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Biochemical Changes Induced due to *Staphylococcal* Infection in Spongy Alphonso Mango (*Mangifera indica* L.) Fruits

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Abstract

Spongy Alphonso mangoes were found to be infected with *Staphylococcus* bacteria. A Gram positive *Staphylococcus* strain was isolated from spongy pulp and identified from CABI Bioscience, UK, by partial 16S rDNA sequence analysis and by morphological and biochemical characterization through IMTECH, Chandigarh, India. Although identification by both of these methods indicated the organism belonged to same genus, different species names were given. Changes in total phenolics, reducing, and non-reducing sugars, respiration rate, total carotenoids, peroxidase (POX), and catalase activities were monitored during ripening of these fruits. The climacteric rise in spongy fruits was marked by an increase in respiration rate and a decrease in sugar content. Total phenolics content increased in spongy fruits as compared to ripe non-spongy fruits. Development of corky white tissue in spongy fruits was associated with about a 2.5-fold reduction in total carotenoids and a concomitant increase in lipoxygenase-mediated, β-carotene co-oxidation. A marked decrease in soluble protein content and about a 1.5-fold increase in POX activity was observed. Maximum POX activity was confined to 50-70% $(NH_4)_2SO_4$ fraction. The intense dark bands visible after POX specific substrate staining of the Native gel indicated a high expression of isoenzymes of POX in spongy fruits. Similarly, changes in levels of catalase activity were also observed in spongy fruits. The results suggest that infection of Alphonso mangoes with *Staphylococcus* bacteria affects the normal ripening processes of the fruit interfering with the carbohydrate and carotenoid metabolism. Also, the studies indicate the expression of POX and catalase enzymes as a plant defense response to microbial invasion.

Key words: Fruits, internal physiological disorder, *Mangifera Indica* L., spongy tissue, *Staphylococcus*, X-ray imaging

Introduction

The mango (*Mangifera indica L.*) is a major fruit crop cultivated in India with an annual production of over 12 million tons. Alphonso, the most delicious variety known for its excellent texture, taste, and aroma, accounts for nearly 60% of the mango export trade from India. Besides being a luscious and exotic-flavored fruit, mango is a rich source of provitamin A (carotenoids) and vitamin C. However, the export trade is plagued by the incidence of a physiological disorder known as 'spongy tissue' (internal breakdown). Fruits affected by this disorder do not show any external symptoms and the malady is detected only after cutting the fruits open or by the non-destructive X-ray imaging technique (Thomas et al. 1993). Diseased fruits are distinguished by soft texture, pale yellow/white color, spongy or leathery tissue associated with an unacceptable off flavor with or without air pockets in certain regions of the mesocarp (pulp) adjacent to the endocarp (stone). Symptoms increase at the ripe stage, associated with blackening of the affected pulp. Spongy tissue occurrence is more prevalent in the coastal Konkan region of Maharashtra, and about 30% of the produce is lost due to this disorder.

Although several investigations carried out in the past relate this disorder to factors as diverse as ecological, nutritional, environmental and physiological (Gupta et al. 1985; Katrodia 1988; Raymond et al. 1998), the actual causative agent has not been satisfactorily determined. Chemical and biochemical studies have shown many compositional and metabolic differences between the healthy and the damaged tissue (Lima et al. 1999; 2001). A recent report by Ravindra and Shivashankar (2004) suggests a shift of the seed in germination mode as a causative factor of spongy tissue. Another report has shown differential expression of some of the enzymes in the spongy tissue-affected fruits (Vasanthaiah et al. 2006). These detailed studies also are

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mainly confined to measuring the effects and the foolproof evidence for the causative agent of this malady is still lacking.

Attempts were made to check whether spongy tissue development in mangoes could be due to microbial infection. In the present paper, I report isolation of a Gram-positive *Staphylococcus* strain from spongy pulp of Alphonso mangoes. The isolation of this bacterium from spongy pulp generated renewed interest in studying biochemical changes as an after effect of *Staphylococcus* infection. As the spongy pulp showed air pocket formation, pale yellow pulp color, and blackening of the pulp upon ripening, attempts were made to study the biochemical changes associated with these symptoms. In the present paper, studies on changes in sugars, respiration pattern, phenolics content, total carotenoids, and changes in POX and catalase levels induced due to *Staphylococcus* infection are reported.

