

## Role of phosphorylase and amylase in accelerated ripening of mutant banana 'Basrai-10Gy'

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'Basrai-10Gy', a mutant developed by  $\gamma$ -irradiation of shoot-cultures of the 'Basrai' banana, ripened much faster compared to its parent. The biochemical mechanism underlying the faster ripening of the mutant was investigated. Ripening was assessed by determining textural changes in the whole fruit, the peel and the pulp, as well as from the physical changes of ripening. The activities of amylase (E.C.3.2.1.3) and starch phosphorylase (E.C. 2.4.1.21), the enzymes that initiate the catabolism of starch, were comparatively evaluated in the parent and mutant variety. They were correlated with the ripening behaviour of the fruit. An increase in the amylase activity, associated with the climacteric and normal ripening behaviour, was observed in 'Basrai' bananas; while, the phosphorylase activity was comparatively insignificant. The mutant variety showed low amylase and very high phosphorylase activities, suggesting the direct availability of sugar phosphate instead of free sugars to accelerate the ripening of 'Basrai-10Gy' fruit.

**Keywords:** amylase, banana ripening, mutant, starch metabolism, starch phosphorylase

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### Introduction

India is the largest producer of fruits in the world and banana tops the list with respect to production and area of cultivation<sup>1</sup>. For the importance of this fruit crop to total world's food production, a number of laboratories are trying to improve the crop<sup>2,3</sup>. Conventional breeding of banana is known to be extremely difficult due to female sterility in the edible triploid varieties and problems in getting viable seeds<sup>4</sup>. In consequence, mutation breeding and micropropagation are employed as a promising technique for the development of diverse varieties of bananas and plantains.

In authors' laboratory, irradiation of multiple shoot cultures from a number of bananas varieties, like 'Basrai', 'Rastali' and 'Lalkela', could lead to the development of stable varieties, which showed improved characteristics. The individual shoots (of Basrai or other varieties) were cultured on a half strength

Murashige and Skoog medium (agar 8%). These shoots, when developed to 3-4 cm size, were exposed to  $\gamma$ -rays in a <sup>60</sup>Co irradiator. Subsequently, the leafy portion was excised and the shoot tips alone were isolated and recultured on the same medium. The plants, developed from such shoot cultures of Basrai banana by exposing to 10Gy dose, were named 'Basrai-10Gy', a new variety. 'Basrai-10Gy' yielded larger sized bunches, having more and bigger fruits<sup>4</sup>.

Studies on the ripening profile of 'Basrai-10Gy' fruits showed them to ripen much faster than the parent variety. One of the most striking changes during banana ripening was the total hydrolysis of starch (20-25% of the fresh weight) to soluble sugars. Earlier studies from this laboratory on various aspects of starch utilization, such as glycolysis and the hexose monophosphate shunt, the regulation of phosphofructokinase, gluconeogenic activities and the glyoxylate pathway, have demonstrated an excellent correlation between the metabolism of carbohydrate and the ripening process in banana<sup>5-12</sup>.

Hence, an evaluation of the status of primary enzymes responsible for the initiation of starch utilization during the ripening of banana, namely starch phosphorylase (E.C.2.4.1.21) and amylase (E.C.3.2.1.3), was undertaken to find out their role, if

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**Abbreviations:** DNSA-Dinitrosalicylic acid; Gy-Gray; HEPES-N-2-Hydroxyethyl piperazine-N'-2-ethane sulphonic acid; PFK-Phosphofructokinase; TCA-Trichloroacetic acid

any, in the faster ripening of the mutant variety, 'Basrai-10Gy'.

## Materials and Methods

### Materials

Freshly harvested banana (*Musa cavendishii*; Family: *Musaceae*), locally known as 'Basrai' and its mutant, 'Basrai-10Gy' were harvested at a maturity of 70-75% from the Gamma Gardens of Plant Biotechnology Section of Nuclear Agriculture and Biotechnology Division, BARC, Mumbai. The substrates, biochemicals, fine chemicals and buffers were of analytical grade and purchased from Sigma Chemical Co., USA; Qualigens, Glaxo Laboratories, India; and SISCO Laboratories, Mumbai.

