

# Impaired starch degradation in sapota fruit (*Manilkara achras*) affected by corky tissue, a physiological disorder

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**Abstract-** Corky tissue of sapota cv. Cricket Ball (*Manilkara achras*) is a major physiological disorder distinguished by the occurrence of starchy lumps coupled with lower sugar levels in ripening pulp. Since sugar content of pulp is one of the major determinants of sapota fruit quality, a reduction in sugar level in CT fruit coupled with the buildup of starch unfavorably impacts fruit quality. In view of this, the present study was carried out with the objective of determining the possible reasons for the disturbance in starch metabolism in corky tissue affected fruits of sapota. Changes in the activities of starch hydrolyzing enzymes, sugar and starch contents, and tissue concentrations of Zn and P in healthy, apparently healthy and CT affected fruit tissues were measured to assess the potential interrelationships among them. Results indicated that the activities of starch phosphorylase and  $\alpha$ -amylase enzymes in CT pulp were significantly lower ( $P \leq 0.005$ ) compared to AH and healthy pulp. Acid phosphatase activity was markedly lower in CT affected fruit pulp in comparison with AH and H pulp. The contents of total soluble sugars and reducing sugars were significantly lower ( $P \leq 0.005$ ) in CT affected pulp while the starch concentration was significantly ( $P \leq 0.005$ ) higher. The concentrations of the major component sugars of pulp like, sucrose and glucose were lower in CT pulp compared to AH and healthy pulp although fructose content was marginally higher. There was a significant ( $P \leq 0.001$ ) rise in the zinc content of CT affected pulp compared to H pulp which, possibly inhibited the activity of acid phosphatase enzyme thus lowering the release of inorganic phosphate needed for the activity of starch phosphorylase. It is concluded from the study that the marked rise in the content of organic acids and a consequent lowering of pH resulted in disturbances in starch metabolism leading to increased electrolyte leakage and disruption of cellular organization in CT pulp.

**Index Terms-** Corky tissue, sapota, *Manilkara achras*, physiological disorder, Starch phosphorylase,  $\alpha$ -amylase, acid phosphatase, starch degradation, inorganic phosphate, zinc, organic acids

## I. INTRODUCTION

Corky tissue in cv. Cricket Ball of sapota is a major physiological disorder distinguished by the presence of starchy lumps in pulp during ripening of fruits thus reducing its edible quality. Past studies showed that the CT fruits have a lower concentration of sugars associated with the accumulation of starch as compared to healthy fruits (Shivashankar *et al.*,

2013). Being a climacteric fruit (Lakshminarayana and Subramanyam, 1970; Broughton and Wong, 1979), sapota ripens rapidly under optimal environmental conditions after harvest. However, the CT fruits exhibit a lack of uniform ripening and the probable cause of such impaired ripening has not been understood so far.

Past studies have shown that ripening of fruits is a complex phenomenon which needs the integration and coordination of several different metabolic pathways within the maturing fruit. It has been reported that the breakdown of stored starch in the pulp of mango is reduced in spongy tissue affected fruits of Alphonso mango affected by the physiological disorder known as spongy tissue is reduced due to a fall in amylase activity (Shivashankar *et al.*, 2007). Work done by Godoy *et al.* (2009, 2010) showed that during banana ripening, degradation of starch granule proceeds in an axial way, as a result of the concerted action of several enzymes. Of the several enzymes involved in degradation of starch in plants,  $\alpha$ -amylase and starch phosphorylase play a dominant role in the conversion of stored starch into simple sugars. Nonetheless, acid phosphatases are believed to function in the production, transport, and recycling of inorganic phosphate, which is a crucial macronutrient for cellular metabolism (Duff *et al.*, 1994) and to sustain the activity of starch phosphorylase. Therefore, this study was initiated to study the changes in the rates of conversion of starch in both H and CT fruits involving the activities of  $\alpha$ -amylase, starch phosphorylase and acid phosphatase enzymes and a few other factors influencing their activities. This study covering a wide and integrative approach is therefore, expected to provide insights into the regulatory points of starch metabolism affecting normal ripening in CT fruits.

## II. MATERIALS AND METHODS

### II.1. Material

“Cricket ball” sapota fruits were collected during the 2013–14 season from 10-year-old trees receiving the recommended supply of fertilizers and plant protection measures and maintained under uniform growth conditions in the experimental orchard of IIHR, Bangalore. Mature fruits of sapota cv. ‘Cricket ball’ were harvested from trees and ripened under ambient conditions at a mean maximum temperature of 29 - 31°C and relative humidity of 55-67%. At optimum ripeness, fruits were weighed and cut open for recording observations on pulp parameters. CT affected fruits were identified based on the presence of a hard lump in the pulp under the skin. The CT

affected tissue (A), the apparently healthy (AH) tissue surrounding the hard lump which remained free from symptoms and healthy (H) tissue from non-affected fruit were collected for analysis.

