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Research Article

Expression Analysis of Amylase Gene and Starch Degradation during Fruit Development and Ripening Stages of Exotic Cultivars of Banana

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Abstract: Trend in expression of amylase gene along with changes in starch and total sugar contents in different banana cultivars during fruit ripening stages were examined. Five exotic cultivars of banana were selected. The ripening stages of fingers were designated by changes in color from green to yellow. RT-PCR analysis revealed that expression of amylase gene was barely detectable during first three stages (6 weeks), but gradually increased at 4th stage and became very strong during 6th to 7th stage (ripening stage). A significant decrease in starch content was observed in all the cultivars at ripening stage while sugar contents increased correspondingly. In all the cultivars, the starch contents ranged from 21.8% to 24.67% at first stage and decreased to almost zero (0.32 to 0.60%) at 11th stage. On the other hand sugar contents were increased from 1% to 1.90% (23.2 to 24.2) from 1st to 11th stage. These findings have future implications in developing functional food products having desired range of sugar and starch contents in the fruit.

Keywords: Banana, fruit, amylase, gene expression, sugar, starch, ripening

1. INTRODUCTION

Fruits and vegetables are the important components of healthy diet [1], and play an important role in human nutrition and health, particularly as sources of vitamin C, thiamine, niacin, pyridoxine, folic acid, minerals and dietary fiber [2]. Normally, fruit are harvested after attaining physiological maturity when the development is accomplished and growth has stopped. Then, ripening is started and fruit attains the sensory, visual and edible properties for consumption [3].

Banana is one of the valuable fruits consumed as raw, ripe or processed into many products. For processing, usually raw banana is used whereas ripe bananas are consumed as such. Banana is climacteric fruit. It is mostly harvested in the unripe stage when the fruit is firm and green. During ripening stage, banana undergoes many important physicochemical changes simultaneously. These complex changes comprise three different physiological stages; pre-climacteric stage, climacteric (ripening) stage and senescence (dying) stage in which the fruits become over-ripe. The physiological stage and nutritional changes in harvested banana varies from variety to variety and may depend on the factors like climate, cultivation methods, post-harvest treatments and storage condition [4, 5]. Technically, ripening process can be initiated by introducing ethylene

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gas as a hormone. Ethylene has many roles in plant metabolism, one of which is to start and enhance the ripening process [5].

During initial stages of senescence a series of changes occur in ripening process. After an active stage of cell division and cell expansion, fruit growth rate stops and ripening phase is started. At this stage physiological, structural and biochemical variations occur which are well coordinated. There are growing evidences that these alterations are governed by the spatial and temporal expression of genes controlling fruit ripening phase [6]. Starch is a key carbon-storage molecule for plants. It is the major energy source for animals and humans. Starch is a product of photosynthesis produced in leaves during the day. This storage starch is vital for plants, and is broken down following specific environmental or developmental signals such as the commencement of spring in roots and bark as well as the beginning of ripening in many fruits. Not much is known about the degradation of starch in plants [7].

Nevertheless, many enzymes involved in starch degradation have been identified. Among them, α-amylase is one of the important enzymes which is an endo-hydrolase capable of quickly degrading the starch into soluble substrates for other enzymes to act. [8]. Evaluation of the status of amylase, showed low activity at the onset of the climacteric and a concomitant enhancement that is parallel to the respiratory climacteric. Changes in amylase activity have also been reported during ripening of mango fruits [19].

Generally, during banana fruit ripening, the disappearance of starch contents is very fast. On the average, starch content decreases from 25% in the pre-climacteric stage to about 1% during the climacteric stage. On the other hand, sucrose increases 12 times and precedes the increase of hexoses [9]. It is observed that only 5% at the most is consumed for respiration. In spite of the fundamental significance of starch to sugar conversion for the fruit physiology and its importance as an eatable not much is known yet about the molecular mechanisms involved [10]. Therefore, unveiling the nutritional values during fruit ripening and its correlation with expression of important genes such as α-amylase is inevitable for studying the molecular physiology of the banana fruit for further genetic improvement programs.

