Do seed VLCFAs trigger spongy tissue formation in Alphonso mango by inducing germination?

SESHADRI SHIVASHANKAR*, MANOHARAN SUMATHI and TAPAS KUMAR ROY

Division of Plant Physiology and Biochemistry, Indian Institute of Horticultural Research, Bangalore, India

**Corresponding author (Email, drsshivashankariihr@yahoo.co.in)*

Spongy tissue is a physiological disorder in Alphonso mango caused by the inception of germination-associated events during fruit maturation on the tree, rendering the fruit inedible. Inter-fruit competition during active fruit growth is a major contributing factor for the disorder which leads to reduced fat content in spongy tissue affected fruits. This study was, therefore, carried out to determine the possible association between seed fats and ST formation. The study of the fat content during fruit growth showed that it increased gradually from 40% fruit maturity. At 70% maturity, however, there was a sudden increase of fat content of whole fruit, leading to acute competition and resulting in differential allocation of resources among developing fruits. As a result, the seed in spongy-tissue-affected mature ripe fruit showed a marked drop in the levels of fats and the two very long chain fatty acids (VLCFAs), tetracosanoic acid and hexacosanoic acid together with an increase of linolenic acid and a fall in oleic acid contents, which are known to be key determinants for the initiation of pre-germination events in seed. Subsequently, a rise in the level of cytokinin and gibberellins in ST seed associated with a fall in abscisic acid level clearly signalled the onset of germination. Concurrently, a significant reduction in the ratio of linolenic acid/linoleic acid in pulp led to the loss of membrane integrity, cell death and the eventual formation of spongy tissue. Based on the above, it is concluded that a significant reduction in the biosynthesis of VLCFAs in seeds during fruit growth might trigger pre-germination events followed by a cascade of biochemical changes in the pulp, leading to lipid peroxidation and membrane injury in pulp culminating in ST development. Thus, this study presents crucial experimental evidence to highlight the critical role played by VLCFAs in inducing ST formation in Alphonso mango during the pre-harvest phase of fruit growth.

[Shivashankar S, Sumathi M and Roy TK 2015 Do seed VLCFAs trigger spongy tissue formation in Alphonso mango by inducing germination? *J. Biosci.* **40** 375–387] **DOI** 10.1007/s12038-015-9515-7

1. Introduction

Spongy tissue (ST) is a physiological disorder characterized by the presence of off-white sponge-like pulp tissue, slightly desiccated, with or without air pockets, originating on the surface of the stony pit, resulting in the production of poor quality fruit (Katrodia 1979). This disorder adversely affects the sensory quality of fruits without showing external symptoms. The primary cause of this disorder was first established in our studies which showed that the Alphonso mango seed switches over to germination phase during fruit maturation in the pre-harvest phase resulting in the development of ST in ripe fruits (Shivashankar 2014).

In order to explain the mechanism of ST formation in only some and not all fruits on a panicle, it was assumed that due to temporal variations in fertilization of flowers and fruit set, developing mango fruits at any given time are likely to be at different physiological stages of maturity and are subject to variable competition during their ontogeny (Shivashankar 2014). Further, it was postulated that the genetically stronger sinks which sustain the competition grow to full maturity producing healthy fruits while the seed from weaker

Keywords. Alphonso mango; cytokinin synthesis; membrane damage; seed germination; spongy tissue; VLCFA

Published online: 17 April 2015

sinks which cannot sustain the competition get separated from the mother plant physiologically as the vascular strands (funiculus) between the peduncle and endocarp (stone) become disconnected, even while on the tree (Wainwright and Burbage 1989), causing a physiological shock to the embryo, thus affecting its dormancy or viability leading to seed germination in the developing fruit. Previous studies in our laboratory had established that the process of seed-germination-associated events led to water uptake from the surrounding mesocarp, resulting in the breakdown of seed reserves followed by changes in pulp composition due to which changes occurred in texture and sensory properties compared to healthy normal tissue (Shivashankar *et al*. 2007; Ravindra and Shivashankar 2004) and resulted in spongy tissue formation. However, the all-important question as to what triggered the seed germination process in some fruits leading to damage to cell membrane and the biochemical mechanism of development of ST had not been established.

Incidentally, Alphonso mango fruit is reported to contain the highest percentage of fats in both pulp and seed compared to other mango varieties (Selvaraj 1996). Past work had shown that the ST-affected Alphonso fruit contained less fats compared to healthy fruit (Shivashankar *et al.* 2007). This suggested a possible link between fat content and sponginess formation. Since the biosynthesis of fats is an energy-intensive process, we speculated that under conditions of limiting assimilate supply during fruit development on the tree, synthesis of fats might be inhibited in some fruits, resulting in such fruits turning spongy after ripening. A review of literature suggested that, in spite of extensive studies on the subject of spongy tissue by several investigators around the world thus far, there was no attempt to connect fatty acid metabolism in seed and pulp to the process of ST formation in Alphonso mango. This study was, therefore, carried out with the aim of examining the possible role of seed fats and fatty acids in ST formation. Based on the results of the study, the sequence of biochemical events leading to cell membrane injury and the eventual formation of spongy tissue in Alphonso mango fruit are discussed in this paper.

2. Materials and methods

2.1 *Tree growth conditions*

'Alphonso' mango fruits were collected during the 2012–2013 season from 25-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.

2.2 *Fat content*

Total fat content in tissue samples was extracted using Soxhlet extractor as described by Osborne and Voogt (1978) and estimated gravimetrically. One gram 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 dry pre-weighed flask (W_1), evaporated to dryness on a boiling water bath and weighed again (W_2). The difference in the weight was used to calculate the percent of fat in sample using the formula, (W_2-W_1)/Weight of sample×100.

2.3 *GC-FID analysis*

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 refluxing for 10 min at 70°C, followed by addition of 14% BF_3 in methanol and 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.

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 0.25 µm film thickness. The temperature program for the column was as follows: The initial oven temperature was 100°C for 4 min, increased by 3°C per min up to 220°C, held for 4 min, temperature increased further at the rate of 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 the 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. Initially injection mode was split-less followed by split mode (1:30) after 1.5 min.

2.3.1 *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 i.d. 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.

2.4 *Intensity of spongy tissue*

Intensity of spongy tissue was quantified as a percentage of the fresh weight (FW) of spongy-tissue-affected pulp compared to the total FW of the pulp. The spongy tissue affected spot showing discoloured and partially dehydrated tissue was considered "ST-affected" (ST) and the tissue surrounding the affected spot which remained free from symptoms was designated as "apparently healthy" (AH). The healthy (H) tissue was collected from non-affected fruit.

2.5 *Enzyme and phytohormone assays*

2.5.1 *Lipase activity:* Lipase activity was determined as described by Jayaraman (1981). Five hundred milligrams of acetone powder was extracted with 10 mL of 0.05M sodium phosphate buffer, pH 7.0 and left standing overnight at 4°C.The extract was centrifuged at 8000 rpm for 10 min and supernatant was used as the enzyme source. The assay medium consisted of 5 mL substrate (a mixture of 1 mL groundnut oil, 1.5 mL of 0.1 M phosphate-citrate buffer, pH 7.0, 0.5 mL of 10% gum Arabica and 5 mL water), 3 mL of 0.01M NaCl and 2 mL of enzyme. The reaction mixture was incubated for 30 min at 37°C. The reaction was terminated after 30 min by the addition of 10 mL absolute alcohol. For the blank, the reaction was stopped at zero time. The reaction mixture was titrated against N/200 NaOH using phenolphthalein indicator. Lipase activity was expressed as mg free fatty acid (FFA) liberated /g/h based upon the standard graph constructed with linoleic acid as standard.