Materials and Methods

Plant material

Mature unripe spongy and non-spongy mango fruits, cv. Alphonso, were purchased within two days of harvest from a commercial market and allowed to ripen at 28-30 °C. Internal flesh breakdown in fruits was detected by X-ray imaging machine (ECIL XBS 9050S). Almost 90% correlation was observed in fruits detected as spongy by X-ray imaging and after cutting of fruits. Pulp samples from healthy and damaged fruits were collected at four ripening stages: unripe, non spongy-ripe, spongy-white and spongy-black and used immediately for microbial isolation. For changes in biochemical studies, samples were either used immediately or kept frozen in liquid nitrogen and stored at -20 °C until further use.

Isolation of the bacteria

Fruits were surface sterilized with ethanol and cut open in a laminar flow. Pulp (10 g) from each healthy fruit and the white corky tissue from spongy fruits was transferred to 90 ml sterile BHI broth (Brain Heart Infusion, HIMEDIA) and homogenized in a sterile Omni mixer cup. One ml homogenate after serial dilution in 0.9% sterile saline was pour plated on PDA (Potato Dextrose Agar) and PCA (Plate Count Agar) plates. PDA plates were prepared containing tartaric acid so as to attain the pH 3.5, thus arresting bacterial growth. PDA plates were incubated at room temperature (28-30 °C) and PCA plates at 37 °C. On the PCA plates, tiny creamy white colonies appeared within 24 h. The total bacterial load was determined per g of pulp. Single colony was subcultured on fresh PCA plates and the pure culture was sent for identification to species level to CABI Bioscience, UK, by partial 16S rDNA sequence analysis and to IMTECH, Chandigarh, India, for morphological, physical and biochemical characterization.

Total phenolics

For estimation of total phenolics, 20 g pulp tissue was homogenized with 80% ethanol in an Omni mixer and centrifuged at 20,000 g for 15 min at 4 °C using Heltich Zentrifugen centrifuge. Total phenolics content in the supernatant was determined by Folin Ciocalteau reagent (Spanos and Wrolstad 1990). The concentration of total phenolics was calculated by comparing the data with the standard graph of gallic acid (0 to 50 μg) and expressed as mg gallic acid equivalents/100 g pulp.

Reducing and non-reducing sugars

For estimation of total sugars, 20 g pulp tissue was transferred to cellulose thimbles and extracted with 80% ethanol on Soxhlet extraction apparatus. After 8-10 h extraction, the ethanol was removed by boiling and aqueous layer was made to a known volume. Reducing sugars and non-reducing sugars were estimated by Nelson-Somoyogi method (Nelson 1944). For non-reducing sugars, the aqueous extract was hydrolyzed with 1:1 HCl overnight (Janave and Sharma 2005) and aliquot after neutralization was used for total sugar estimation as above. The sugar content was expressed as mg glucose/100 g pulp after generating a standard graph for glucose (0 to 500 μg).

Respiration rate

Four fruits of identical maturity from the diseased and healthy fruits were selected after scanning through X-ray imaging machine. The respiration experiment was carried out using one single fruit at a time kept in a dessicator attached to respiratory assembly and connected to a peristaltic pump operated at constant pressure. Respiration rate was determined by trapping the CO_2 evolved by the fruits in a Pattenkoffer tube containing 75 ml of 0.1 N $Ba(OH)_2$ solution and calculating the amount of 0.1 N HCl required to titrate the unused $Ba(OH)_2$ (Thomas et al. 1971). For the control, the experiment was run without fruits for the same time as above. When the experiment was terminated, the fruits were cut open to confirm if they were really non-spongy or spongy. The respiration rate was expressed as mg CO_2 kg^{-1} fruit h^{-1}.

Total carotenoids

Pulp tissue (20 g) at various stages: unripe, ripe non-spongy, spongy-white, and spongy-black was homogenized with 75 ml of 75:60 acetone/hexane mixture in an Omni mixer and vacuum filtered (Janave and Sharma 2004a; Janave and Thomas 1979). The residue was repeatedly extracted with acetone/hexane mixture until the filtrate was colorless. The filtrate was transferred to a separating funnel and diluted excessively with distilled water. The contents were shaken and allowed to separate into two layers. The hexane epiphase was removed and the lower phase was again extracted with 20 ml of hexane. The hexane layers were pooled, washed with water to remove traces of acetone, and dried over anhydrous sodium sulfate overnight. The hexane layer was made to a known volume and an aliquot was evaporated by passing a stream of N_2 and then transferred to a known volume of acetone. Absorbance readings at 470, 661.6, and 644.8 nm were recorded and total carotenoid content was determined by employing the formulas of Lichtenthaler (1987).