In present study trend in expression of amylase gene in different exotic banana cultivars during the development and ripening stages were examined using RT-PCR, and the changes in starch and total sugar contents were observed. The results obtained allow us to infer that expression of amylase gene was barely detectable during initial stages but gradually increased and became stronger at later stages of fruit ripening. A significant decrease in starch content was observed in all varieties at ripening stage while sugar contents increased reciprocally.

2. MATERIALS AND METHODS

2.1. Plant Material and Growing Conditions

Five introduced varieties that are being grown in Pakistan were selected and tagged at field station of Agriculture Research Institute, Tandojam, Pakistan. The cultivars included were Williams, Grand-naine (G-naine), Brazillian, Pisang and Basrai. Banana fruit (10-12 fingers) were collected every two weeks after the emergence of fruit till the harvesting stage (12 weeks). To assure the integrity of RNA, at each stage the picked fruits were transported to NARC Islamabad under very cold conditions using coolers having ice. These fruits were harvested and allowed to ripen naturally in the laboratory under cold environment (12 °C) at Food Science and Product Development Institute, NARC, Islamabad. During ripening, fruit color changed from green to yellow and samples were collected on daily basis. The ripening stages have been judged visually through changes in colour using the banana ripening colour chart. A total of 11 stages were analyzed including developmental and harvesting stages. At each stage, banana fingers were picked and stored at -80 °C for total RNA extraction.

2.2. Total RNA Extraction

For total RNA extraction, plant tissues collected at different fruit ripening stages for all the 5 cultivars were utilized. Total RNA extraction was carried out through improved CTAB-LiCl method. This

protocol is primarily based on integrating improved CTABII method and hot borate method of [11, 12]. Quality of the extracted RNA from banana fruit was evaluated on 1.5% agarose gel and photographed using gel documentation system. The RNA was also quantified with Nano-Drop (Thermo Fisher Scientific) to make sure good quality and quantity of RNA is available for further gene expression analysis.

2.3. Gene Selection and Primer Designing

Nucleotide coding sequences of the selected α-amylase (GenBank: AF533648.1) and Actin (GenBank: AB022041.1) genes were retrieved from the National Center for Biotechnology Information (NCBI) database. Actin gene was used as an internal control in this study. The forward and reverse primers were designed from the coding sequences of the above mentioned genes using Primer 3 online package.

2.4. cDNA Synthesis

Complementary DNA or cDNA was synthesized from RNA in a reaction catalysed by reverse transcriptase. Best quantity RNA with 190 OD and nucleotide concentration of 800 ng μL^{-1} was used for cDNA synthesis. A total of 20 µL reaction mixture was prepared for cDNA synthesis using Revert Aid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific). Master mix was prepared in a nuclease free micro centrifuge tube containing 4 µL of RNA, 1 µL of OligodT primer and 9 µL of double distilled water. Master mix was prepared for each sample, in case where RNA was not of good quality (~600 ng μL^{-1}) then the volume of RNA was adjusted. Master mix was incubated at 65 °C for 5 min to denature RNA and then the reaction tube was chilled in ice cold water for 2-3 min. In the following step, 1 µL of reverse transcriptase enzyme, 1 µL of 10 mM dNTPs and 4 µL of 5X reaction buffer were added to each tube. The mixtures were mixed by vortexing and short spinning to bring all contents into the bottom of the tube. The reaction mixture was placed in PCR machine for final incubation at 42 °C for 60 min followed by reaction stoppage at 70 °C for 5 min.

2.5. Multiplex Semiquantitative RT-PCR

For gene expression analysis of α-Amylase RT-PCR was performed. Reaction mixture was prepared containing 2.5 µL cDNA template, 3 µL $MgCl_2$, 1 µL of 10 mM dNTPs, 1 µL each of forward and reverse primers for both the α-Amylase and Actin genes, 5 µL of Taq buffer and 0.5 µL of Taq polymerase enzyme. The final reaction volume was raised to 20 µL with PCR water. The PCR profile consisted of an initial denaturation phase of 95 °C for 5 min, followed by 37 cycle of 94 °C for 30 sec, annealing at 58 °C and extension at 68 °C for 1 min and a final extension at 68 °C for 7 min.. PCR products were run on 2% agarose gel and observed under GelDoc system.