2.5.2 *Amylase activity:* α-Amylase was extracted by homogenizing 1.0 g of pulp in 10 mL of 16 mM sodium acetate buffer, pH 4.8, containing 0.5 M NaCl and centrifuged at 10,000*g* for 10 min at 4°C. Five-hundred µL of the supernatant was added to a reaction mixture containing 0.5 mL of 1% (w/v) starch dissolved in the same extraction buffer and incubated for 30 min at 20°C. A zero time blank containing all the above components was also maintained. The reaction was terminated by adding 0.5 mL dinitro salicylic acid (DNS) reagent and heated for 5 min on a boiling water bath. The mixture was made up to a final volume of 20 mL and its absorbance was read at 540 nm (Bernfeld 1955). α-Amylase activity was expressed as mg maltose liberated/g/h

2.5.3 *Malate dehydrogenase:* Malate dehydrogenase (MDH) activity was determined as described by Selvaraj *et al*. (1995). The assay mixture consisted of 0.4 mL 0.2 M Tris-HCl buffer, pH 7.5, 0.5 mL 1 mM NADH, 0.5 mL 15 mM oxaloacetate, and 0.1 mL enzyme extract. Absorbance at 340 nm was measured at 30°C, and MDH activity was expressed as change in absorbance at 340 nm/mg protein/min.

2.5.4 *Succinate dehydrogenase:* Succinate dehydrogenase (SDH) was assayed in a reaction mixture containing 0.45 mL 0.5% (w/v) 2,3,5 triphenyltetrazolium chloride (TTC), 0.55 mL 0.2 M sodium succinate, 1.3 mL 0.1 M sodium phosphate buffer, pH 7.2 and 0.2 mL enzyme extract, in a total volume of 2.5 mL. The enzyme activity was expressed as the change in absorbance at 460 nm/mg protein/min (Baqui *et al*. 1974).

2.5.5 *Estimation of gibberellins, abscisic acid and cytokinins:* Extraction and purification of seed hormones: Seeds (10 g) were ground in liquid nitrogen, mixed with 20 mL of 80:20 (v/v) mix of methanol and water containing 20 ng of each internal standard [Gibberellic acid (GA), abscisic acid (ABA) and kinetin], and incubated at 4°C on a shaker at 300 rpm for 24 h in the dark. Samples were centrifuged at 300*g* for 10 min and the supernatant was transferred to a clean test tube. The pellet was re-suspended in 5 mL of extraction solvent mix of methanol: water [80:20 (v/v)] and centrifuged again at 300*g* for 10 min at 4°C. After centrifugation, the two supernatants were combined and concentrated in a rotary flash evaporator at 35°C, dissolved in double distilled water, pH adjusted to 3.0 and re-extracted three times with diethyl ether. The ether layer was collected and mixed with sodium sulphate to remove traces of moisture. The purified extract was concentrated under a continuous stream of nitrogen and the residue was re-dissolved in 200 µL HPLC-grade methanol, centrifuged at 12,740*g* for 10 min at 4°C, filtered through a 0.22-µm PTFE filter (Waters, Milford, MA, USA) and separated by HPLC for the quantification of gibberellins and ABA.

The aqueous layer obtained after separating the ether layer was adjusted to pH 8.0 and extracted three times with water-saturated butanol. The butanol layer was collected and concentrated under a continuous stream of nitrogen. The residue was re-dissolved in 200 µL HPLC-grade methanol, centrifuged at 12,740*g* for 10 min at 4°C, filtered through a 0.22 µm PTFE filter (Waters, Milford, MA, USA) and used for separation of cytokinins.

High-performance liquid chromatography (HPLC): The method of Kelen *et al*. (2004) with some modifications was used for identification and quantification of seed hormones by HPLC.Separation was performed using a Shimadzu LC-10 AD VP liquid chromatograph, equipped with a stainless steel analytical column, (Synergi 4μ Hydro RP 80A; 250 mm ×4.6 mm id; Phenomenex, Torrance, CA, USA). Samples (10 μL) were injected into the HPLC system and separation of ABA and GA was carried out by isocratic elution using a 26:74 (v/v) mix of acetonitrile:water, pH 4.0, as the mobile phase, at a flow rate of 0.8 mL/min. Cytokinins were separated using a 14:86 (v/v) mix of acetonitrile:water containing 0.5%(v/v)acetic acid at a flow rate of 0.2 mL/min. The mobile phase was filtered through a 0.22-μm nylon membrane and degassed before use. PGR concentrations were monitored by recording the absorption at 200 nm, 220 nm and 270 nm for GA, ABA and cytokinins respectively and calculated using calibration curves prepared using 10 μL each of the standard (Sigma, St. Louis, MO, USA).

2.6 *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 content was measured according to Schopfer *et al*. (2001) and expressed as ng H_2O_2 generated /g FW of tissue.

2.7 *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 hydroxytoluene, was added before heating the samples. After cooling on ice, samples were centrifuged at 840*g* 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.

2.8 *Electrolyte leakage*

One gram 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.

2.9 *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).

3. Results

3.1 *Rate of fat synthesis during fruit growth*

As shown in table 1, the levels of fats in both pulp and seed increased gradually with fruit maturity. However, at 70%, there was an abrupt increase in the rate of fat accumulation by the fruit which continued until full maturity. A striking feature of the study was that there was a significant increase in the fat content of whole fruit ($P \leq 0.005$) at 70% maturity at which time it was 7-times higher compared to 40% mature fruit and subsequently increased by about 15-times at 80% maturity and 21-times at 90% maturity.

3.2 *Commencement of seed germination events*

Lipase activity in seed of ST fruit was higher than the H fruit ($P \leq 0.005$) while on the contrary, pulp of ST fruit had lower lipase activity compared to H pulp. Lipase activity increased rapidly in seed with increasing intensity of ST ($P \leq 0.005$) while registering a rapid decline in pulp (figure 1). The activities of amylase, SDH and MDH measured in seed were also higher in ST compared to H fruit ($P \leq 0.005$) while the converse was true for pulp (figure 2).

The fat content of seed in mature healthy fruit was 8.5% as against 7.7% in ST-affected fruit while fat content in pulp of healthy fruit was 1.5% compared to 0.8% in ST-affected fruit. The content of total free fatty acids was significantly lower in ST pulp and seed compared to healthy pulp and seed ($P \leq 0.005$) respectively (table 2).

3.3 *Generation of free radicals*

As a first step towards analyzing the effect of seed germination on the possible changes in pulp, free radical production was estimated in healthy, apparently healthy (AH) and spongy tissue affected (ST) pulp. H_2O_2 levels showed a marked increase in AH (70.3%) and ST (114.8%) compared to the control ($P \leq 0.005$). The level of hydroxyl radicals (•OH) increased significantly in AH (587.6%) and further

Table 1. Fat content[1] of Alphonso mango during fruit growth

Parameter	Fruit maturity (%)							*F*- value
	40	50	60	70	80	90	100	
Pulp fat (g/100 g DW)	0.41±0.03[2]	0.53±0.02	0.66±0.01	0.77±0.02	0.94±0.02	1.40±0.03	1.50±0.19	35.62
Seed fat (g/100 g DW)	1.60±0.15	2.48±0.19	4.00±0.21	5.95±0.22	7.40±0.27	8.00±0.34	8.50±0.76	65.53
Fruit fresh wt (g)	76.9±2.4	125.5±7.2	164.8±3.9	185.1±5.3	238.3±9.1	252.1±7.5	260.3±2.4	137.00
Total fat (g/FW fruit)	0.06±0.004	0.14±0.013	0.29±0.009	0.42±0.018	0.91±0.069	1.27±0.086	1.34±0.152	56.31

[1]Fat content in pulp and seed were extracted using Soxhlet extractor and estimated gravimetrically.