### Preparation of Fruits

The maturity of the fruits was determined from the peel to pulp ratio of the fruits<sup>5</sup>. Hands having identical maturity (having fruits with a peel to pulp ratio of 0.6-0.66) were cut from both varieties of the fruits and the latex was allowed to drain off. The cut portion of the fruit was smeared with the antifungal agent, Bavistin (Bayer, India). The hands of identical maturity, cut from the same variety of fruits, were placed together in perforated polythene bags. 'Basrai' and 'Basrai-10Gy' fruits were separately stored at 25°C in properly ventilated boxes. The average weight, length, and circumference of the banana fingers in the selected hands were 170-190 g, 16-18 cm, and 13-14.5 cm, respectively.

### Evaluation of Ripening

#### Determination of Respiratory Profile of Fruits

The respiratory profile of fruits was determined by Patenkofer's method by measuring the respiratory carbon dioxide output of the fruits as detailed in Surendranathan and Nair<sup>13</sup>.

#### Determination of Texture

Changes in the texture consistency of the whole fruit, the pulp and the peel were determined as an index of ripening using an Instron Universal Testing Machine TM Model (Instron Engineering Corp., Canton, Massachusetts, USA), using cylindrical brass probes of 1 mm diam for the whole fruit and 6 mm diam for the peel and the pulp as detailed in Bhushan and Thomas<sup>14</sup>. Fruits of identical weight, length and circumference were taken for the studies and the pressure needed to puncture the whole banana through its diameter, and the peel and pulp were independently recorded. The instrument was calibrated each day of

the test. The crosshead and chart speeds were 2 and 10 cm min<sup>-1</sup>, respectively.

### Visual Evaluation of Ripeness

The colour of the peel and ripeness was assessed from the appearance of the fruit, aroma and hand feel.

### Enzyme Assays

#### Isolation of Crude Enzyme

The crude enzyme was isolated as detailed in Iyer *et al.*<sup>5</sup>. Unless mentioned, all operations were carried out at 2-4°C. Fruits of identical maturity were selected in triplicate from both the varieties; the peel and pulp were separated and cut into small pieces. Ten grams of peel or pulp tissues were weighed into 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 50 mM Hepes, pH 7.5, 5 mM MgCl<sub>2</sub>, 0.2% Triton X 100 and 20 mM β-mercaptoethanol. The slurry obtained was filtered through a double-layer of cheesecloth, centrifuged for 20 min at 15000 rpm in a Sorvall RC-5 refrigerated centrifuge (USA). The clear supernatant obtained was used as the crude enzyme.

#### Amylase Activity

It was determined by assaying the formation of reducing sugars in the reaction mixture. The reaction mixture consisted of 0.2 ml 0.1% starch in 50 mM acetate buffer, pH 5.6, 0.2-1.0 µg of crude enzyme extract (depending upon the enzyme activity) and water to a final volume of 0.3 ml. The reaction mixture was incubated at 50°C and after 10 mins, 1 ml of 0.5 M acetic acid was added to stop the reaction. Acetic acid was added to the reference tube prior to the addition of the enzyme.

The reducing sugar formed during the reaction was determined by the DNSA method<sup>15</sup>. A unit of enzyme activity was defined as the µ moles of reducing sugar formed per minute per ml and the specific activity as the units of activity per mg protein.

#### Phosphorylase Activity

The phosphorylase activity was determined as detailed in Surendranathan and Nair<sup>13</sup>, by assaying the inorganic phosphate formed in the reaction mixture. The reaction mixture consisted of 50 mM Hepes buffer, pH 7.5, 5 mM MgCl<sub>2</sub>, 10 mM β-mercaptoethanol, 0.4 ml 0.5% starch, 20 mM potassium phosphate buffer, 0.2-2 µg protein of crude enzyme extract and water to a final volume of 1ml. To the reference tube 0.2 ml of 20% TCA was added prior to the addi-

tion of the enzyme. The reaction mixture was incubated at 30°C for 20 min. The precipitate formed, if any, was removed by centrifugation at 15000 rpm for 10 min and the supernatant was assayed for enzyme activity by the method of Fiske and Subbarow<sup>16</sup>. Protein content was determined by the method of Bradford<sup>17</sup> using BSA as the standard.