## II.2. Enzyme assays

### II.2.1. Acid phosphatase (EC 3.1.3.2)

Acid phosphatase enzyme was extracted according to Murray (1980). 5g of the sample was ground in a chilled mortar with acid – washed sand and 20 mL of chilled 50 mM Tris – HCl buffer (pH 7.6) containing 1 mM EDTA. The homogenate was filtered through double layers of cheesecloth and centrifuged at 20,000 g for 20 min. The supernatant was used for the enzyme assay. Acid phosphatase activity was assayed by adding 0.2 mL of enzyme extract to 1 mL of 5.5 mM *p* – nitrophenyl phosphate (*p* – NPP) in 55 mM citrate buffer (pH 4.8). The reaction mixture was incubated at 37°C and the reaction was terminated by the addition of 10 mL of 200 mM NaOH. The amount of *p*–nitrophenol released was estimated by recording the absorbance at 405 nm. The assay was performed in triplicate and acid phosphatase activity expressed as  $\mu\text{mol } p\text{-nitrophenol released min}^{-1} \text{g}^{-1}$  fresh wt

### II.2.2. $\alpha$ -Amylase (EC 3.2.1.1)

$\alpha$ -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  $\times$  g for 10 min at 4°C. Five-hundred  $\mu\text{L}$  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).  $\alpha$ -Amylase activity was expressed as mg maltose liberated  $\text{g}^{-1} \text{h}^{-1}$ .

### II.2.3. Starch phosphorylase (EC 2.4.1.1)

The crude enzyme preparation was carried out at 2 - 4°C. 5g of pulp tissues were weighed in to a precooled pestle and mortar and ground into a fine powder in liquid nitrogen. The crude enzyme was prepared using an extraction medium (1:1 w/v) consisting of 50mM Hepes, pH 7.5, 5mM  $\text{MgCl}_2$ , 0.2% Triton X 100 and 20mM  $\beta$ -mercaptoethanol. The slurry obtained was filtered through a double-layer of cheesecloth, and centrifuged at 15000  $\times$  g for 20 min. The clear supernatant obtained was used as the crude enzyme

Starch phosphorylase activity was determined following Surendranathan and Nair (1973) by assaying the inorganic phosphate formed in the reaction mixture. The reaction mixture consisted of 50 mM HEPES buffer, pH 7.5, 5 mM  $\text{MgCl}_2$ , 10 mM  $\beta$ -mercaptoethanol, 0.4 mL 0.5% starch, 20 mM potassium phosphate buffer, 0.1mL of crude enzyme extract and water to a final volume of 1 mL. To the reference tube 0.2 mL of 20% TCA was added prior to the addition of the enzyme. The reaction mixture was incubated at 30°C for 20 min. The supernatant was assayed for enzyme activity following Fiske and Subbarow (1925).

## II.3. Protein

Protein content was determined by the method of Bradford (1976) using BSA as the standard. 25 $\mu\text{L}$  of extract and 75 $\mu\text{L}$  of distilled water were added into a test tube. Then 5 mL of Bradford solution was added, the mixture was vortexed and incubated at room temperature. Absorbance at 595 nm was recorded using a spectrophotometer. Protein content in the sample was estimated based on the standard curve prepared using BSA.

## II.4. Total and reducing sugar

Total soluble sugars from mature ripe fruits were extracted in boiling 80% (v/v) ethanol, centrifuged at 3,000  $\times$ g for 10 min. at 25°C, and the supernatant was evaporated to dryness on a boiling water bath. The sugars in the dry residue were then re-dissolved in 2 ml of distilled water. Reducing sugars in the extract were estimated following Miller (1972). 200  $\mu\text{L}$  of each extract was mixed with 800  $\mu\text{L}$  of water and 500  $\mu\text{L}$  of dinitro salicylic acid (DNS) reagent and heated on a boiling water bath for 5 min. The final volume was made up to 20 mL with water and absorbance was read at 540 nm using a DU-64 spectrophotometer (Beckman Instruments Inc., Fullerton, CA, USA). The concentration of reducing sugars was calculated from a standard curve and expressed as mg maltose equivalents  $\text{g}^{-1}$  fresh weight (FW). The concentration of total soluble sugars in the extract was estimated after acid hydrolysis. One mL of concentrated HCl was added to 10 mL of each extract, mixed and incubated overnight at 37°C. The hydrolysate was neutralised with 10 M NaOH, using phenolphthalein as the indicator. The final volume was made up to 20 mL with water, and the concentration of reducing sugars in the extract was measured, as described above and expressed as mg maltose equivalents  $\text{g}^{-1}$  FW.