Note the 7-, 15- and 21-fold increase of fat accumulation at 70, 80 and 90% fruit maturity.

Respectively indicating increased competition for resources.

[2] ± denotes standard error of mean.

up in ST (1211.6%) compared to healthy control. Superoxide radical ($O^{2\bullet-}$) levels also increased rapidly in AH (167.6%) and shot up further in ST (404.4%) (figure 3).

3.4 *Changes in free fatty acids and membrane degradation in pulp*

The influence of seed germination on the composition of fatty acids in pulp and membrane structure was examined. The levels of short and medium chain fatty acids, nonanoic acid and lauric acid were higher in both seed and pulp of ST-affected fruit compared to healthy fruit while the levels of long chain fatty acids, palmitic acid, palmitoleic acid, stearic acid, oleic acid and linoleic acid were lower in both pulp and seed of ST-affected fruit. There was a significant fall in oleic acid and the levels of the two very long chain fatty acids (VLCFA), tetracosanoic acid and hexacosanoic acid in both seed and pulp of ST-affected fruit while linolenic acid level increased in ST seed with a steep fall in ST pulp. The levels of the two long chain fatty acids, erucic acid and eicosanoic acid increased in ST pulp (table 3). The ratio of linolenic acid/linoleic acid decreased significantly from 0.622 in healthy pulp to 0.059 in ST pulp ($P \leq 0.005$) while the concentration of MDA in ST pulp (2.48 µg/100 g FW) was higher compared to AH (1.64) and H tissue (1.39). The pH of ST pulp was significantly lower (4.3) compared to AH (4.9) and H pulp (5.4) ($P \leq 0.05$) while the EC was significantly higher in ST pulp (0.76) compared to AH (0.61) and H pulp (0.54) ($P \leq 0.005$) (figure 4).

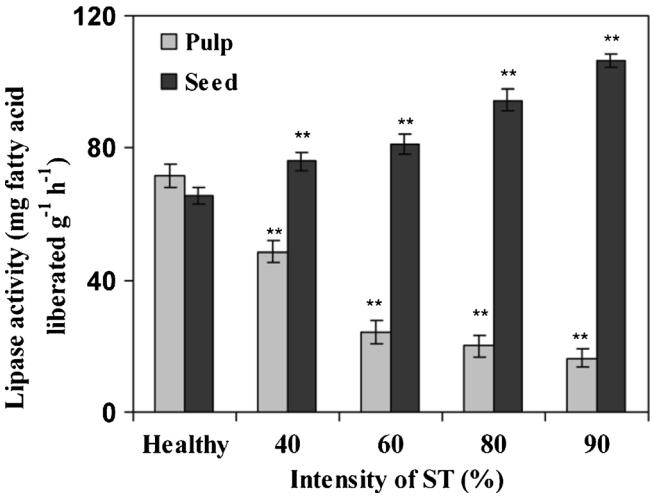

Figure 1. Changes in lipase activity of Alphonso mango pulp as a function of intensity of ST (**$P \leq 0.005$). Fruits affected by increasing intensity of ST were monitored for lipase activity by titrimetric estimation of the liberated free fatty acids. The rapid increase of activity in ST seed with increasing intensity of spongy tissue indicated the increased rate of conversion of stored fats during ST development.

3.5 *Changes in the levels of cytokinin, gibberellins and abscisic acid in seed*

The levels of cytokinins, abscisic acid (ABA) and gibberellins (GAs) in seed of healthy and ST-affected fruit are presented in figure 5. The concentrations of cytokinins namely, Zeatin(Z), Zeatinriboside (ZR), Dihydrozeatinriboside (DHZR), isopentenyl adenine(iP) and isopentenyl adenosine (iPA) were significantly higher in seed of ST fruit compared to H fruit. Gibberellins also increased significantly in seed of ST fruit while ABA content was lower in seed of ST fruit compared to H fruit.

4. Discussion

4.1 *Competition for resources leads to reduced fat synthesis*

There was a steady increase in the fat content of both pulp and seed in developing fruits up to 60% fruit

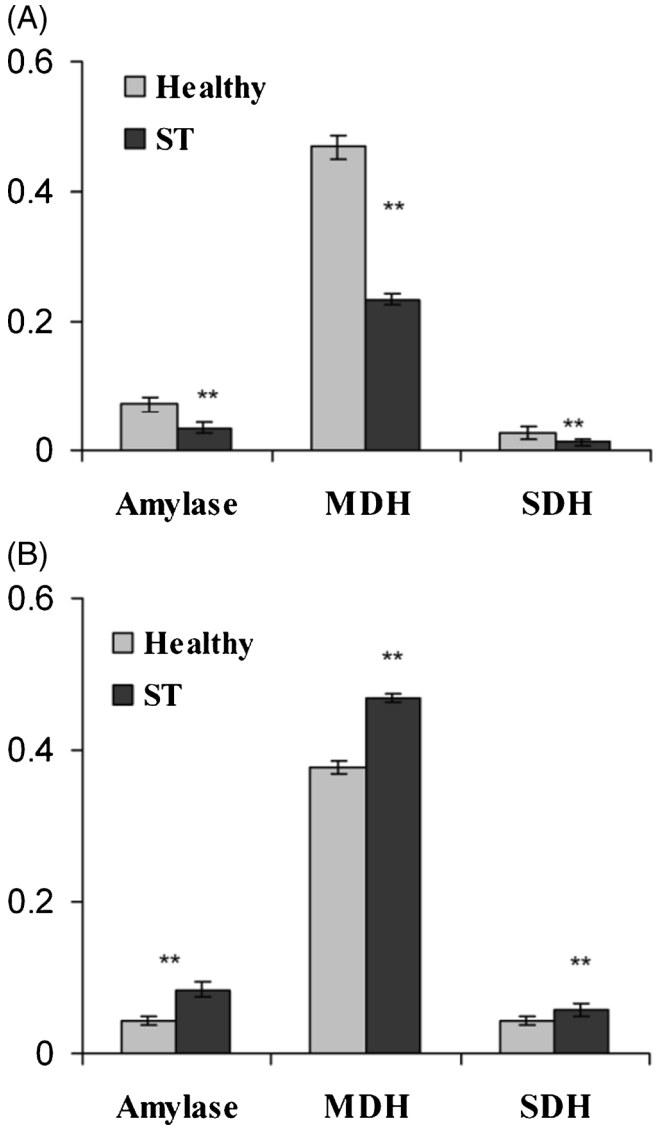

Figure 2. Activities of amylase (mg maltose liberated g^{-1} h^{-1}), MDH (A_{340} nm mg^{-1} protein min^{-1}) and SDH ((A_{460} nm mg^{-1} protein min^{-1}) enzymes in healthy and ST-affected pulp (A) and seed (B) of Alphonso mango fruit (**$P \leq 0.005$). The higher activities of amylase, MDH and SDH in ST seed associated with a reduction in the pulp of ST confirmed the progress of germinatin events in seed coupled with a reduced rate of starch conversion and generation of reducing power in pulp tissue during ST formation.