Enzyme extraction

20 g fresh pulp was powdered in liquid nitrogen and homoge-

nized in 0.05 M potassium phosphate buffer, pH 6.5 (buffer A), containing 2% PVPP (polyvinylpolypyrrolidone), 0.01% ascorbic acid, and 0.01% PMSF (Phenylmethanesulfonyl fluoride). PVPP was allowed to hydrate in the buffer for at least 3 h prior to extraction. The frozen mass was allowed to thaw and passed through two layers of muslin cloth. The filtrate was centrifuged at 32,700-g for 15 min. The clear supernatant was used as crude enzyme. Soluble protein content was determined by Branford's dye-binding assay (Bradford 1976) using bovine serum albumin (BSA) as a standard.

Acetone precipitation and $(NH_4)_2SO_4$ fractionation

To the crude enzyme, three volumes of chilled acetone were added and the precipitated proteins were sedimented by centrifugation at 32,700-g for 15 min as described above. The pellet was dissolved in buffer A and re-centrifuged as above. The supernatant was used for $(NH_4)_2SO_4$ fractionation.

The acetone purified enzyme was fractionated into 0-20%, 20-50% and 50-70% $(NH_4)_2SO_4$ fractions. The pellets after centrifugation at 32,700-g for 15 min were dissolved in buffer A and dialyzed against the same buffer overnight with three changes of the dialysate buffer. After dialysis, the enzyme fractions were again centrifuged and tested for POX activity and protein content.

β-carotene co-oxidation

Lipoxygenase mediated *β*-carotene co-oxidation was carried out by measuring the change in absorbance of *β*-carotene at 455 nm (Ben-Aziz et al. 1971). *β*-carotene was extracted from carrots in 75:60 acetone/hexane mixture and the hexane layer was separated as detailed in total carotenoids extraction. The hexane layer after concentration was subjected to silica gel TLC in petroleum ether/iso-propanol/water (100/12/0.5, v/v) (Janave 1997). *β*-carotene band (Rf. 0.89) was eluted with acetone and an aliquot was made into an aqueous solution by adding 0.5 ml Twin 20. The solvent was removed by passing a stream of N_2 and the residue was dissolved in 0.05 M sodium phosphate-citrate buffer, pH 6.0 (buffer B), containing 0.25% ethylenediaminetetraacetic acid (EDTA) (Bhushan and Thomas 1990). For *β*-carotene co-oxidation assay, one ml reaction mixture contained 0.05 M buffer B, 0.5 mM linoleic acid, 0.3 ml of aqueous *β*-carotene, and 0.2 ml of acetone fractionated enzyme, and decrease in absorbance at 455 nm with time was recorded. The blank contained all the reaction mixture components except linoleic acid. The activity was expressed as $\wedge A_{455}$ nm/min/mg protein.

POX Assay and activity staining

POX activity was determined by using *o*-dianisidine as substrate (Mica and Lüthje 2003). One ml reaction mixture contained 0.05 M sodium phosphate-citric acid buffer pH 5, 0.03% H_2O_2, 0.25 mM *o*-dianisidine, and enzyme protein. The reaction was followed at 460 nm with time and the initial rate was determined. The activity was expressed as $\wedge A_{460}$ nm/min/mg protein. Native PAGE was carried out in the presence of Triton X-100 as detailed in a previous paper (Janave and Sharma 2004b). About 20 μg proteins from each sample were loaded and electrophoresis under undenaturing conditions was carried out at 2-4 °C and at a current of 15 mA/gel. After electrophoresis, the gels were first stained with 5 mM *o*-dianisidine in 0.05 M citrate-phosphate buffer, pH 5.0 for 30 min. Then H_2O_2 was added at the final concentration of 0.03%. After POX bands appeared, the gels were scanned and then stabilized with Coumassie Blue staining.

Catalase assay

The catalase activity was determined by following the change in absorbance of H_2O_2 at ^240 nm with time (Beers and Sizer 1952). One ml reaction mixture contained 0.05 M potassium phosphate buffer, pH 6.5, 0.03% H_2O_2, and enzyme fraction. The reaction was followed up to 10 min or until constant rate was obtained. The enzyme activity is expressed as îA_{240} nm/min/mg protein.