## Results and Discussion

### Textural Changes during Ripening

In order to correlate the physiological stage of the fruit to the enzyme activity, a comparative evaluation of the change in texture of the whole fruit, pulp and peel of the 'Basrai' and 'Basrai-10Gy' fruits during ripening was made. Physico-chemical parameters, like change in colour, feel of softness and development of aroma were also correlated to the ripening (Table 1). Fig. 1 presents the textural changes of the fruit, pulp and peel of 'Basrai' fruit during ripening. The firmness of the whole fruit was determined as the pressure required to radially puncture the banana with a 1 mm brass probe (---). The pressure required to puncture the 'Basrai' fruit did not considerably change until the onset of climacteric (4<sup>th</sup> day). During the climacteric (7-8<sup>th</sup> day), there was a 70% decrease in the pressure required to puncture the fruit. The changes in puncture pressure required showed an excellent correlation with the respiratory profile of the fruit (...\*), confirming that textural changes are an excellent indicator of the stage of ripening of the fruit. An identical relationship was observed for the pressure required to puncture the peel (-●-) as well as pulp (-○-) during ripening of 'Basrai' fruits. In contrast, 'Basrai-10Gy' fruits, though of identical maturity of 'Basrai' fruits and harvested on the same day, did not show a typical post-harvest respiratory profile during ripening (Fig. 2; ...\*). The pressure required to puncture the fruit also showed a drastic decrease, from 6 kg cm<sup>-2</sup> initially to 2 kg cm<sup>-2</sup> on the 4<sup>th</sup> day. On the 6<sup>th</sup> day, the pressure requirement decreased to 0.1 kg cm<sup>-2</sup> (---).

The puncture pressure of the peel (-●-) and the pulp (-○-) showed similar trends. The 'Basrai-10Gy' fruit, having identical maturity like 'Basrai', was unripe on the 1<sup>st</sup> day after harvest, was turning yellowish and soft on the 2<sup>nd</sup> day when it reached the climacteric peak and on the 3<sup>rd</sup> day it was a ripened fruit. Thus these data showed that the textural changes of the fruit measured in terms of puncture pressure could be used as an index for the ripening. Furthermore, these studies showed that 'Basrai 10Gy' fruits did not follow the normal ripening pattern of its parent variety.

### Amylase Activity Profile in 'Basrai' and 'Basrai-10Gy' Fruits

A comparative profile of amylase activity in ripening 'Basrai' and 'Basrai-10Gy' varieties has been given in Fig. 3. 'Basrai' fruit pulp (-□-) showed low amylase activity during the preclimacteric and, at the onset of the climacteric, a concomitant enhancement parallel to the respiratory profile (Fig.1, ...\*). A similar enhancement of enzyme activity parallel to the respiratory climacteric has been reported for a number of glycolytic and other enzymes during ripening<sup>5,7,9,10,18,20</sup>. The peel enzyme activity also showed a

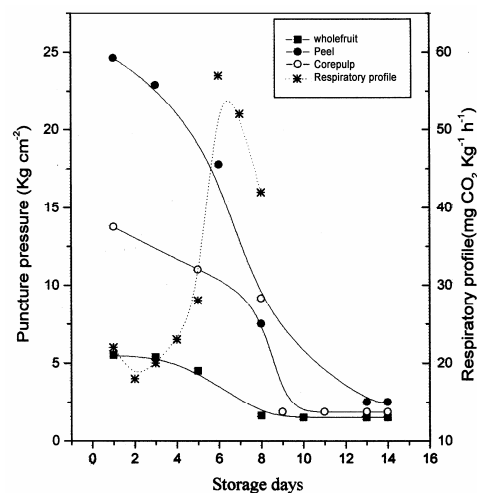


Fig. 1—Textural changes in 'Basrai' banana during ripening

Table 1—Sensory evaluation of ripeness of 'Basrai' and 'Basrai-10Gy' bananas

|                    |      |      |           |           |      |
|--------------------|------|------|-----------|-----------|------|
| Basrai             |      |      |           |           |      |
| Post-harvest days: | 1    | 2    | 4         | 6         | 8    |
| Colour             | G    | G    | GY        | Y         | Y    |
| Feel               | Hard | Hard | Semi-soft | Semi-ripe | Ripe |
|                    | R    | R    | S-R       | S         | S    |
| Basrai-10Gy        |      |      |           |           |      |
| Post-harvest days  | 1    | 2    | 3         | 4         |      |
| Colour             | G    | GY   | Y         |           |      |
| Feel               | R    | S    | F-R       | O-R       |      |