maturity. However, at 70% fruit maturity, there was a rapid 7-fold increase in the fat content of whole fruit, which further increased to about 15-fold at 80% maturity and further up to 21-fold at 90% maturity as compared to 40% maturity fruits. The steep increase of fruit weight during the same period also contributed for the marked increase of fat content of whole fruit. From this account, it was evident that at 70% fruit maturity, the demand for supply of assimilates for fat synthesis in Alphonso fruit increased rapidly and continued to rise further until full maturation. Similar results were reported by Abou-Aziz *et al.* (1973) in avocado pear fruits supporting the present findings. Thus, it was apparent that 70% fruit maturity was the critical stage in Alphonso mango at which fruits on a panicle began to face strong competition for resources from neighbouring fruits for the synthesis of a greatly increased proportion of fats. Incidentally, the Alphonso mango fruit is reported to contain the highest fat content among all mango varieties examined so far (Selvaraj 1996) and is, therefore, thought to be a major sink for assimilates. Since carbohydrates provide the carbon skeleton for the synthesis of fats in plants (Kikuta 1969; Rawsthorne 2002), it could be expected that a short supply of carbohydrates in Alphonso mango would lead to intense competition for resources among developing sinks at 70% maturity. Since dry matter partitioning among sinks is determined by the sink strength of various organs (Marcelis 1994, 1996), the allocation of assimilates to weak sinks would be reduced (Bertin 1995) due to acute competition among sinks as fruit growth in mango mainly occurs from the limited stored carbohydrate reserves of the previous year (Bustan *et al*. 1996). This presumably could result in reduced synthesis of fats in some fruits on a panicle. Data presented in table 2 confirmed this speculation, when it was found that the contents of total fats and free fatty acids in both seed and pulp of ST fruit were significantly lower compared to seed and pulp of healthy fruit respectively. The causal nature of reduced seed fat content leading to ST formation was clearly apparent when it was noted that some fruits of 80% maturity showing lower fat content (0.64–0.85%) than normal (0.91–1.27%) had higher seed moisture content (42–54%) than normal (32%), a characteristic distinguishing feature of spongy-tissue-affected fruits. Interestingly, the earliest stage at which the increase of seed moisture was observed was at the 80% stage of fruit maturity. This finding was of great significance as it provided positive proof for the fact that the flow of moisture from pulp to seed and the associated biochemical changes in the ST-affected seed were initiated around 70% fruit maturation stage although symptoms of the disorder became apparent only after fruit ripening (Shivashankar *et al*. 2007). Considering the fact that the onset of seed germination caused the ST disorder (Ravindra and Shivashankar 2006), it was obvious that the reduction in the content of seed fat signalled the beginning of the sequence of events leading to ST development in Alphonso mango.

Table 2. Total fat, free fatty acids and lipase activity in pulp and seed of healthy (H) and spongy-tissue-affected (ST) Alphonso mango fruit

Parameter measured	Pulp			Seed		
	H	ST	*t*-Value	H	ST	*t*-Value
Total fat[1] (g/100 g FW)	1.5±0.33	0.8±0.20	5.14	8.5±0.27	7.7±0.20	6.69
Lipase activity[3] (mg) fatty acid liberated/g/h)	71.5±2.1	48.6±3.9	13.32	65.6±3.5	76.0±2.7	−7.69
Free fatty acids[2] (mg/100 g)	193.6±5.3	157.9±2.8	16.97	326.3±4.3	232.4±3.8	46.02

[1] Note the marked reduction in the total fat content of both pulp and seed of ST fruit compared to H fruit showing a reduction in the synthesis of fat in ST fruit.

[2] Free fatty acid composition determined by GC MS showed a significant reduction in both pulp and seed of ST fruit indicating that the synthesis of fatty acid was reduced in ST fruit.

[3] Lipase activity in seed increased in seed indicating the initiation of seed germination while it reduced in pulp denoting slower degradation of fat in ST fruit pulp.

4.2 *Changes in the fatty acid composition of seed and its effect on seed germination*

From the data presented in table 3, it is worthwhile mentioning that there was a significant decline in the levels of the two very long chain fatty acids (VLCFAs), tetracosanoic acid and hexacosanoic acid in seed from ST-affected fruit. VLCFAs are fatty acids containing 20 to 36 carbons which perform a wide range of physiological functions depending on their chain length and the level of unsaturation, making them crucial for many vital processes such as cell expansion, cell proliferation or differentiation (Bach and Faure 2010). Bach *et al*. (2008) showed that VLCFAs are essential for cell viability. High levels of VLCFAs in seed are known to suppress biosynthesis of cytokinins (Nobusawa *et al*. 2013a) which are crucial for pre- and early post-germination events (Letham and Bollard 1961; Miller 1961; Fosket *et al*. 1977). On the contrary, a lower VLCFA content in seed led to an increase of cytokinin level (Nobusawa *et al*. 2013b) which in turn countered the action of germination inhibitors (Khan 1971, 1975) and overcame ABA-suppressed seed germination (Wang *et al*. 2011). Based on these reports, it was apparent that a reduced level of VLCFAs in ST seed would favour early germination. Further, data presented in table 3 also showed a decrease in palmitic acid content coupled with an increase of linolenic acid in ST seed compared to healthy seed. The analogous changes in these two fatty acids in the embryonic axes of seeds have been correlated with faster germination in sunflower (Munshi *et al*. 2007). Accordingly, changes in the levels of linolenic acid, palmitic acid and VLCFAs in ST seeds in comparison with the healthy seed clearly showed the onset of pre-germination events in ST seed.

Further, measurement of the changes in the levels of seed hormones showed a significant increase in the levels of cytokinins and gibberellins coupled with a reduction in ABA content of seed of ST-affected fruit compared to H fruit (figure 5).It is a well known fact that gibberellins (GA) and cytokinins promote germination (Taylorson and Hendricks 1977; Nikolić *et al*. 2006) while, ABA is a potent inhibitor. Considering the changes in hormones and the activities of lipase (table 2), amylase and the respiratory enzymes (figure 2) in ST seed, it was evident that germination events had begun in ST seed. It was thus apparent that a fall in the VLCFA content of seed could have provided the primary trigger for the onset of pre-germination events in seed as reported by Munshi *et al*. (2007).

4.3 *Effect of seed germination on pulp characteristics*

Data presented in table 2 showed that the fat content, FFA and lipase activity in pulp of ST-affected fruit were lower compared to H fruit confirming that both the rate of synthesis and breakdown of fats were reduced in ST pulp. Lipase activity in pulp decreased rapidly with the increasing intensity of ST in pulp (figure 1). The levels of short and medium chain fatty acids, nonanoic acid, lauric acid and myristic acid increased in ST pulp, while levels of long chain fatty acids, palmitic acid, palmitoleic acid, stearic acid and oleic acid and the two VLCFAs, tetracosanoic acid and hexacosanoic acid, registered a decline. Among the changes occurring in the FFA levels, the increase in the contents of two long chain fatty acids, erucic acid and eicosanoic acid in ST pulp were of significance as these fatty acids are known to exhibit higher transition temperatures, leading to decreased membrane fluidity, permeability and stability of membrane (Bangham 1975). Additionally, another notable finding was that while linoleic acid registered a marginal decline in ST pulp, linolenic acid level dropped markedly. Consequently, the ratio of linolenic/linoleic acid in pulp reduced significantly, indicating a higher rate of lipid