Results and Discussion

Spongy tissue characteristics of the fruit

The fruits affected with spongy tissue did not show any external symptoms of internal pulp spoilage (Fig. 1A). These fruits could be detected as spongy only after passing through an X-ray imaging machine (Fig. 1B) or after cutting (Fig. 1C). As seen in the Figure (Fig. 1B), the X-ray image of spongy fruits showed white patches throughout the internal flesh due to a difference in density. When the fruit was cut open, a white unripe pulp affected by spongy tissue was found firmly adhered to the seed epicoat surrounded by the ripe brown yellow pulp. As ripening progressed further, the white pulp became soft and the ripe pulp turned brownish black with air cavities (Fig. 1D). The development of black color indicated changes in phenolics content and either POX or polyphenol oxidase (PPO) enzymes.

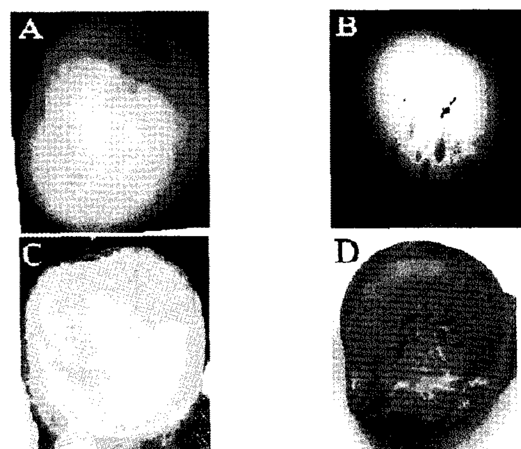

Fig. 1. Symptoms of spongy tissue development in Alphonso mango fruits: A. Spongy uncut fruit, B. X-ray image of Spongy fruit, C. Spongy cut fruit and D. Spongy fruit with air cavities.

Bacteria isolation and characterization

When pour plated on PCA plates, and incubated at 37 °C, tiny colonies appeared within 24 h in the homogenate from spongy pulp. Further sub-culturing resulted in pure creamy white colonies. These colonies were smooth, raised, circular, glistering, and entire of size 0.5-0.8 °m in diameter. The organism was catalase positive and Gram-positive and after staining and viewing under a microscope, showed the shape of Gram-positive bunch of grapes belonging to the genus *Staphylococcus* (Part of this work was presented in the "47th Annual Meeting of the Association of Microbiologists of India", Janave 2006a). After serial dilution and pour plating on PCA plates, the bacterial load was found to be 1.38 X 10^5 cfu/g pulp. No any other bacteria were detected apart from the one reported above in spongy fruits, indicating the absence of any other microorganisms. Also, no growth was observed on PDA plates prepared containing tartaric acid indicating the absence of fungus or mold in spongy pulp. Non-spongy pulp did not show any growth either on PDA or PCA plates, and the organism was not detected by staining. The presence of this bacterium only in spongy pulp indicates that it could be associated with the development of spongy tissue in Alphonso mangoes.

The organism was identified to species level from IMTECH, and CABI Bioscience. The identification, based on biochemical and morphological characteristics, indicated it belonged to *S. xylosus* (Accession No. MTCC 7441). Identification by partial 16S rDNA sequence analysis showed the same genus (Accession No. IMI 393389), but indicated it belonged to *S. epidermidis*. The sequence homology of 483 nucleotides was compared with different strains from the EM-PRO database and most of the top 50 sequence matches showed >-99.79% identity to *S. epidermidis* (data not presented). *S. xylosus*, identified by biochemical and morphological characteristics, did not appear at all in the top 50 matches. Identification of this organism from two different sources although confirmed the genus as *Staphylococcus*, but differed in species identification. Hence, at present the correct species name cannot be positively confirmed. Further studies are in progress to identify this organism to nearest correct species by further DNA sequencing.

