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Physiological, Biochemical and Cellular Changes Associated With the Ripening Of Bitter Less Bitter Gourd (*Momordica Dioica* Roxb. Ex Willd.) Fruits

[1,]Payal T. Shah and [2,]T. V. Ramana Rao
[1,]*Department Of Biosciences Sardar Patel University VALLABH VIDYANAGAR- 388 120 Gujarat, INDIA*
[2,]*Asst. Professor Shree P. M. Patel Collage, ANAND, Gujarat: INDIA*

ABSTRACT

Fruits of Momordica dioica were selected at seven sequential developmental stages, starting from very young stage to post ripened stage and were analyzed. From the study physiological and biochemical changes it was concluded that gradual decrease was found in chlorophyll-a (5.25 fold), chlorophyll-b (13.0 fold), total chlorophyll (8.23 fold), starch (6.5 fold) and free amino acids (14.4 fold), while in case of total proteins decrease was observed from pre-mature stage to post ripened stage 67.2 percent. In case of RNA, decrease was found 55.1 percent from mature stage to post ripened stage. However, gradual increase level was found only in carotenoids (2.5 fold) and in total sugars (209 percent), non-reducing sugars (317 percent) and phenol (2.9 fold) increase was found from very young stage to pre-ripened stage. In contrast the amount of anthocyanins, reducing sugars and DNA was unstable.Hydrolytic enzymes viz., Amylase, Invertase and Peroxidase; the activity of Amylase decreased from mature stage to post ripened stage (86.73 percent). While activities of Invertase and Peroxidase was noticed in 94.3 percent decreased and 44.64 percent increased from very young stage to mature stage respectively.In contrast, the activities of cell wall degrading enzymes (such as cellulase, polygalactouronase (PG) and pectin methyl esterase (PME)) 6.38 fold increase activity was noticed in cellulase activity from very young stage to ripened stage while during ripening PG activity gradually increased with 2.37 fold, whereas the activity of PME was noticed inconsistent. These biochemical changes also reflect on cellular changes such as increasing cell number, enlargement of cell, decreasing cell content, separation of middle lamella etc., were observed during the mature stage to post ripened stage. The low production of ethylene and an increased rate of respiration indicates that the bitter less bitter gourd fruit falls under the category of climacteric fruits.

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I. INTRODUCTION

Momordica dioica (Bitter less bitter gourd, kakrol, Teasle gourd) is a perennial, dioecious climber with thickened roots, which belongs to cucurbitaceae family occurring in Southern Asia. Bitter less bitter gourd is relatively small and oval in shape. This fruit is in demand for internal as well as external markets due to its medicinal properties and is also rich in calcium, phosphorus, iron and carotenoids. Its immature fruits are berries; softly echinate which are used as a vegetable (More and Nayar, (1998). Luo, et al., (1998) found three triterpenes and two steroidal compounds were isolated from the dry root of *Momordica dioica*. Their structures were elucidated by spectral analyses (MS, IR, 1HNMR, 13CNMR and DEPT) and chemical methods.Ali and Srivastava (1998) found two new chemical constituents for the first time from the fruit of *Momordica dioica* along with the known sterol compound and an unknown pentacylic triterpene isolated from the seeds. A more or similar study on phyotochemical constituent was previously studied by Shantha, and Radhakrishnaiah (1993) in the *Momordica* sps. From the above report it was derived that this plant is not only helpful for the medicinal purpose but is also commercially important. There are very few reports cited on physiological studies such as increase in respiration, ethylene production, carotenoids synthesis, chlorophyll degradation and hormonal changes was previously reported (Kays and Hayes (1978); Zheng, (1986) Tan *et al*., (1999), Rodriguez *et al.,* (1976), Huong & Raymundo (1999), Basu *et al*., (1994), in *M. charantia* but not in *M. dioica* as a result this fruit was selected for present study.For giving idea about fruit ripening this study was alienated in five divisions (i) Changes in pigments (ii) Changes in primary metabolites (iii) Changes in hydrolytic and cell wall degrading enzymes (iv) Changes in cellular structure & (iv) Changes in respiration rate and ethylene production.