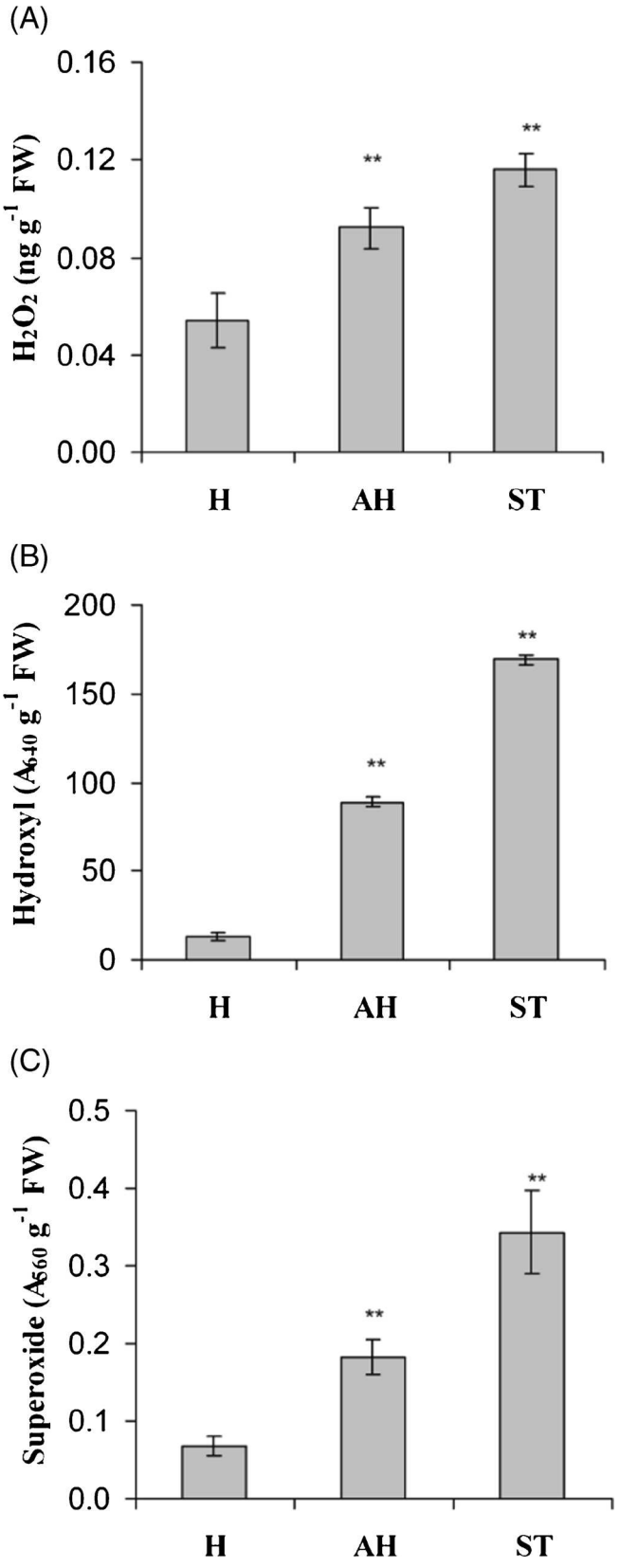

Figure 3. Pattern of free radical production in healthy, apparently healthy and ST-affected Alphonso mango pulp (**$P \leq 0.005$). ST-affected pulp, pulp surrounding the ST-affected portion remaining free of ST symptoms and pulp from healthy fruit were analysed. Note the significant increase in free radical production, particularly the hydroxyl radical in AH and ST pulp compared to H pulp coupled with a drastic reduction in antioxidant enzyme activities, indicating rapid destruction of mesocarp tissue during ST formation.

peroxidation. Earlier studies on Alphonso mango following seed germination concurrent with the climacteric ripening of fruit had indicated development of hypoxia in the pulp (Ravindra and Shivashankar 2004), which is also known to favour increased rate of lipid peroxidation (Blokhina *et al*. 2003).

4.4 *Generation of free radicals and lipid peroxidation of membranes*

The significantly higher levels of •OH and $O^{2\bullet-}$ radicals generated in pulp of AH and ST compared to healthy tissue (figure 3) are considered to be highly detrimental as excessive levels of these two free radicals have been implicated in the destruction of plant cells through peroxidation (DaCosta and Huang 2007). Hydroxyl radical is one of the most destructive free radicals responsible for modifications of macromolecules and cellular damage (Jiang Ming-Yi 1999). As the level of free radicals increase, the rate of oxidation also increases unless the activity of antioxidant enzymes increases proportionately. In a previous report by Nagamani *et al.* (2010), the activities of anti-oxidative enzymes such as, catalase (CAT), peroxidase (POX) and superoxide dismutase (SOD), which together constitute a mutually supportive defence system against ROS, were found to be lower in ST compared to healthy tissue of Alphonso mango. A reduction in the activities of anti-oxidative enzymes was reported under hypoxic conditions (Ushimaru *et al.* 1997) as observed in ST pulp by the increased accumulation of CO_2 (Ravindra and Shivashankar 2004). Considering the fact that 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, the greatly increased production of free radicals in ST pulp coupled with a reduction in the activities of anti-oxidative enzymes indicated increased peroxidation of lipids. Data presented in figure 4B showed that the level of MDA in ST pulp increased markedly compared to AH and H tissue.MDA is the end product of peroxidative decomposition of polyenic fatty acids in the lipid peroxidation process and its accumulation in tissues is indicative of the extent of lipid peroxidation. It was thus evident from the data that the production of a disproportionately higher level of free radicals coupled with lower activities of anti-oxidative enzymes

Table 3. Fatty acid composition of healthy (H) and spongy-tissue-affected (ST) pulp of Alphonso mango fruit

Fatty acid	Seed (mg/100 g)			Pulp (mg/100 g)		
	Healthy	Spongy	*t*-Value	Healthy	Spongy	*t*-Value
Nonanoic acid	2.37±0.12	2.77±0.23	−1.58	0.25±0.01	2.01±0.16	−25.57
Lauric acid	4.11±0.15	4.97±0.17	−3.62	0.99±0.09	6.28±0.37	−27.32
Myristic acid	0.48±0.07	0.35±0.07	1.50	0.21±0.01	0.29±0.10	−2.23
Palmitic acid	143.0±1.15	106.4±1.89	16.45	76.6±1.59	58.7±1.98	24.28
Palmitoleic acid	82.7±1.87	47.6±1.42	14.81	36.4±1.49	34.6±2.61	1.83
Stearic acid	46.3±1.97	33.4±0.88	5.93	32.7±1.15	26.9±2.78	4.74
Oleic acid	25.5±1.72	19.1±1.20	3.01	17.4±0.50	11.3±1.57	11.35
Linoleic acid[1]	8.73±1.14	7.48±1.02	0.85	3.99±0.36	2.67±0.31	6.81
Linolenic acid[2]	0.10 ±0.03	0.57±0.11	−3.98	2.48±0.18	0.16±0.03	30.13
Eicosanoic acid	1.79 ±0.16	1.84±0.17	−0.17	3.39±0.35	3.94±0.12	−4.17
Erucic acid	2.20±0.23	1.64±0.16	2.01	1.91±0.27	3.56±0.42	−9.67
Behenic acid	2.02±0.18	1.82±0.18	0.99	1.89±0.28	1.89±0.22	0
Tetracosanoic acid[3]	6.02±0.29	3.62±0.33	5.50	8.17±0.29	4.92±0.36	19.52
Hexacosanoic acid[4]	1.04±0.20	0.88±0.15	0.69	7.14±0.29	0.69±0.20	52.87

[1,2] Note the marginal reduction in linoleic acid content and a significant reduction in the linolenic acid content of the ST seed which accounted for a major change in the ratio of linolenic acid to linoleic acid of the ST seed compared to H seed representing membrane injury in ST seed.

[3,4] Note the marked reduction in the levels of the two VLCFA in ST seed triggering cytokinin biosynthesis and seed germination.

(Nagamani *et al.* 2010) contributed to a large accumulation of malondialdehyde leading to increased rate of lipid peroxidation in AH and ST pulp. Increase in lipid peroxidation has been reported during leaf senescence, ozone injury, anoxia, drought stress and wounding (Kepler and Novacky 1986).