Total phenolics, sugar and soluble protein content

Spongy tissue development in Alphonso mangoes was associated with various biochemical changes (Part of this work was presented in BARC Golden Jubilee & DAE-BRNS Life Sciences Symposium on "Trends in Research and Technologies in Agriculture and Food Sciences", Janave 2006b). The total phenolics content of Alphonso fruits during ripening and in the spongy tissue-affected fruits is reported in Table 1. As the fruits ripened, the initial total phenolics content of unripe non-spongy fruits decreased by about 30%. In spongy-white pulp, the phenolics content decreased still further. However, as the spongy tissue-affected pulp turned brownish black, the levels increased by about 16% over that in the non-spongy ripe fruits. Gupta et al. (1985) also found higher phenolics content in spongy pulp than in the healthy fruits. A four-fold increase in reducing sugar content was observed in ripe non-spongy fruits over that in the unripe non-spongy fruits. The total sugar content also increased in ripe non-spongy fruits. However, in spongy-white pulp, a decrease in both reducing and non-reducing sugars was observed (Table 1). At the spongy-black stage, the levels of both reducing and non-reducing sugars showed a slight recovery due to the unripe spongy-white pulp turned ripe, but the levels were comparatively lower than that at the ripe non-spongy stage. Katrodia (1988) and Lima et al. (2001) also observed an appreciable reduction in reducing and non-reducing sugars in the damaged fruits. The reduction in sugar content could be attributed to a lower invertase activity and an increase in amylase activity in spongy tissue-affected fruits (Lima et al. 2001). In unripe fruits, the initial soluble protein content was comparatively low and as the fruits turned fully ripe, a several-fold increase in protein content was observed (Table 1) due to the synthesis of ripening-associated proteins. On the contrary, in the spongy-white pulp, a ca. 70% decrease in protein content was observed. This could be due to the fact that the affected pulp was unripe and ripening-associated protein synthesis was affected. As the spongy-white pulp turned black, the protein content increased over that in the spongy-white stage, however, the values were about 50% less than those at the ripe non-spongy stage. The lower levels of protein could be due to the utilization of protein by the *Staphylococcus* bacteria observed in spongy pulp.

Changes in respiration rate

Continuous loss in fruit weight was observed from the day of harvest to the fully ripe stage (Fig. 2A). However, per day loss in the fresh weight increased up to day seven and thereafter decreased. In non-spongy fruits, the initial respiration rate decreased up to three days of storage. As the fruits started ripening,

Table 1. Total phenolics, total sugar, and soluble protein content in spongy and non-spongy Alphonso mango fruits. The data is replicate of four experiments ± SD and repeated in 2005 and 2006 harvesting seasons. Total phenolics were determined by the method of Spanos and Wrolstad (1990) and total sugars by the method of Nelson (1944). Soluble protein content was determined by the method of Bradford dye binding assay (1976).

Sample	Total phenolics		Total sugars g/100 g			Soluble protein	
	mg gallic acid/100 g	Change %	Reducing	Non-reducing	Total	mg/100 g	Change %
Unripe	98.63 ± 5.4	-	1.42 ± 0.15	8.75 ± 0.25	10.17 ± 1.6	48.5 ± 8.4	-
Ripe-NSP	66.85 ± 7.2	- 32.2	5.73 ± 0.26	10.05 ± 0.36	15.78 ± 0.1	568.2 ± 40.0	+ 13
Sp-White	59.36 ± 5.1	- 11.2	2.64 ± 0.14	6.38 ± 0.42	9.02 ± 1.6	.173.7 ± 16.9	- 70
Sp-Black	77.5 ± 7.4	+ 16	4.64 ± 0.26	6.95 ± 0.31	11.59 ± 1.7	240.1 ± 8.6	- 58