## II.5. Starch

Starch content was estimated according to Mc Cready *et al.* (1950). The 80% (v/v) ethanol-insoluble residue obtained after extraction of sugars was mixed with 6 mL of 52% (v/v) perchloric acid. The mixture was incubated at 0°C for 20 min and centrifuged at 3000  $\times$  g for 10 min at room temperature. The volume of the supernatant was made up to 20 mL with water and a 10 mL aliquot was acid hydrolysed, as described above. The concentration of reducing sugars was measured as described above. Starch concentrations were expressed as mg  $\text{g}^{-1}$  FW.

## II.6. HPLC analysis of sugars

The identification and quantification of sugars was performed by HPLC according to the method described by Galdón *et al.* (2009). One g of the frozen sapota fruit pulp was directly transferred to polypropylene tubes and mixed with 2 mL of 4:1 ethanol: water. The tubes were put in an ultrasound bath for 5 min and centrifuged for 5 min at 1090 $\times$ g. The supernatant was carefully collected so as to prevent contamination. Two mL of 4:1 ethanol: water was added to the pellet and placed in the ultrasound bath and centrifuged again. The two supernatants were mixed and concentrated under a continuous stream of nitrogen until all the ethanol was removed and the residue was dissolved in 5 mL of 50% acetonitrile and passed through a

0.45  $\mu\text{m}$  filter GHP (Waters, Millford, MA, USA) prior to HPLC analysis.

The analysis was performed by Shimadzu LC-10 AD VP high performance liquid chromatography system under isocratic conditions at  $25 \pm 1$  °C. The mobile phase consisted of (A) acetonitrile; (B) water (70:30). Flow rate was set at  $1 \text{ mL min}^{-1}$  and injection volume was 10  $\mu\text{L}$ . The analytical column Alltech-NH2 (4.6 mm $\times$ 250 mm $\times$ 5  $\mu\text{m}$ ) was used for separation and detected by refractive index detector (RID-10A). Total time of analysis was up to 30 min. The identification of sugars in sample was done by comparison of retention times of individual sugars with reference standards. The quantification of sugars was done based on comparison of peak areas obtained from the reference standards. Concentrations were expressed as  $\text{g } 100\text{g}^{-1}$  FW.

## II.7. HPLC analysis of organic acids

The organic acids were determined using a HPLC method with diode array detector described by Suárez *et al.* (2008) with slight modifications. The organic acids of sapota pulp samples were extracted by distilled water (1:10, w/v), clarified by centrifuging at  $3150 \times g$  for 10 minutes and the supernatant was membrane filtered (0.45  $\mu\text{m}$ ) before injection.

Organic acid was analyzed by Shimadzu LC-10 AD VP high performance liquid chromatography (HPLC) system containing quaternary pump, autosampler, and diode array detector with Zorbax SB-Aq C18 column (150 mm  $\times$  4.6 mm ID, 5 $\mu\text{m}$ ) (Agilent Technology, Santa Clara, CA, USA). Chromatography separation was performed at 25°C with a flow rate of 0.4 mL/min. The mobile phase was carried out with 1% monopotassium phosphate ( $\text{KH}_2\text{PO}_4$ , pH 2.5). Absorbance was measured at 214 nm. The detected organic acids calculated using calibration curves prepared using 10  $\mu\text{L}$  each of the standard (Sigma, St. Louis, MO, USA) and expressed as  $\text{g } 100\text{g}^{-1}$  FW.

## II.8. Zinc and phosphorous

One g of oven-dried seed was digested with 10 mL of 9:4 (v/v) mix of 16 M nitric acid: 11.6 M perchloric acid. Zinc and phosphorous concentrations were estimated according to Jones *et al.* (1991) using an AAnalyst-200 atomic absorption spectrometer (Perkin-Elmer, Waltham, MA, USA).