4.5 *Changes in the linolenic/linoleic acid ratio and membrane damage*

As a consequence of increased lipid peroxidation, the ratio of linolenic/linoleic acid dropped significantly in ST pulp (figure 4A). The ratio of the two fatty acids, linolenic acid and linoleic acid is considered to be critical for membrane structure and function. α-linolenic acid is reported to be 10 times more susceptible to oxidation than linoleic acid and, therefore, readily undergoes conversion to the unstable hydroperoxides. Accordingly, it was evident that the drastic fall by over 90% in the ratio of linolenic/linoleic acid in ST pulp, strongly signified the breakdown of membrane structure and a loss of membrane fluidity (Girotti 1990). The free fatty acids liberated during ST development in the pulp also act as substrates for lipid peroxidation and as uncouplers of mitochondrial electron transport chain (Skulachev 1998), thus accelerating the pace of cell damage and death. Increased lipid peroxidation is reported to lead to an increase of electrolyte leakage (Kepler and Novacky 1986) due to irreversible alteration to cell membranes (Dhindsa *et al.* 1981). In the present study, this was evident from the increased EC of ST pulp (0.76) compared to AH (0.61) and H pulp (0.54) while the pH was lower (4.3) compared to AH (4.9) and H pulp (5.4) (figure 4). Accordingly, it was apparent that increased lipid peroxidation in ST pulp led to loss of cell membrane integrity resulting in leakage of cellular contents. An acidic environment was thus created reducing the pH of the pulp, which, in turn, progressively damaged the surrounding tissues due to osmolysis, leading to death of cells. Scandalios (1993) reported that leakage of cellular contents following peroxidation of plasmalemma leads to rapid desiccation and cell death. Light microscopic observations by Raymond *et al*. (1998) showing extensive disintegration of walls of most cells in 'Tommy Atkins' and 'Van Dyke' mango fruits affected by jelly seed, a disorder similar to spongy tissue, is in agreement with our findings and supports the results of the present study. It appears likely that the manifestation of large air spaces and starch grains interspersed among the mass of broken cell walls in the advanced stage of the spongy tissue disorder could occur, possibly, by a reduction in the activity of amylase in pulp following the death of cells.

Summing up, the present results have established that a greatly increased competition among fruits for assimilate supply at 70% fruit maturity significantly decreased the rate of fat synthesis in both pulp and seed of weak sinks.

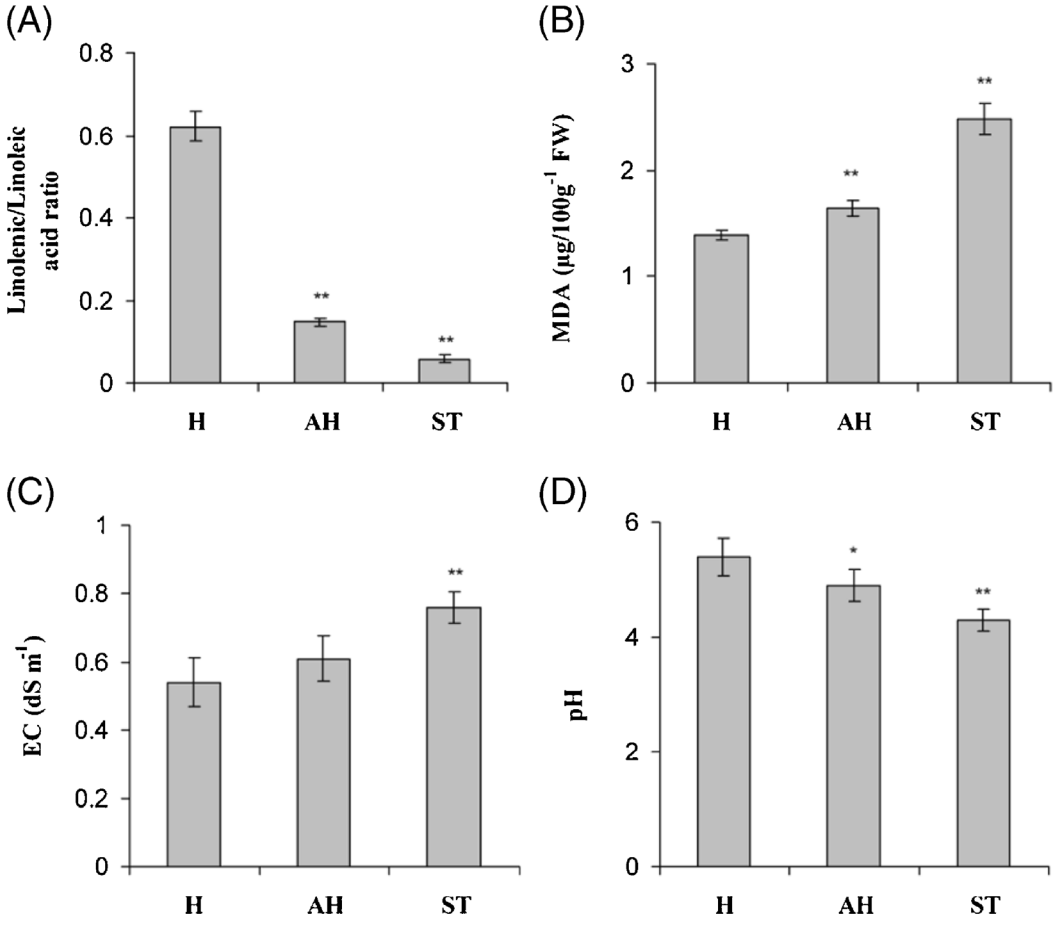

Figure 4. Changes in linolenic/linoleic acid ratio (**A**), MDA content (**B**), EC (**C**) and pH (**D**) of healthy (H), apparently healthy (AH) and ST-affected Alphonso mango pulp (*$P \leq 0.05$,**$P \leq 0.005$). ST-affected pulp, pulp surrounding the ST-affected portion remaining free of ST symptoms and pulp from healthy fruit were analyzed. Note the drastic fall in the ratio of linolenic acid to linoleic acid, increased accumilation of MDA associated with an increase in the EC. and a fall in the pH of the ST pulp compared to H pulp signifying membrane damage in ST pulp.

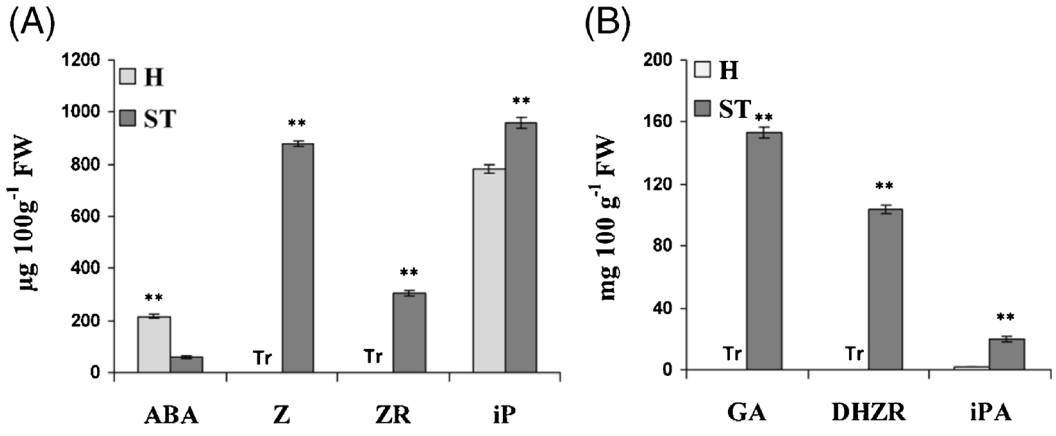

Figure 5. Levels of ABA, Z, ZR and iP (**A**) and GA, DHZR and iPA (**B**) in seed of healthy and ST-affected Alphonso mango fruit (**$P \leq 0.005$). Tr refers to traces. Seed hormones in healthy and ST fruit were analysed by HPLC. Note the rapid increase in the levels of the cytokinins (Z, ZR, iP, DHZR and iPA), gibberellin (GA) and a drop in the levels of ABA in ST seed compared to H seed signifying the initiation of seed germination events in ST seed.