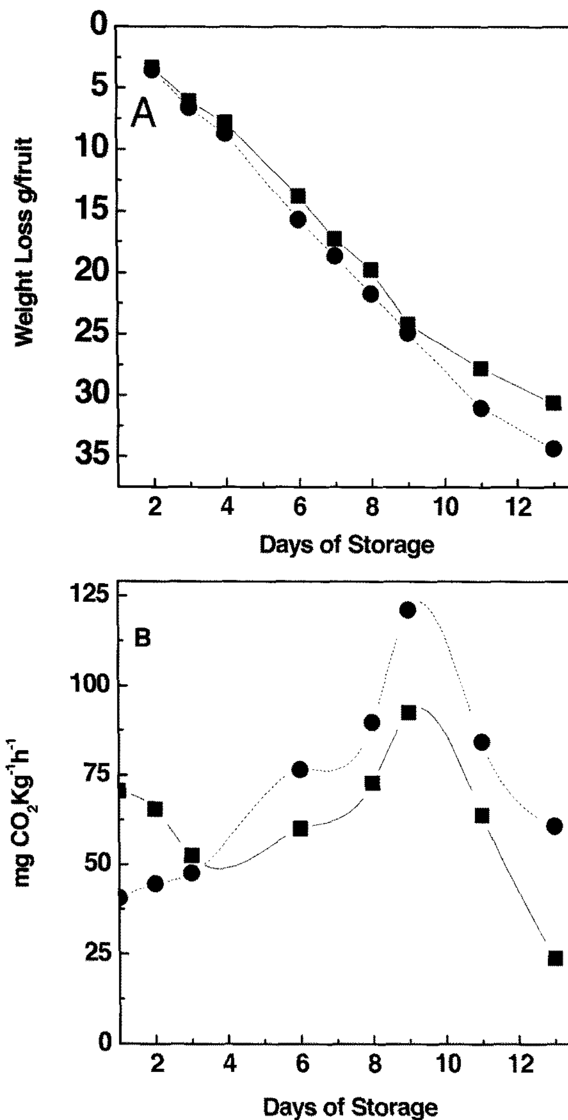

Fig. 2. Fruit weight loss and respiration rate of Alphonso mango fruits: A. Fruit weight loss and B. the respiratory pattern of ripe non-spongy (-■-) and ripe spongy (-●-) fruits during post-harvest ripening. The respiration rate was measured by trapping the CO_2 generated by the fruits in 0.1 N $Ba(OH)_2$ and titrating the unused $Ba(OH)_2$ with 0.1 N HCl as detailed in Materials and Methods. The data is the average of four different experiments ± SD in 2005 and 2006 harvesting seasons.

a sudden rise in the respiratory burst was observed reaching a peak by day nine and declined further up to 13 days of storage (Fig. 2B). In spongy fruits however, the initial rate was comparatively lower and reached a maximum after the fruits ripened. A ca. 2-fold higher respiration rate was observed in spongy fruits throughout the storage period as compared to that in ripe non-spongy fruits. The respiration rate was in opposite trend to loss in fresh weight indicating the biomass consumption in respiratory process. Mango is a climacteric fruit exhibiting a typical pattern of respiration (Rhodes 1971). The post-harvest storage of mango is generally associated with a gradual increase in the rate of CO_2 evolution that reaches a maximum followed by a decline. The results of respiration experiments with the control fruits are in agreement with the reported observations. Microbial infection is known to cause an alteration in the regular respiratory profile of fruits (Sommer 1982). The increased rate of respiration in spongy fruits could be due to infection by *Staphylococcus* strain (Janave 2006a) thereby resulting in an air-cavity formation in the diseased fruits. Ravindra and Shivashankar (2004) have reported the development of anaerobiosis due to the build up of CO_2 in the mesocarp tissue near the endocarp arising out of climacteric respiration.

Changes in total carotenoid content and *β*-carotene co-oxidation

The white-unripe pulp adhered to seed epicoat in spongy fruits (Fig. 1C) indicated that the carotenoids synthesis was affected. At the unripe stage, very low levels of total carotenoids were observed and as ripening progressed, it resulted in a ca. 3-fold increase (Table 2). In the spongy-white pulp, a drastic reduction in carotenoids was observed showing a ca. 2.5-fold less carotenoids than that in ripe non-spongy fruits. This could be due to the inability of the affected part to synthesize normal ripening-associated carotenoids since the pulp remained unripe. In the late spongy-black stage, although the fruit was ripe, the carotenoids levels remained almost constant as in the spongy-white stage. Even though at the spongy-black stage the fruit was

Fig. 3. NATIVE PAGE and activity staining of peroxidase after acetone fractionation of crude enzyme from unripe, ripe non-spongy and ripe spongy fruits: A. Unripe, B. Ripe non-spongy crude, C. Ripe non-spongy acetone fractionated and D. Ripe spongy acetone fractionated enzyme fractions. The gel after electrophoresis was first stained for POX with o-dianisidine in 0.05 M Citrate phosphate buffer, pH 5.0 containing 0.03% H_2O_2 and then stabilized with Coumassie Brilliant blue staining as detailed in Materials and Methods.

Table 2. Total carotenoids and lipoxygenase mediated β-carotene co-oxidation activity in non-spongy and spongy Alphonso mango fruits. Total carotenoids content was measured by the phase separation method as detailed in the previous paper (Janave and Sharma 2004a) and LOX activity by the method of Ben-Aziz et al. (1971). The data is a replicate of four experiments ± SD and repeated in 2005 and 2006 harvesting seasons.