## II.9. Electrolyte leakage and pH

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

## II.10. 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  $\pm$  standard error (SE).

## III. RESULTS

Results presented in Figure 1 showed that the acid phosphatase activity decreased from 2.13 in H pulp to 1.98 in

AH and 1.80 in CT pulp.  $\alpha$ -amylase activity showed a reduction of 47.8% in CT pulp compared to H pulp. Similarly, starch phosphorylase activity was significantly lower in CT pulp (0.14) compared to AH (0.30) and H pulp (0.52).

There was a 42.9% increase in the concentration of zinc in AH pulp and 79.2% in CT pulp compared to H. The phosphorus content showed a decrease of 42.9% in CT pulp and 23.1% in AH pulp as compared to H pulp. The level of organic acids increased significantly in AH (18.42) and further up in CT (20.85) pulp compared to healthy pulp. The pH of CT pulp was lower (5.30) compared to AH (5.42) and H pulp (5.81) while the EC was higher in CT (0.92) pulp compared to AH (0.87) and H (0.77) pulp (Table 1).

The level of total sugar, reducing sugar and starch in pulp of healthy, AH and CT affected fruit presented in Figure 2 showed that there was a significant decrease of 37.8% and 30.7% respectively of total sugar and reducing sugar in CT pulp in comparison with AH and H pulp while starch content was significantly higher in CT (63.7%) pulp and AH (38.7%) compared to healthy pulp.

HPLC analysis of sugars showed that the concentration of glucose and sucrose declined by 13.7% and 19.9% respectively in CT pulp compared to H pulp while fructose content increased by (7.1%) in CT (Figure 3).

## IV. DISCUSSION

### IV.1. Inhibition of starch degradation in CT pulp

As shown in Figure 1, the activities of both  $\alpha$ -amylase and starch phosphorylase declined significantly in CT pulp compared to healthy pulp indicating that starch breakdown was affected in CT pulp. Since amylase is primarily involved in breaking down starch molecule, the reduction in amylase represented a disturbance in starch metabolism in CT tissue. Besides, the starch phosphorylase enzyme activity also reduced drastically in CT pulp showing that starch breakdown was significantly affected in CT pulp. Starch phosphorylase activity is dependent on acid phosphatase activity for supply of inorganic phosphate and since acid phosphatase activity also reduced in CT pulp, it was clear that the short supply of inorganic phosphate resulted in a reduction of starch phosphorylase. As a consequence, starch metabolism was hampered in CT pulp

Incomplete ripening is a serious problem in CT affected sapota fruit which leads to the appearance of starchy lumps within the pulp mass. It is a well established fact that the process of fruit ripening is associated with various biochemical processes, the foremost being the conversion of starch to sugars (Marriott *et al.*, 1981). Accordingly, activities of several enzymes involved in the conversion of starch to sugars have been reported to increase during ripening. One of the key enzymes responsible for the starch-sugar conversion is starch phosphorylase which catalyzes the phosphorolytic cleavage of starch resulting in the production of sugar phosphates that are needed for the increased respiratory and biosynthetic reactions that occur during ripening (Iyare and Ekwukoma, 1992). The phosphorolysis of starch by phosphorylase occurs in the presence of inorganic phosphate obtained from the hydrolysis of esters of orthophosphate by phosphatases which help to maintain the phosphate pool needed for fruit ripening (Fernandez and Jouve, 1990; Vincent *et al.*,

1992). Hence, the metabolism of inorganic phosphate is of critical importance in fruit ripening (Julie *et al.*, 2000; Bozzo *et al.*, 2002). In view of this, studies were carried out on the changes in amylase, starch phosphorylase and acid phosphatase in both healthy and CT affected sapota fruits.

Results presented in Figure 1 showed that acid phosphatase activity in the pulp of CT fruit recorded a steady decline from H to AH and a rapid decline in CT. Acid phosphatase catalyzes the hydrolysis of inorganic phosphate from a broad range of phosphate monoesters and anhydrides with a pH optimum ranging from 4-7 (Vincent *et al.*, 1992). It may be noted that the content of organic acids in CT fruit showed a marked increase. This might have possibly accounted for the decrease of pH in CT pulp compared to H fruit. However, since the decrease of pH was marginal, ranging from 5.81 in H to 5.30 in CT, it was unlikely that the decrease in acid phosphatase activity of CT fruit was related to the lower pH of pulp, as acid phosphatase shows optimum activity over a broad range of pH.