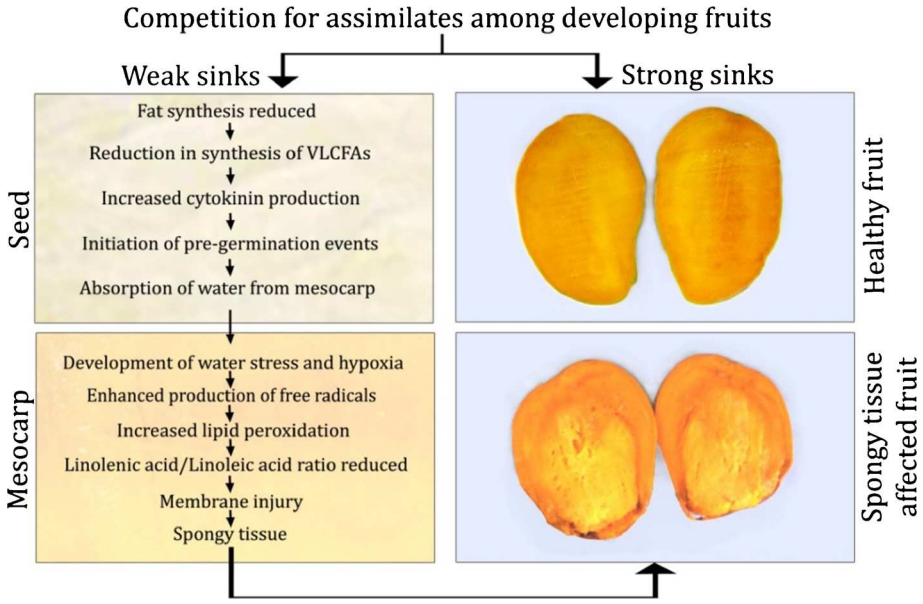

Figure 6. Proposed biochemical sequence of events leading to spongy tissue development in Alphonso mango fruit. The reduced rate of synthesis of VLCFAs in weak sinks leads to cytokinin production, which triggers seed germination followed by subsequent changes in pulp culminating in the formation of spongy tissue.

Following this, the changes occurring in the profile of fatty acids and the reduced synthesis of the two VLCFAs, tetracosanoic acid and hexacosanoic acid, in seed led to a rise in the level of cytokinin, resulting in initiation of seed germination events in the maturing fruit. The sustained flow of pulp moisture to the germinating seed created water-deficit stress in the pulp followed by hypoxia leading to the excessive production of free radicals (Chirkova *et al*. 1998). Free radicals then acted upon the fatty acid components of cell membranes, leading to peroxidation of unsaturated fatty acids. In this process, linolenic acid was more rapidly degraded compared to linoleic acid, and as a result, the ratio of linolenic/linoleic acid was significantly reduced, thus decreasing membrane fluidity. This resulted in membrane injury, tissue damage (Porter *et al*. 1995; Gutteridge 1995) and cell death, eventually leading to spongy tissue formation (figure 6). Thus, the present study has shown, for the first time, that a reduced synthesis of VLCFAs in the seed during the pre-harvest fruit growth phase may provide the primary trigger for seed germination as demonstrated in *Arabidopsis* by Nobusawa *et al.* (2013b), thus setting in motion the initiation of spongy tissue development in the Alphonso mango fruit. Incidentally, reduced synthesis of VLCFAs in some seeds may also help explain the occurrence of vivipary in Alphonso mango.

Acknowledgements

We thank the Director, Indian Institute of Horticultural Research, Bangalore for providing access to facilities. Grateful thanks are due to Dr KK Upreti, Principal Scientist, and Mr HL Jayaram, Chief Technical Officer, for help in the analysis of seed hormones.

References

Abou-Aziz AB, Rizk AM, Hammouda FM and El-Tanahy MM 1973 Seasonal changes of lipids and fatty acids in two varieties of avocado pear fruits. *Plant. Mater. Veg. Qual.* **XXII** 253–259

Bach L and Faure JD 2010 Role of very-long-chain fatty acids in plant development, when chain length does matter. *C. R. Biol.* **333** 361–370

Bach L, Michaelson L, Haslam R, Bellec Y, Gissot L, Marion J, Da Costa M, Boutin J-P, *et al*. 2008 The plant very long chain hydroxy fatty acyl-CoA dehydratase PASTICCINO2 is essential and limiting for plant development. *Proc. Natl. Acad. Sci. USA* **105** 14727–14731

Bangham AD 1975 Models of cell membranes. In cell membranes: Biochemistry, cell biology and pathology, Hospital Practice, New York. *Nature* **247** 438–441

Baqui SM, Mattoo AK and Modi VV 1974 Mitochondrial enzymes in mango fruit during ripening. *Phytochem.* **13** 2049–2055

Bernfeld P 1955 Amylases; in *Methods in enzymology, Vol I* (eds) NO Kaplan and SP Colowick (NY, USA: Academic Press) pp 149–158

Bertin N 1995 Competition for assimilates and fruit position affect fruit set in indeterminate greenhouse tomato. *Ann. Bot.* **75** 55–65

Blokhina O, Violainen E and Fagerstedt KV 2003 Antioxidants, oxidative damage and oxygen deprivation stress: a review. *Ann. Bot.* **91** 179–194

Bustan A, Goldscmidt EE and ErnerY 1996 Carbohydrate supply and demand during fruit development in relation to productivity of grapefruit and "Murcott" mandarin. *Proc 4th Intl. Sym. Comp. Mod. fruit research ISHS* (ed) R Habib and P H Blaise. *Acta Hort.* **416** 23–26

Chirkova TV, Novitskaya LO and Blokhina OB 1998 Lipid peroxidation and antioxidant systems under anoxia in plants differing in their tolerance to oxygen deficiency. *Russ. J. Plant Physiol.* **45** 55–62

DaCosta M and Huang B 2007 Changes in antioxidant enzyme activities and lipid peroxidation for bentgrass species in response to drought. *J. Am. Soc. Hortic. Sci.* **132** 319–326

Dhindsa RS, Plump-Dhindsa P and Thorpe TA 1981 Leaf senescence: correlated with increased levels of membrane permeability and lipid peroxidation, and decreased levels of superoxide dismutase and catalase. *J. Exp. Bot.* **32** 93–101

Doke N 1983 Involvement of superoxide anion generation in the hypersensitive response of potato tuber tissues to infection with an incompatible race of *Phytophthorainfestans* and to the hyphal wall components. *Physiol. Plant Pathol.* **23** 345–357

Draper HH and Hadley M 1990 Malondialdehyde determination as index of lipid peroxidation. *Methods Enzymol.* **186** 421–431

Folch J, Lees M and Stanley GSH 1957 A simple method for the isolation and purification of lipids in animal tissues. *J. Biol. Chem.* **226** 497–509