Sample	Total carotenoids		Lipoxygenase activity		
	mg /100 g	fold	Time min	ΔA_{455}nm	Change %
Unripe	5.90 ± 1.0	-	0.5	0.592 ± 0.02	0
Ripe-NSP	18.36 ± 1.67	3.1	1.0	0.552 ± 0.01	6.8
Sp-White	7.20 ± 1.01	- 2.6	1.5	0.536 ± 0.01	9.5
Sp-Black	7.94 ± 0.89	- 2.3	2.0	0.529 ± 0.01	10.6
			2.5	0.524 ± 0.005	11.5
			3.0	0.516 ± 0.003	12.8
			5.0	0.509 ± 0.007	14.0

completely ripe, the very low levels of carotenoids suggested that the carotenoids may be lost by some enzymatic mechanisms involving lipoxygenase-mediated oxidation.

Lipoxygenase-mediated β-carotene oxidation revealed a gradual decrease in β-carotene resulting in a ca. 15% reduction within 5 min as compared to the control (Table 2). The increased activity also could be correlated with decreased levels of total carotenoids in spongy fruits. The use of lipoxygenase in the oxidation system described in this study was based on the fact that the enzyme is a free-radical-generating biocatalyst bleaching fat-soluble pigments. Owing to its partial vitamin A activity, β-carotene is of nutritional importance. Oxidizing enzymes due to their effect on color and flavor of plant foods are of interest. Lipoxygenase (EC 1.13.11.12, LOX, linoleate:oxygen oxidoreductase) is of special interest in fruits because it may also catalyze the co-oxidation of carotenoids resulting in loss of natural color and essential nutrient (Biacs and Daood 2000). However, further studies are essential to correlate the increased LOX activity and loss of carotenoids associated with spongy tissue development in Alphonso mango.

Changes in POX and catalase activities

The data in Table 3 represents changes in peroxidase levels in the crude enzyme extract at various stages of ripening and spongy fruits. In unripe non-spongy fruits, a very low POX activity was observed. POX activity increased by several fold in non-spongy fruits upon ripening, however, in spongy fruits there was a further 1.5-fold increase in activity over that in ripe non-spongy fruits. High POX activity is thought to scavenge the

Table 3. Peroxidase and catalase activities in Alphonso mango fruit during ripening and spongy tissue development. POX and catalase activities were determined as detailed in Materials and Methods. The data is the average of four different experiments ± SD from 2005 and 2006 harvesting seasons.

Sample	Peroxidase activity			Catalase activity	
	Activity/20 g ΔA_{460}nm/min	Sp. Activity ΔA_{460}nm/min/mg	Fold	Activity/20 g ΔA_{460}nm/min	Sp. Activity ΔA_{460}nm/min/mg
Unripe Crude	3.5 ± 0.6	0.031 ± 0.002	-	11.27 ± 1.1	1.35 ± 0.05
Acetone fr.	6.9 ± 0.5	0.22 ± 0.03	-		
Ripe-NSP Crude	65.6 ± 7.2	0.63 ± 0.03	20.3	183.6 ± 3.5	3.13 ± 0.1
Acetone fr.	99.5 ± 6.6	4.48 ± 0.06	20.4	-	
Sp-White Crude	145.5 ± 4.7	1.02 ± 0.1	35.2	40.3 ± 3.1	1.18 ± 0.05
Acetone fr.	245.6 ± 13.2	8.2 ± 0.14	36.2	-	
Sp-Black Crude	-	-	-	110.03 ± 3.3	2.34 ± 0.04

H_2O_2 evolved during the ripening of fruits and peroxidases present in many plants are known to consume H_2O_2 (Saunders et al. 1964). Figure 3 represents the NATIVE-PAGE and staining with *o*-dianisidine patterns of proteins in crude and acetone-fractionated enzyme. The staining of the gel with Coumassie brilliant blue showed a lower number of bands, however, staining first with *o*-dianisidine exhibited some isoenzymes of POX, which were poorly stained with Coumassie blue. In ripe spongy fruits, staining with o-dianisidine produced intense red-colored bands due to peroxidase giving a visible indication of increase in POX activity (Fig. 3). The results indicated that in spongy fruits the isoenzyme showing an intense red color development after o-dianisidine staining was overexpressed compared to that in ripe non-spongy fruits. The data on enzyme activity and gel pattern suggested that this antioxidant enzyme is differentially expressed in non-spongy and spongy fruits.