II. MATERIALS AND METHODS

The fruits of bitter gourd bitter less were collected from local fields at seven sequential stages, starting from very young stage to post-ripening stage. The length and breadth were measured for the collected fruit samples before they were subjected for their physiological and biochemical analyses and their remarkable data were tabulated (Table 1, Pl. 1.1). Biochemical analysis of pigments (Chlorophyll, Carotenoids & Anthocyanins) and primary metabolites (Sugars, Starch, Total Proteins, Total Phenols & Free Amino Acids) were measured from the collected fruit samples by using different methods cited by Thimmaiah (1999). The estimation of nucleic acids (DNA & RNA) was performed by the methods of Devi (2002). Amylase, Invertase, Peroxidase, Polygalactrouronase and Pectin Methyl Esterase were extracted and assayed according to the procedure described by Selveraj and Pal (1984) & Thimmaiah (1999). Data presented at each stage were the mean of three replicates (± Standard error, S.E.). Data of the experiment were subjected to statistical analysis using Duncan's multiple range test (DMRT) (Bliss, 1967).The histological and ultra structure studies were carried out from the last four stages (including mature stage to post ripening stage) of bitter gourd fruits. The collected fruits were fixed in Formalin Acetic Acid (FAA) (Berlyn & Miksche, 1976). Customary methods described by Johansen (6) were followed for the dehydration and embedding of these FAA fixed materials. The sections of 8 - 10 µm thick were cut with the AO Spencer's rotary microtome from the paraffin embedded materials and then they were stained with safranin - fast green combination. The stained sections of fruit were observed under Carl-Zeiss MP3 400 Microscope and photomicrographs were taken with same microscope with an Image Analyzer Facility. For the TEM studies, the preparation of fruit materials has been done as per the methods cited by Dawes (1971) and the ultra thin sections were observed under the Philips Tecnai (High Tension 200 KV) TEM. The amount of ethylene synthesis and the rate of respiration were measured from mature, pre- ripening, ripening and post ripening stages with the Perkinelmer Autosystem XL Gas Chromatography as per the procedures cited by Thimmaiah (1999).

III. RESULTS AND DISCUSSION

3.1.Changes in Pigments:-

The quantitative analysis of the Chlorophylls revels that the amount of Chlorophyll a (0.042 mg/gm), Chlorophyll b (0.065mg/gm) and Total chlorophyll (0.107 mg/gm) were very high in very young stage, but as the fruit proceeds towards the maturation, ripening and post ripening stages the amount of chl. a (0.008 mg/gm), chl. b (0.005 mg/gm) and total chl. (0.013 mg/gm) were found declined 5.25 fold, 13.0 fold and 8.23 folds respectively. Carotenoids (0.067 mg/gm) were found gradually increasing from very young stage to almost 2.5 folds at post ripening stage (0. 170mg /gm). Anthocyanins dose not show consistency in their quantity during the maturation, ripening and post ripening stages of fruit. The maximum amount of anthocyanins (6.365 mg/gm) found in the young stage of fruit.Merzlyak *et al.,* 2003 found that during the analysis of pigments, amount of chlorophyll were decline, carotenoids increases due to the pigments changes at terminal stages of leaf and fruit development in many plant species. At these stages plant tissues retain certain amounts of carotenoids or carotenoids synthesis was stimulated on the background of chlorophyll degradation. The increasing level of caroetnoids and decreasing level of chlorophyll also recently noticed by Wang and Zhong (2005) during ripening of tomato fruit. The decreasing level of chlorophyll a, chlorophyll b and Total chlorophyll and increased level of carotenoids were also previously reported in the cucurbits during ripening and storage period by Tan *et al.,* (1999), Rodriguez *et al*., (1976) and Huong & Raymundo(1999). Anthocyanins were found unstable during ripening which was observed by Underhill and Critchley (1993) in Litchi fruit due to polyphenol oxidase degradation.