2.6. Determination of starch and sugar contents:

Starch contents in the banana samples were measured by acid hydrolysis method of FAO described by [13]. Sample was hydrolysed in the presence of water and HCl to convert all the starch into sugars. The converted sugar was then analysed by Lane and Eynon method described by [14], and the result was then multiplied by a starch factor (0.90) to get the starch contents. Sugar was first extracted by water ethanol mixture (1:1), ethanol was allowed to evaporate and the sample was diluted with distilled water to 250 ml; it was the neutralized. The Fehling's solution was then titrated with the sample solution till the appearance of brick red colour.

3. RESULTS AND DISCUSSION

3.1. Amylase Transcripts Exhibit Variable Expression at Different Developmental and Ripening Stages

For the expression studies of amylase and its relation to sugar accumulation the banana fruit during ripening were selected because in banana the ripening stages have easily been identified and secondly the banana is considered to contain high sugar and a good candidate for the study of amylase expression and sugar accumulation during ripening .In order to discern the expression patterns of α-amylase gene transcripts at different fruiting stages of banana cultivars RT-PCR analyses

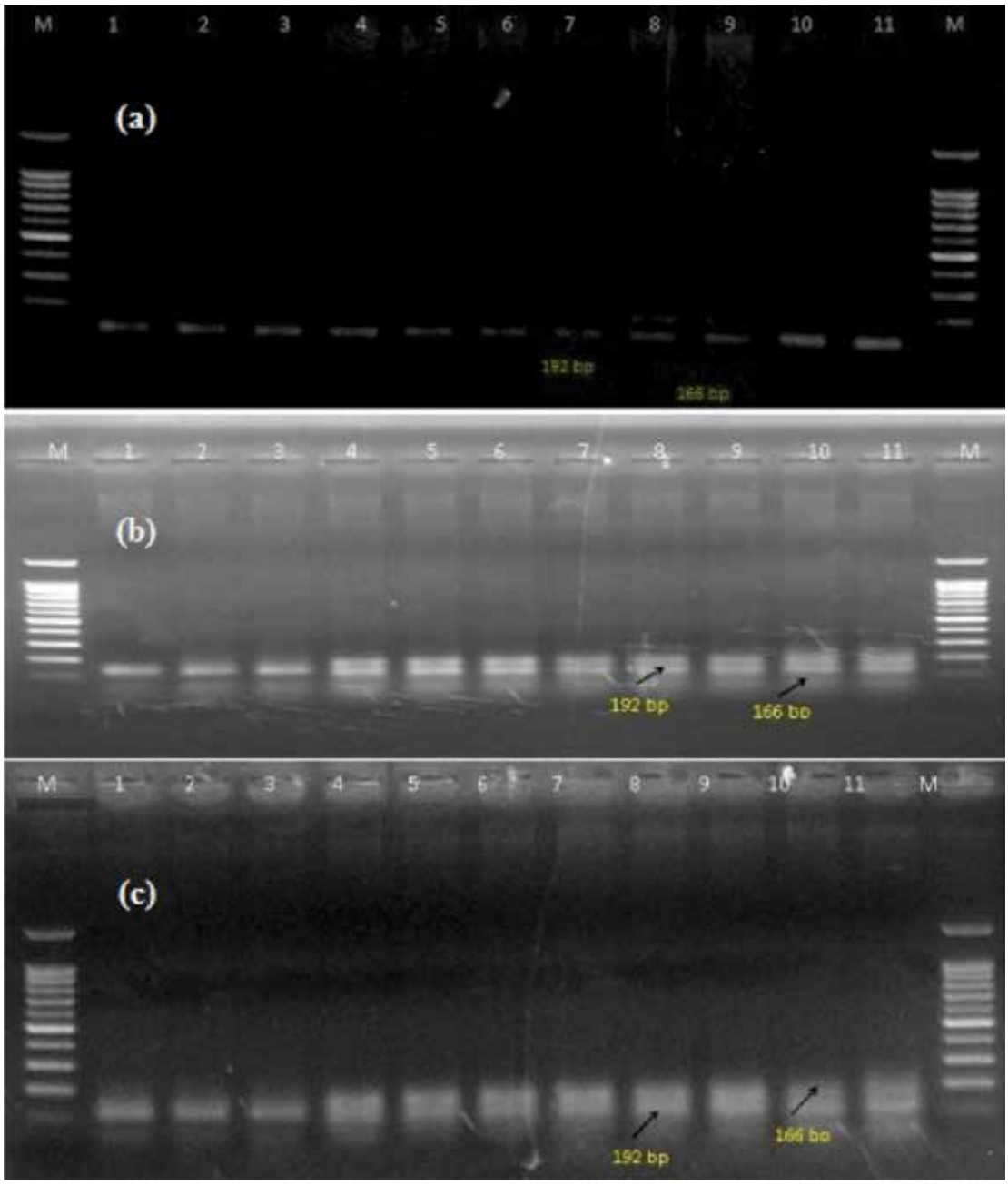

Fig. 1. Gene expression analysis of Amylase during development and ripening of: **(a)** William; **(b)** Pisang; **(c)** G. naine using RT-PCR. Actin gene served as endogenous control. The number indicates stages of development and ripening. The product size is also indicated as bp. M, stands for marker.

were done. Fig. 1-2 demonstrate the significant variations in the expression level of the α-amylase gene transcripts during development and ripening stages of Williams'. Transcription of the gene is initiated at pre-climacteric stage 4. Before this i.e. first three stages the transcript signals are undetectable. Gradually the RNA accumulation (gene expression) increases as evident from the intensity of the amplified products during middle to later stages. At stage 6, the α-amylase gene exhibits the maximum expression signals as shown by bright bands. This might indicate the ripening stage when starch breakdown is at the highest stage. After this maximum expression phase there is a uniform trend in gene expression in the next stages of ripening indicating continuous decrease in starch content. It is clear from the above data that amylase gene expression is increased during fruit