Ammonium sulfate fractionation indicated a very low activity of POX in all three fractions in unripe fruits (Table 4). In ripe non-spongy fruits, 50-70% fraction exhibited a several-fold increase in POX activity as compared to that in unripe fruits. However, in ripe spongy fruits a ca. 2-fold increase in POX activity was observed in this fraction over that in ripe non-spongy fruits. Activity staining after native PAGE also confirmed this increase as intense bands of peroxidase were observed. The protein content pattern in the three $(NH_4)_2SO_4$ fractions indicated a turnover of proteins during ripening. In unripe fruits, the protein content in the 0-20% and 20-50% fractions was higher and as ripening progressed, an increase in protein content in 20-50% fraction and a decrease in 0-20% fraction was observed with minor changes in POX activity. In the 50-70% fraction however, an increase in protein content and very high levels of POX activity were observed.

Table 4. Changes in peroxidase levels in $(NH_4)_2SO_4$ fractionated enzyme fractions in unripe, ripe non-spongy and ripe spongy Alphonso fruits. POX activity is expressed as ΔA_{460}nm/mg protein.

Sample	Volume ml	Total Protein mg/20 g	Peroxidase activity in $(NH_4)_2SO_4$ fractions					
			0-20%		20-50%		50-70%	
			Protein mg	ΔA_{460}nm/mg	Protein mg	ΔA_{460}nm/mg	Protein mg	ΔA_{460}nm/mg
Unripe	9 0	51.8 ± 1.5	13.73 ± 0.9	3.26 ± 0.06	17.57 ± 0.13	0.19 ±0.02	0.72 ± 0.04	1.12 ± 0.03
Ripe-NSP	9 0	60.4 ± 2.3	3.83 ± 0.08	3.43 ± 0.08	49.29 ± 4.2	0.25 ± 0.03	1.5 ± 0.17	5.9 ± 0.5
Sp-White	9 0	35.8 ± 1.7	4.37 ± 0.3	0.42 ± 0.03	32.67 ± 1.9	0.06 ± 0.01	3.46 ± 0.09	9.62 ± 0.1

In ripe-spongy fruits, the 50-70% $(NH_4)_2SO_4$ fraction showed about a 2-fold increase in protein content associated with a 1.5-fold higher POX activity over that in ripe non-spongy fruits.

Unripe non-spongy fruits showed very low catalase activity in the buffer soluble enzyme fraction (Table 3). As fruits became fully ripe, a ca. 3-fold increase in activity was detected. In spongy white fruits, catalase activity was slightly decreased and at the spongy black stage, catalase activity was restored showing almost identical levels as in non spongy-ripe fruits. The increased levels of POX and catalase activities indicate generation of H_2O_2 from oxidative burst induced by infection by *Staphylococcus* bacteria. Parallel studies show very high levels of catalase in the proteins isolated from the *Staphylococcus* strain isolated from spongy pulp (data not presented). Hence, the increased levels of POX and catalase activities in spongy tissue-affected mangoes indicate that these two enzymes are expressed as the defense mechanism against *Staphylococcus* infection. Increased rates of production and accumulation of reactive oxygen species (ROS) including H_2O_2, organic peroxide, and superoxide, are important components of active plant defense responses to microbial invasion (Lagrimimini et al. 1993). For successful plant invasion, these ROS must be rapidly detoxified. Plant catalases and peroxidases are ubiquitous enzymes that are believed to play a vital role in the plant's defensive mechanisms that guard against attack by pathogens.

The occurrence of spongy tissue in Alphonso mango is a detrimental factor in the potential for marketing this commercial important fruit crop of India. Several studies in the past have indicated that the spongy tissue development is an internal physiological disorder or ripening-associated disorder. Since the present studies indicate the biochemical changes in spongy tissue-affected fruits could be due to staphylococcal infection (Janave 2006a), spongy tissue development in mangoes may not be either physiological or ripening-associated disorder. Also, the air pockets developed in spongy fruits can be correlated with an increased respiration rate and a higher catalase activity present in the proteins of *Staphylococcus* bacteria observed in the spongy pulp. The data presented here on various biochemical changes and enzyme activities suggest that the fruits affected by this disorder resulted in poor-keeping quality. To obtain a commercially-successful cultivar, it has to match both the organoleptic and storage qualities. A long-term solution is to maintain proper hygienic conditions during inflorination and fruit development to control bacterial infection by controlling the spongy tissue formation.

Abbreviations

BHI - Brain Heart Infusion; LOX - Lipoxygenase; PAGE - Polyacrylamide gel electrophoresis; PCA - plate count agar; PDA - potato dextrose agar; PMSF -Phenylmethanesulfonyl fluoride; POX - Peroxidase; PPO - Polyphenol oxidase; PVPP - Polyvinylpolypyrrolidone; ROS -Reactive oxygen species; S. - *Staphylococcus*.

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