Fosket DE, Volk MJ and Goldsmith MR 1977 Polyribosome formation in relation to cytokinin induced cell division in suspension culture of *Glycine max* (L.) Merr. *Plant Physiol.* **60** 554–562

Girotti AW 1990 Photodynamic lipid peroxidation in biological systems. *Photochem. Photobiol.* **51** 497–509

Gutteridge JM 1995 Lipid peroxidation and antioxidants as biomarkers of tissue damage. *Clin. Chem.* **41** 1819–1828

Jayaraman J 1981 *Laboratory manual in biochemistry* (New Delhi: Wiley Eastern Ltd)

Ming-Yi J 1999 Generation of hydroxyl radicals and its relation to cellular oxidative damage in plants subjected to water stress. *Acta Bot. Sin.* **41** 229–234

Katrodia JS 1979 The study into the cause of the development of spongy tissue in mango (*Mangiferaindica* L.) fruit of cultivar Alphonso. Ph.D. Thesis, Marathawada Agricultural University, Parbhani

Kelen M, Demiralay EC and Ozakan SG 2004 Separation of abscisic acid, indole-3-acetic acid, gibberellic acid in 99 R (*Vitisberlandieri* × *Vitisrupestris*) and rose oil (*Rosa damascene* Mill.) by reversed-phase liquid chromatography. *Turk. J. Chem.* **28** 603–610

Kepler LD and Novacky A 1986 Involvement of lipid peroxidation in the development of a bacterially induced hypersensitive reaction. *Phytopathol.* **76** 104–108

Khan AA 1971 Cytokinins: permissive role in seed germination. *Science* **171** 853–859

Khan AA 1975 Primary, preventive and permissive roles of hormones in plant systems. *Bot. Rev.* **41** 391–420

Kikuta Y 1969 Lipid metabolism in the fruit of *Perseaamericana* Mill. III. Lipogenesis and complex lipid synthesis. *J. Facul. Agr. Hokkaido Univ. Sapporo* **56** 2–6

Letham DS and Bollard EG 1961 Stimulants of cell division in developing fruits. *Nature* **191** 1119–1120

Liu K 1994 Preparation of fatty acid methyl esters for gas chromatographic analysis of lipids in biological materials. *J. Am. Oil Chem. Soc.* **71** 1179–1187

Marcelis LFM 1994 A simulation model for dry matter partitioning in cucumber. *Ann. Bot.* **74** 43–52

Marcelis LFM 1996 Sink strength as a determinant of dry matter partitioning in the whole plant. *J. Exp. Bot.* **47** 1281–1291

Miller CO 1961 A kinetin-like compound in maize. *Proc. Natl. Acad. Sci. USA* **47** 170–174

Morrison WR and Smith LM 1964 Preparation of fatty acid methyl esters and dimethyl acetals from lipids with boron-fluoride-methanol. *J. Lipid Res.* **5** 600–608

Munshi SK, Sandhu S and Sharma S 2007 Lipid composition in fast and slow germinating sunflower (*Helianthus annuus* L) seeds. *Gen. Appl. Pl. Physiol.* **33** 235–246

Nagamani JE, Shivashankara KS and Roy TK 2010 Role of oxidative stress and the activity of ethylene biosynthetic enzymes on the formation of spongy tissue in 'Alphonso' mango. *J. Food Sci. Technol.* **47** 295–299

Nikolić R, Mitić N and Miletić R 2006 Effects of cytokinins on *in vitro* seed germination and early seedling morphogenesis in *Lotus corniculatus* L. *J. Plant Growth Regul.* **25** 187–194

Nobusawa T, Okushima Y, Nagata N, Kojima M and Sakakibara H 2013a Synthesis of very-long-chain fatty acids in the epidermis controls plant organ growth by restricting cell proliferation. *PLoS Biol.* **11** e1001531. doi:10.1371/journal.pbio.1001531

Nobusawa T, Okushima Y, Nagata N, Kojima M, Sakakibara H and Umed M 2013b Restriction of cell proliferation in internal tissues via the synthesis of very-long-chain fatty acids in the epidermis. *Plant Signal. Behav.* **8** e25232

Osborne DR and Voogt P 1978 The analysis of nutrients in foods (Academic Press) pp 155-156

Panse VG and Sukhatme PV 1978 *Statistical methods for agricultural workers* (New Delhi: ICAR). 108 pp

Porter NA, Caldwell SE and Mills KA 1995 Mechanisms of free radical oxidation of unsaturated lipids. *Lipids* **30** 277–290

Ravindra V and Shivashankar S 2004 Spongy tissue in Alphonso mango I. Significance of *in situ* seed germination events. *Curr. Sci.* **87** 1045–1049

Ravindra V and Shivashankar S 2006 Spongy tissue in Alphonso mango II. A key evidence for the causative role of seed. *Curr. Sci.* **91** 1712–1714

Rawsthorne S 2002 Carbon flux and fatty acid synthesis in plants. *Prog. Lipid Res.* **41** 182–196

Raymond L, Schaffer B, Brecht JK and Crane JH 1998 Internal breakdown in mango fruit: symptomology and histology of jelly seed, soft nose and stem end cavity. *Postharvest Biol. Technol.* **13** 59–70

Scandalios JG 1993 Oxygen stress and superoxide dismutases. *Plant Physiol.* **101** 7–12

Schopfer P, Plachy C and Frahry G 2001 Release of reactive oxygen intermediates (superoxide radicals, hydrogen peroxide and hydroxyl radicals) and peroxidase in germinating radish seeds controlled by light, gibberellin, and abscisic acid. *Plant Physiol.* **125** 1591–1602

Selvaraj Y, Pal DK, Singh R and Roy TK 1995 Biochemistry of uneven ripening in Gulabi grape. *J. Food Biochem.* **18** 325–340

Selvaraj Y 1996 "Research on the biochemistry of tropical fruits at IIHR", Tech Bulletin, Indian Institute of Horticultural Research, Bangalore. 122 pp

Shivashankar S 2014 Physiological disorders of mango fruit. *Hortic. Rev.* **42** 313–347

Shivashankar S, Ravindra V and Louis L 2007 Biochemical changes in seed and mesocarp of mango (*Mangiferaindica* L.) cv. 'Alphonso' and their significance during the development of spongy tissue. *J. Hortic. Sci. Biotechnol.* **82** 35–40

Skulachev VP 1998 Cytochrome c in the apoptotic and antioxidant cascades. *FEBS Lett.* **423** 275–280

Taylorson RB and Hendricks SB 1977 Dormancy in seeds. *Annu. Rev. Plant Physiol.* **28** 331–354

Ushimaru T, Maki Y, Sano S, Koshiba K, Asada K and Tsuji H 1997 Induction of enzymes involved in the ascorbate dependent anti oxidative system, namely ascorbate peroxidase, monodehydroascorbate reductase and dehydroascorbate reductase after exposure to air of rice (*Oryza sativa*) seedlings germinated under water. *Plant Cell Physiol.* **38** 541–549

Von Tiedemann A 1997 Evidence for a primary role of active oxygen species in induction of host cell death during infection of bean leaves with *Botrytis cinerea. Physiol. Mol. Plant Pathol.* **50** 151–166

Wainwright H and Burbage MB 1989 Physiological disorders in mango (*Mangifera indica* L.) fruit. *J. Hortic. Sci.* **64** 125–135

Wang Y, Li L, Ye T, Zhao S and Liu Z 2011 Cytokinin antagonizes ABA suppression to seed germination of *Arabidopsis* by downregulating ABI5 expression. *Plant J.* **68** 249–261

MS received 12 August 2014; accepted 24 February 2015

Corresponding editor: Man Mohan Johri