3.2.Changes in primary metabolites:-

The quantitative analyses of sugars (i.e. total sugars, reducing sugars and non reducing sugars) indicate that the amount of total sugars was gradually increased from very young stage (0.986 mg/gm) to pre-ripened stage (3.047 mg/gm) but there after their amount declines which displayed 209 percent increase. However, the quantum of reducing sugars does not show consistency. In contrast non-reducing sugars was gradually increased from very young stage (0.686 mg/gm) to pre-ripened stage (2.864 mg/gm) which displayed 317 percent increase, but thereafter declining nature was seen in remaining stages.A more or similar observation was also noticed in the apple fruit during there ripening process (Sabir *et al.,* 2004). From the data it was concluded that increase in sugar might be due to hydrolysis of polysaccharides or due to less acidity and ascorbic acid contents. The contents of reducing sugar and non-reducing in spongy tissue may be also due to lower activities of invertase and amylase (Luiz *et al.,* 2001).The amount of starch was observed 1.380 mg/gm at very young stage but thereafter a steady decline was found in the post ripened stage 0.211 mg/gm which displayed 6.5 fold decrease. According to Luiz *et al.,* 2001 during development stage of mango, starch was hydrolyzed. However, as the fruit becomes over-ripe, only traces of starch were detected and amylase activity was substantially reduced.

The sugars in the pulp spongy tissue were accounted by the presence of higher content of starch, which remained unhydrolysed due the low activity of amylase.The quantitative analyses of the total proteins, free amino acids and total phenols in *M. dioica* reveal that initially the amount of total protein does not show consistency during their growth development and ripening process. The amount of total protein was decrease from pre-mature stage (7.644 mg/gm) to post ripened stage (2.500 mg/gm), which showed 67.2 percent decrease. The amount of total phenol was gradually increasing from very young stage (2.074 mg/gm) to pre-ripened stage (6.123 mg/gm) which showed 2.95 fold increase thereafter it declines in later stages. In, contrast of proteins and phenols the amount of free amino acids shows 14.4 fold gradual decrease.Hulme *et al.,* (1968) and Dilley (1972) reported that in the early stages of fruit protein synthesis is stimulated and reaches a peak value of climacteric thereafter it declines slowly and gradually during ripening process. An increase in protein synthesis during early climacteric period has been measured in many fruits observed by Sharma (2000). The sharp decrease in free amino acids towards maturity and onset of ripening has been attributed to their incorporation into protein required for the synthesis of various ripening enzymes (Frunkel, Klein and Dilley, 1965). This declining trend of amino acids has been noticed by a number of workers during the approach of fruit maturity (Sharma, (2000)) According to (Dilley, 1970 and Sharma, 2000) the phenolics content of most fruits declines from high levels during early growth to low levels when the fruit is considered to be physiologically mature and therefore, susceptible to the induction of ripening. A more or similar observation was found by Kumar and Goswami (1985) that the presence of higher concentration of phenolics during early stage of development provides protection mechanism to the phytohormones like auxins, gibberellin and cytokines, which play an important role in cell division and cell enlargement. The quantitative analysis of nucleic acids (i.e. DNA & RNA) has been carried out in the seven sequential developmental stages of fruit. The amount of DNA remains inconsistent during ripening while, RNA does not shows consistency in earlier stages. RNA was found decrease from mature stage (0.261 mg/gm) to post ripened stage (0.117 mg/gm), which displayed 55.1 percent decrease.

During the climacteric period the RNA was increased and there after its decline up to ripening stage, while in the case of DNA it is fluctuated during the ripening noticed by (Sharma 2000).Amylase activity was found to be gradually declining from very mature stage (0.377 activity) to the post ripened stage (0.050 activity), which displayed 86.73 percent decrease. In contrast, Invertase shows increase in first stage (0.037 activity) and declines upto mature stage (0.019 activity), which displayed 94.3 percent decrease and remains unstable for remaining stages. In case of Peroxidase was gradually increased from very young stage (0.274 activity) to mature stage (0.495 activity) that displayed 44.64 percent increase. These hydrolytic enzymes were observed very high in the early stage of development and thereafter they declined towards the ripening due to starch disappearance, hydrolysis of sugar, climacteric periods or related to growth development process respectively. The activities of hydrolytic enzymes increased from early mature stage to peel colour turning stage and declined in later ripening stage of pineapple fruit during ripening was observed by Selveraj (1993). A more or similar observation also noticed by previously Hulme (1970) and Seymour *et al.,* (1993).

