by inhibiting the conversion of ACC to ethylene catalysed by ACC oxidase (De Wild *et al.* (2003). Consequently, a significant reduction of ethylene production during climacteric phase led to incomplete ripening of Alphonso mango and production of off-flavour (Selvaraj and Kumar 1989). The differential regulation of fruit flavour components by ethylene was demonstrated by Dandekar *et al.* (2004) who showed that transgenic apple silenced for ethylene synthesis had led to a dramatic suppression of the synthesis of volatile esters in fruit without affecting the aldehyde and alcohol precursors. Thus, the study has clearly established that the rapid surge of ethylene evolution by the premature seed triggered a series of biochemical changes in the mature fruit pulp during ripening climacteric leading to alterations in the composition and quality of ripe pulp characteristic of spongy tissue affected Alphonso mango fruit.

4.6 *Conclusions*

Stress imposed by the vascular breakdown at the peduncular end in 70% mature fruit of Alphonso mango induced the rapid burst of ethylene evolution by the immature seed leading to the formation of black spot on the seed coat. Subsequently, the initiation of germination associated events in the premature seed led to a host of biochemical changes in the mesocarp coupled with a sudden spurt in CO_2 concentration. Following this, the inhibition of ethylene production at the climacteric stage led to incomplete breakdown of starch and pectin polymers in the pulp, coupled with the production of off-flavour compounds, which are characteristic of spongy tissue affected Alphonso mango fruits. Thus, the study has firmly established that the burst of ethylene evolution by premature seed is the precursor to spongy tissue formation in Alphonso mango, which could be used as a non-destructive biomarker to distinguish between ST and healthy fruit at an early stage.

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