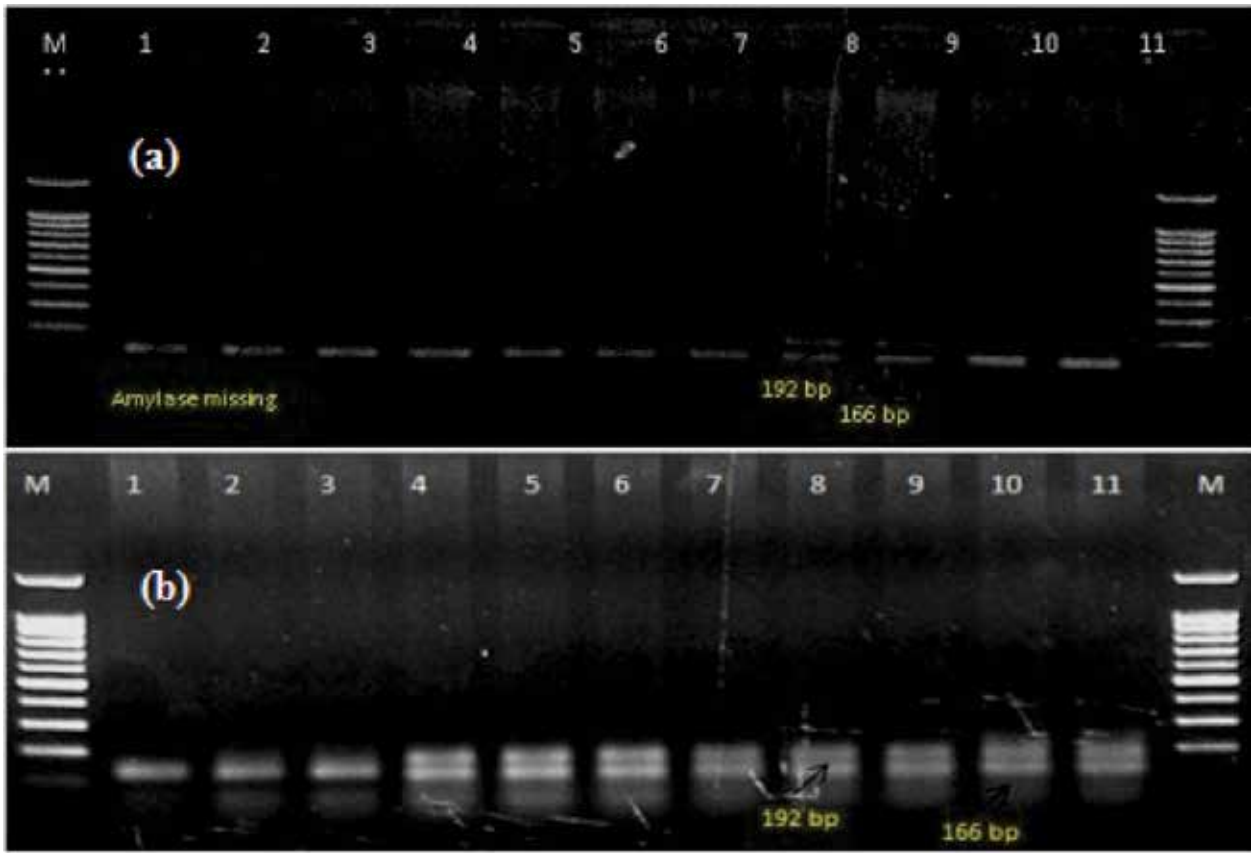

Fig. 2. Gene expression analysis of Amylase during development and ripening of: **(a)** Basrai; and **(b)** Brazillian using RT-PCR. Actin gene served as endogenous control. The number indicates stages of development and ripening. The product size is also indicated as bp. M, stands for marker.

ripening stages as compared to other developmental stages. The overall trend of the gene expression in all the varieties (Williams, Grand-naine (G-naine), Brazillian, Pisang and Basrai) was similar with slight variations.

The above gene expression results are in complete agreement with [15] wherein the authors reported significant increase in amylase transcript during ripening. In control fruit, peak of transcripts was recorded on day 16th, while in ethylene-treated fruit abundance of the mRNA was observed on day 2, and transcript accumulation in fruits was delayed by the 24th day. Similar results about amylase gene were also obtained by [16]. The aroma and taste of tomato fruits were found to be influenced by the accumulation of sugars and organic acids. During fruit ripening a conversion of starch to sugars takes place; this alters the taste, and eventually, the quality of the ripe tomato fruits. β-amylases, a group of major starch hydrolytic enzymes involved in starch degradation were examined in developing cherry tomatoes. Accumulation of the gene transcripts at the plastidial isoenzymes were observed, this was associated with later activation of the β- amylases enzyme at development and maturation of tomato fruits, contributing to the depletion of starch and the increase of the total soluble solids.

3.2. Starch Contents Reduces while Sugar Content Increases during Development and Ripening Stages

Values of starch and sugar contents in different varieties of banana (Fig. 3-4) were determined to correlate the α-amylase gene expression with biochemical alterations. As the fruit ripening progress, there was a reduction in starch content of the fruit in all the varieties. Because of the breakdown of starch and its conversion into sugars, the total sugar content of fruit increased (Fig. 3-4). Consequently, the fruit becomes sweet as ripening is progressed. Though level of starch breakdown and sugar content varied in different varieties but