#### IV.2. Increased zinc levels inhibit acid phosphatase activity

From the data presented in Table 1, it was also noted that the concentration of zinc in AH and CT were significantly higher compared to H pulp. Incidentally, a higher level of  $Zn^{2+}$  is known to inhibit acid phosphatase activity. Inhibition of acid phosphatase isolated from red kidney bean (Cashikar *et al.*, 1997) and banana fruit (Turner & Plaxton, 2001) by  $Zn^{2+}$  have been reported. It was therefore, likely that the decrease in acid phosphatase activity from H to CT was possibly related to the increase in the concentration of Zn. Higher zinc content in plants is also known to reduce the uptake of P and Fe and enhance the production of free radicals causing toxicity symptoms (Prasad *et al.*, 1999; Vitosh *et al.*, 1994; Teige *et al.*, 1990; Ruano *et al.*, 1988). In this study also, it was found that the level of P was lower in both AH and CT fruit compared to H pulp (Table 1) clearly supporting the previous findings. Further, the ability of Zn to increase free radical production might lead to membrane damage which, in turn, would result in an increase of electrical conductivity as evidenced from the results of the present study in which EC rose from 0.772 in H pulp to 0.922 in CT pulp. Thus, the increase of EC indicated the breakdown of cell structure and loss of tissue integrity, possibly due to higher level of Zn in CT fruits. Further, starch phosphorylase activity also decreased in CT fruit due to a reduction in the supply of inorganic phosphate. A concurrent reduction in the activity of amylase in CT fruit resulted in the accumulation of starch in pulp associated with a lower level of total and reducing sugars in pulp. Thus, a reduction in the activities of amylase, acid phosphatase and starch phosphorylase together with increased concentration of Zn and lower pH in CT fruit mainly contributed to incomplete conversion of starch into sugars. This was reflected in the marked reduction of glucose and sucrose in CT fruit (Figure 2) although the fructose content of CT fruit showed a marginal increase.

#### V. CONCLUSION

Summing up, the results clearly showed that starch metabolism into sugars was disturbed in CT due to a combination of factors involving the reduction in the activities of

starch degrading enzymes and a decrease of acid phosphatase activity, possibly induced by high levels of Zn in CT pulp. Increased Zn level could also lead to the production of free radicals damaging cell organelles and an increase of electrical conductivity signifying destruction of cellular structure. The concurrent accumulation of organic acids results in a fall in the pH of the cells thus leading to further disturbances in metabolism and disruption of cellular organization in CT pulp.

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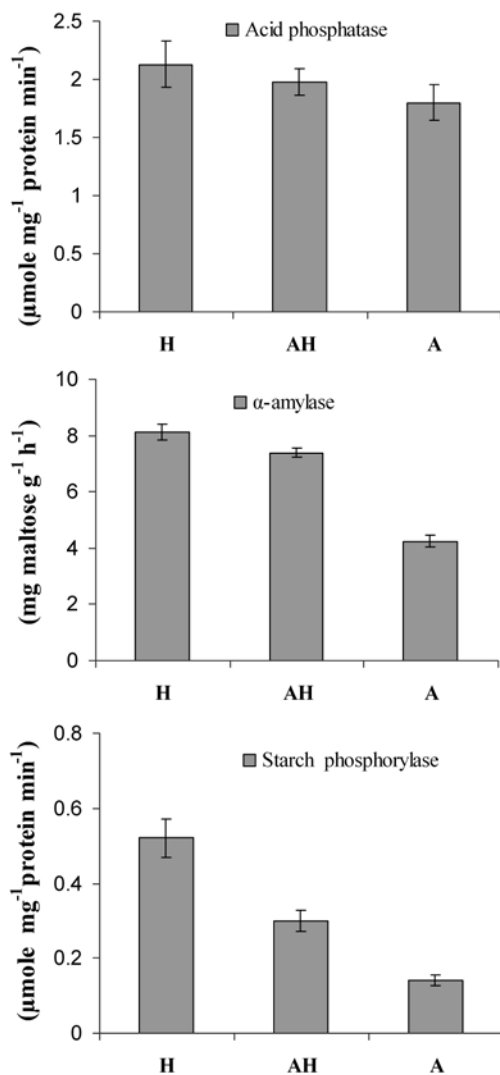
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**Figure 1: Activities of acid phosphatase,  $\alpha$ -amylase and starch phosphorylase in healthy, apparently healthy and CT affected sapota fruit. Values are the means  $\pm$ SE of five replicates**