G: green; GY: Greenish yellow; Y: Yellow; R: raw; S-R: Semi-Raw; F-R: Fully ripe; O-R: Over-ripe; S: Soft

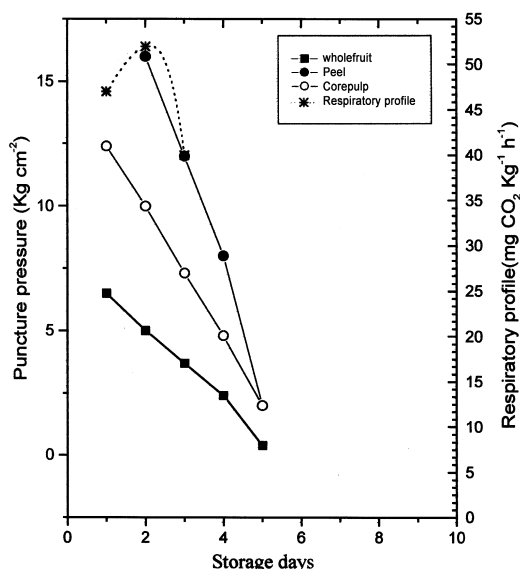


Fig. 2—Textural changes in 'Basrai-10Gy' mutant variety during ripening

similar trend. However, the activity of the enzyme in the peel was three times higher compared to the pulp (-■-). At the climacteric peak this difference in activity was much lesser. Comparatively, the amylase activity in the 'Basrai-10Gy' pulp was very low (·○·). Furthermore, the fruit did not show an upsurge in activity concomitant with the respiratory climacteric. The activity in the peel as in the case of 'Basrai' fruits, however, was much higher than in the pulp tissue (·●·). Thus, the amylase activity during climacteric was lesser in the pulp and peel tissues in the fruits of 'Basrai-10Gy' and the increase in activity along with the climacteric peak was absent. This suggests that the hydrolysis of starch to glucose by amylase is not sufficient to meet the energy requirements during the ripening process.

#### Phosphorylase Activity in 'Basrai' and 'Basrai-10Gy' Fruits

Fig. 4 shows the comparative activity profiles of starch phosphorylase in the peel and pulp of 'Basrai' and 'Basrai-10Gy' banana. The peel and pulp of 'Basrai' fruits showed low enzyme activity. Furthermore, there was no climacteric related upsurge in activity in these fruits (-■-, -□-), suggesting that unlike amylase, phosphorylase does not catalyse ripening related to the hydrolysis of starch in 'Basrai' fruits. 'Basrai-10Gy' pulp, on the other hand, showed a 1.7-fold enhancement by the second day and 3-fold by the 4<sup>th</sup> day of harvest (-○-). In case of the peel tissue, the increase in activity was about 3-fold by the 2<sup>nd</sup> day and on the 4<sup>th</sup> day of harvest it increased sharply to 8-fold