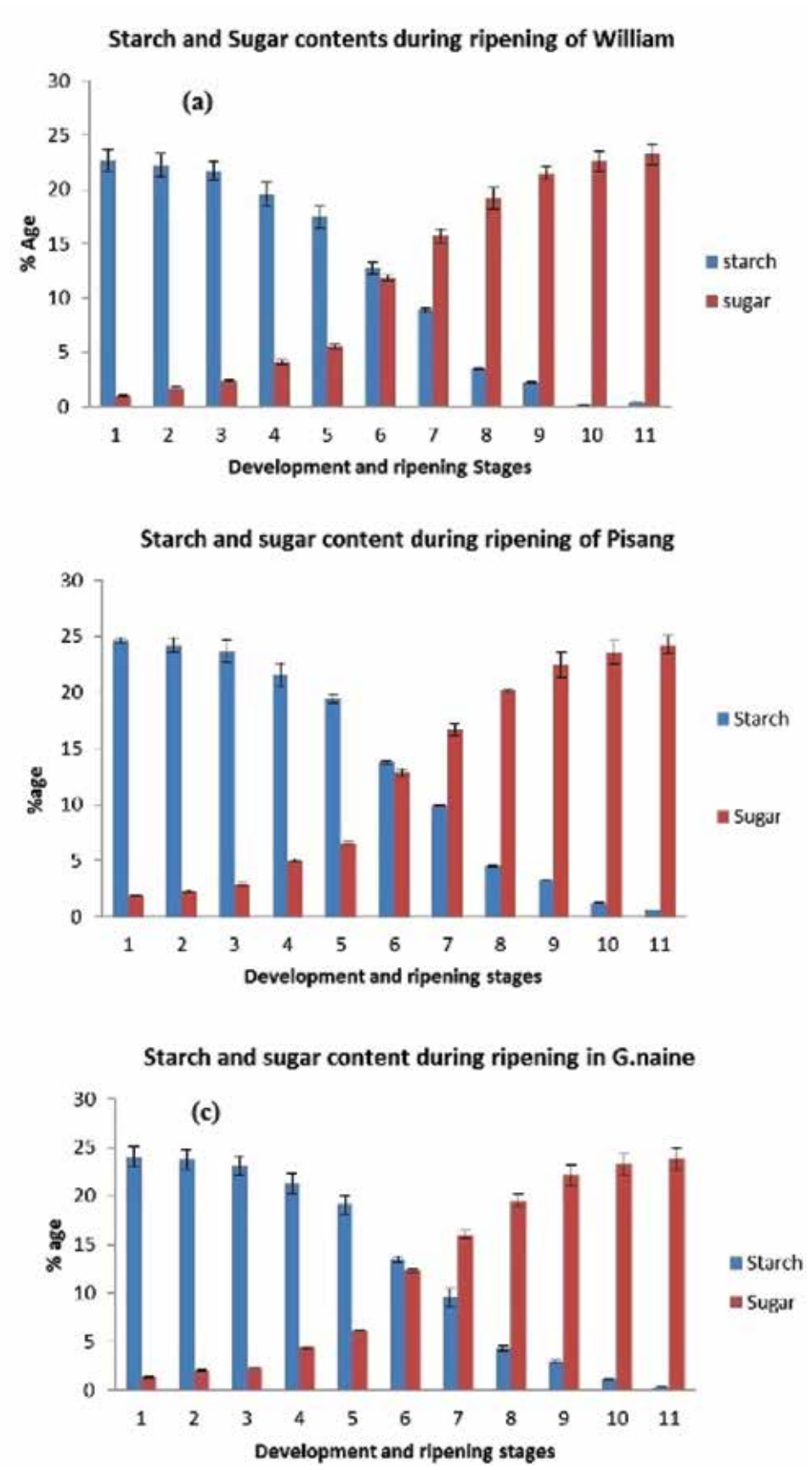

Fig. 3. Starch and sugar contents in: **(a)** William; **(b)** Pisang; and **(c)** G. naine during development and ripening.