The activities of cell wall degrading enzymes viz., Cellulase, Polygalactrouronase (PG) and Pectin Methyl Esterase (PME) during the ripening of fruit were presented in (Table 1.5A, Fig. 1.5B). From analyzed of cell wall degrading enzymes indicate that there was an increment noticed in the activities of cellulase from very young stage (0.013 activity) to ripened stage (0.083 activity) which displayed 6.38 fold increase. PG enzymes were gradually increase 2.37 fold during ripening. While the activity of PME was gradually decline from mature stage (1.009 activity) to post ripened stage (0.800 activity) which displayed 20.7 percent decrease.It has been widely recognized that cell wall changes are related to fruit softening and that these modification are due to the action of cell wall enzymes. The accumulation of these enzymes during ripening and their often-concerted activity will result, eventually, in degradation to many cell wall polymers leading to cell separation and associated softening. During ripening increasing activities of cell wall softening enzymes were reported by Selveraj & Raju (2000) in Kagzi Lime fruit.

3.3.Changes in cellular structure:-

Histological Studies:-

The development anatomy of fruit has provided information about the prepositional cell division and cell enlargement in various parts of fruit. For better understanding of histological changes the fruits were selected from the mature stage to post ripened stage. It was found that mesocarps cells were divided into three categories (i) Outer mesocarps (ii) Middle mesocarps (iii) Inner mesocarps cell. Middle mesocarps cells and inner mesocarps cells are parenchyma cell, which contain abundant starch grains, as the middle mesocarps cells were comparatively thick; hence they were distinguished from the inner ones.

At mature stage in the formation of inner mesocarps cells parenchyma cells were very small in size and contained abundant starch due to cell division and cell enlargement. In the starting process of pre-ripened and ripened stage the cell number and cell size were increased while, starch grain was decrease. However, in the last stage the cells were collapsed and empty or contained very less amount of starch grains. During the different developmental stages of *Momordica dioica* fruit more or similar histological changes was reported by Thanki(1978) (Plate 2.2).White (2002) concluded that "Anatomically, fruit are swollen ovaries that may also contain associated flower parts. Their development follows fertilization, and occurs simultaneously with seed maturation. Initially, fruits enlarge through cell division and then by increasing cell volume".

3.4.ULTRA STRUCTURE STUDIES:-

This study helps in understanding the cell wall softening or changes in cell wall. For this reason the fruit were selected from mature stage to post-ripened stage. In the earlier development stages the cell wall becomes very hard but when the ripening process starts the cell wall becomes soft due to presence of cell wall degrading enzymes polygalactouronase(PG), pectin methyl esterase(PME) and cellulase. The investigations of plasma lamella was observed at mature stage, which was cited near cell wall, while, in pre-ripened stage the cell wall enlarged in their position, which contains granular cytoplasm, ribosome like bodies and nucleus. However, during the ripening and post ripening stages cell wall becomes despoiled. These structural patterns were also previously noticed by Thanki (1978) and Whitaker and Davis(1962) in cucurbitaceae fruit.

Brummell *et al.,* (2004) concluded that cell wall softening occur due to differences in cell wall thickness and composition, cell size, cell shape, packing, contents and turgor.

Changes in respiration rate and ethylene production:-

3.5.ETHYLENE AND RESPIRATION:-

The ethylene production was increase from pre-ripened stage to the post-ripened stage of the fruit, while the rate of the respiration was increased from mature stage to ripened stage, their after decline nature observed in the post ripened stage (Fig 2.6).According to Hardenuburg *et al.,* (1986), Zong *et al.,* (1995), Kays & Hays (1978) and Zheng (1986) conclude that the fruits of bitter gourd (*M. charantia*) was climacteric fruit due increasing rate of respiration and low production of ethylene. A more or similar observation was also noticed in *M. dioica* fruit.

3.6.ANALYSIS OF VARIANCE:-

The analysis of variance revels that the changes of fruit ripening such as Chl. a, Chl. b, Total chlorophyll, carotenoids, amino acids and the activities of amylase and peroxidase were highly significant (Tables 2.1, 2.3 & 2.4), while less significance was observed in total sugars, starch and cellulase activity (Tables 2.2 & 2.5). However, no significance was found in case of anthocyanins, reducing sugars, non- reducing sugars, total proteins, total phenols, nucleic acids, invertase, PG and PME activities (Tables 2.1, 2.2, 2.3 and 2.5)

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