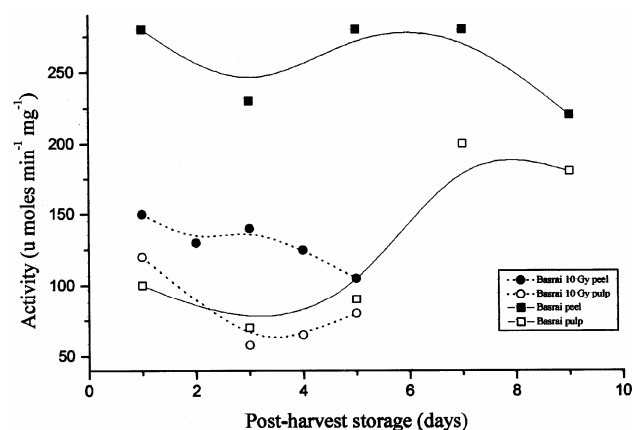


Fig. 3—Amylase activity profiles in 'Basrai' and 'Basrai-10Gy' banana fruits

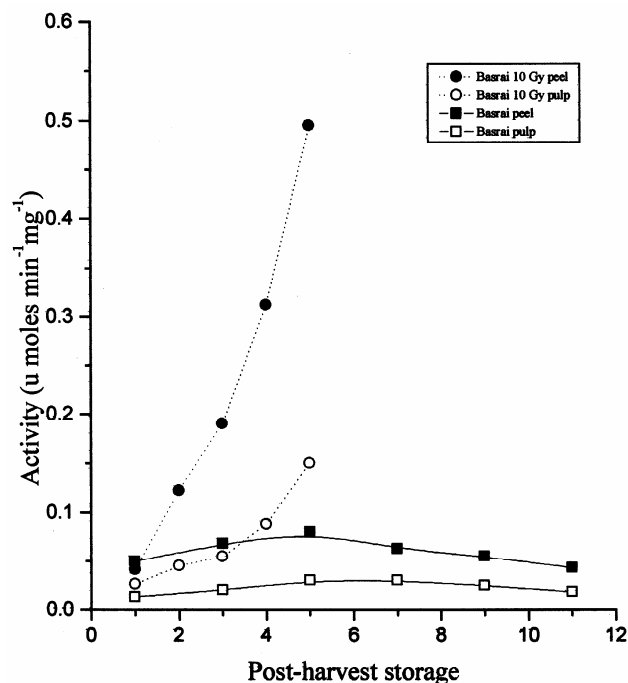


Fig. 4—Starch phosphorylase activities in 'Basrai' and 'Basrai-10Gy' banana fruits

(-●-). Thus, a comparative evaluation of activities of both the enzymes in both the cultivars (Figs 3 and 4) suggested that while amylase facilitated starch hydrolysis in 'Basrai' fruits, starch phosphorylase is the enzyme that catalysed ripening related starch hydrolysis in 'Basrai-10Gy' fruits.

The activity enhancement of enzymes of starch catabolism parallel to the respiratory climacteric has been reported earlier for a number of enzymes. Iyer *et al.*<sup>5</sup> reported several-fold enhancement of PFK activity parallel to the respiratory climacteric during the ripen-

ing of bananas. Isaac and Rhodes<sup>18</sup> reported an identical result in ripening tomatoes. 'Basrai' bananas showed a similar trend in the activity profiles of amylases (Fig. 3). Hence, it may be concluded that the metabolism of starch utilization and ripening in 'Basrai' banana is identical to that of other fruits. It also suggests that phosphorylase does not contribute substantially to starch hydrolysis in 'Basrai' fruits as the activity was minimal and did not exhibit a climacteric related upsurge (Fig. 4).

On the other hand, phosphorylase activity in 'Basrai-10Gy' showed a 3-fold enhancement in pulp and an 8-fold enhancement in the peel during ripening. Also, correlation of these activities to the ripening index (Fig. 2) and to the physical changes in the fruit suggests that phosphorylase made available starch for further oxidation and the generation of energy for the ripening process. Starch phosphorylase facilitates the single step conversion of starch to glucose 1-phosphate. This avoids the utilisation of a high-energy molecule like ATP for the phosphorylation of glucose (formed by the amylase reaction), which is a prerequisite for further oxidation reactions. The direct supply of glucose 1-phosphate instead of free glucose could be one of the reasons for the quicker ripening of the 'Basrai-10Gy' fruits.

Another interesting observation in these studies is that both the enzymes are several-fold more active in the peel than in the pulp (Figs 3 & 4). It may be because the ripening process is initiated in the peel before the pulp. This is further supported by the fact that the endogenous ethylene generated in the peel tissue is responsible for triggering the ripening process, at least in climacteric fruits<sup>21,22</sup>. However, this could only be confirmed after an assessment of the key enzymes of ripening and the correlation of their activity profiles with endogenous ethylene levels in peel and pulp tissues.

## Conclusions

An evaluation of the activities of amylase and starch phosphorylase in the pulp and peel tissues of 'Basrai' and its mutant 'Basrai-10Gy' (obtained by irradiating shoot cultures of the 'Basrai' variety) during the post-harvest storage period suggested that at least one of the factors responsible for the quicker ripening of 'Basrai-10 Gy' banana was the enhanced activation of starch phosphorylase. The peel tissue of the fruit showed a 2- to 7-fold increase in the activity of starch phosphorylase when compared to the pulp tissues. The ripening stage of the fruits could be de-

termined from an assessment of the change in texture of the whole fruit, the pulp and the peel during storage, the post-harvest respiratory profile and the colour and feel of the fruit.

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