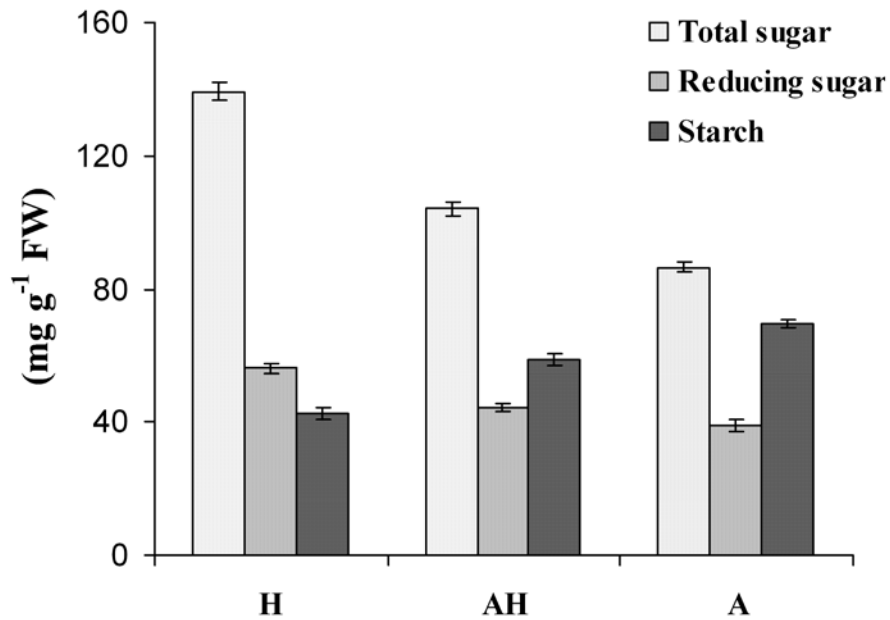


Figure 2: Changes in the concentrations of sugar and starch in pulp of healthy, apparently healthy and CT affected fruits. Values are the means  $\pm$ SE of five replicates

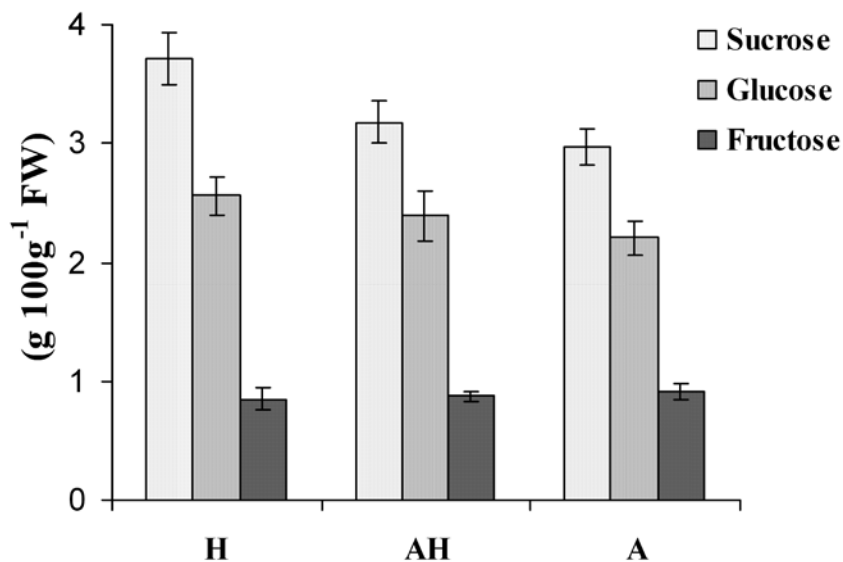


Figure 3: Changes in the levels of component sugars in pulp of healthy, apparently healthy and CT affected fruits. Values are the means  $\pm$ SE of five replicates.

Table 1: Mineral composition, organic acids, pH and conductivity of healthy, apparently healthy and CT affected pulp

Parameters	H	AH	A
Phosphorus (%)	0.091 $\pm$ 0.0045	0.070 $\pm$ 0.0024	0.052 $\pm$ 0.0032
Zinc (ppm)	2.4 $\pm$ 0.14	3.4 $\pm$ 0.16	4.3 $\pm$ 0.11
pH	5.81 $\pm$ 0.23	5.42 $\pm$ 0.13	5.30 $\pm$ 0.18
Electrical conductivity (dS/m)	0.772 $\pm$ 0.039	0.873 $\pm$ 0.017	0.922 $\pm$ 0.027
Total organic acids(g 100g <sup>-1</sup> FW)	17.54 $\pm$ 0.70	18.42 $\pm$ 0.42	20.85 $\pm$ 0.34

Values are the means  $\pm$ SE of five replicates.