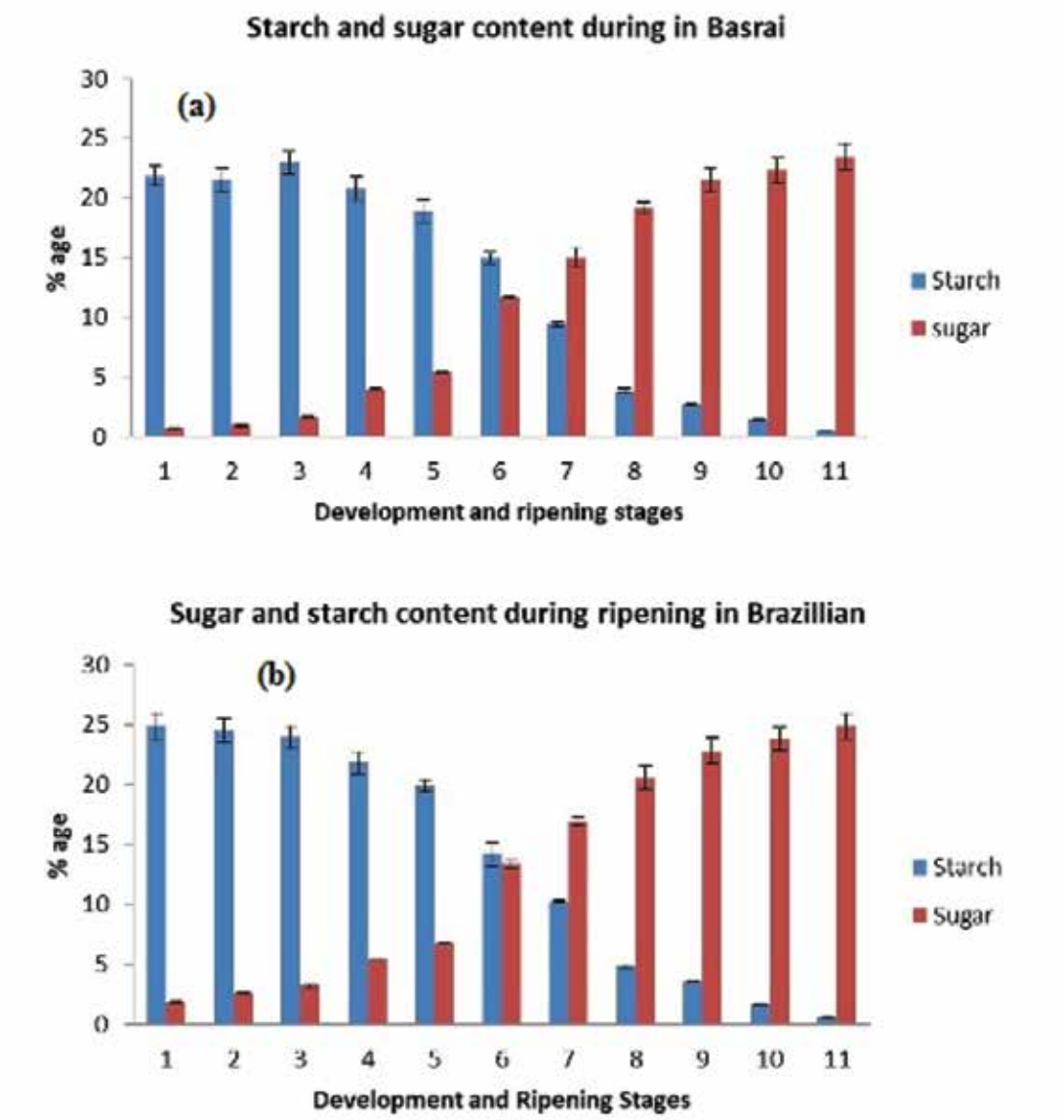

Fig. 4. Starch and sugar contents in: **(a)** Basrai; and **(b)** Brazillian during development and ripening.

the overall trend was similar. This may be due to varietal differences and expression divergence of amylase gene in different banana varieties. The highest sugar contents are recorded in Psang (24.23) and Brazillian (24.20%) whereas the lowest sugar contents are found in Williams' (23.23%) at the final stage. Starch content is higher in Brazillian (24.90) and lowest in Basrai (21.88%) cultivar. Our results are in corroboration with [17] who indicated a major increase in total sugar content and decrease in starch content during ripening of banana. This could be due to the hydrolysis of starch into sugar during ripening. Similar findings were also reported by Garcia & Lajolo [18].

The starch and sugar contents at particular stage can be administered to produce a food product having desired range of sugar and starch. That can also be helpful in developing food products for diabetic patients.

4. CONCLUSIONS

This study revealed that transcript expression for α-amylase gene is weaker in the earlier stages but eventually becomes stronger as the ripening stages progress. Overall trend was similar in all banana cultivars, with slight divergence. Similarly there was a significant decrease in starch content

in all varieties at ripening stage but there was also simultaneous increase in sugar contents due to breakdown of starch. The results of this study will help those who seek to produce food products from these banana cultivars. A particular stage of banana ripening can be selected to produce products having desired level of sugars. Expression analysis will also help to control the accumulation of sugars at a particular growth stage.

5. ACKNOWLEDGEMENT

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