Chapter 3

Physiological Role of Bioactive Compounds during Development of Mango Ginger Rhizomes

All great truths begin as blasphemies
— George Bernard Shaw
Participation of bioactive compounds in an array
teleological functions as precursors in imparting characteristic flavour, color, defense
intermediaries and health benefiting factors in fruits, vegetables, and rhizomes were well
documented [Tholl, 2006]. Temporal variation in the concentration of bioactive molecules
is regulated by a complex interaction between intrinsic plant factors and external factors,
both abiotic and biotic [Herms and Mattson, 1992; Beckman, 2000; Booij-James et al.,
2000]. Interestingly, they showed site and cells specificity in accumulation, as a function
of maturity [Kause et al., 1999; Samanani, 2002]. Lack of such studies in Mango ginger is
apparent despite its pharmaceutical importance and exotic mango flavour.

Coordinated biochemical alterations during development and growth mango ginger
plant determine the quality of rhizomes in terms of maturity, peak accumulation of
bioactive compounds in rhizome. Extensive review of literature showed lack of any
maturity index for harvest. This is critical in agriculture to know when to harvest the plant.
Successful identification of three bioactive compounds in mango ginger gave an impetus
to test teleological role of these bioactive compounds to define the maturity of rhizome for
harvest with preferred nutritional or nutraceutical or pharmaceutical quality. Among the
isolated compounds Difurocumenonol exhibited the highest antimicrobial and antioxidant
activity along with other functional property. Since it is highly stable and easy to estimate
accumulation pattern of difurocumenonol and phenolics along with changes in solubles
and storage components as a function of physiological maturity in mango ginger were
studied. A time course study of these changes from the time of planting to harvest, which
ranged from 0 to 240 days at a time interval of 30 days, were carried out. The details
were presented in this chapter.
Materials and Methods

Sample collection

The rhizomes were collected from commercial plot near Hassan, Karnataka, India. The first sampling time [60 days after planting] was conducted when the rhizomes initiated their growth. Subsequently, the samples were collected at 90, 120, 150, 180, 210 and 240 days after planting. Each sample was prepared from rhizomes obtained from five mango ginger plants that were harvested randomly from five different beds. All the biochemical analysis and other experiments were carried out in triplicates.

Chemical composition of mango ginger

Sample preparation

About 500 g of mango ginger rhizomes were sliced, homogenized, and squeezed in two-layered muslin cloth, to extract the complete juice. The juice was centrifuged at 8,000 rpm for 20 min at 4°C and used to determine pH, titrable acidity, total soluble solids [TSS], sugar content, protein content, and phenolic content and antioxidant activities.

The pulp [residue] left after the extraction of juice is still a rich source of bound phenolic compounds. Hence, the pulp was homogenized with 80% methanol to extract the phenolics completely. The extraction was repeated till it became colorless. The methanol extract was filtered, and evaporated using a rotary evaporator. The extract was dissolved and diluted to a final volume of 100 mL with 80% methanol. The mixture was centrifuged at 8000 rpm at refrigerated temperature [4°C] for 20 min and used for determination of total phenolic content, DPPH radical scavenging activity and total reducing power.

Extraction and quantification of difurocumenonol by HPLC

To study the accumulation and quantification of difurocumenonol, the fresh rhizomes [10 g] were homogenized with chloroform till they became colorless. The extract was filtered and concentrated using a rotary evaporator and freeze dried before using the sample for HPLC analysis. Difurocumenonol [the isolated compound] and chloroform extracts obtained during different developmental stages were tested using a LC-10AT liquid chromatograph [Shimadzu, Singapore] equipped with 300 x 4.6 mm i.d., 5 µ, Thermo Hypersil C-18 column [Bellefonte, PA, USA]. The gradient programme used for mobile phase was, methanol: water, as follows; 0 min, 25:75, v/v; 5 min, 40:60, v/v;
10 min, 50:50, v/v; 20 min, 70:30, v/v; 40 min, 90:10, v/v; 60 min, 100:0, v/v; with a flow rate of 1 ml/ min. UV detection was carried out with a SPD-M10A VP diode array detector [Shimadzu, Singapore], operated at 240 nm

**Determination of phenolics**

The total phenolic content in mango ginger juice as well as pulp was determined with the modified method of Taga et al. [1984]. In brief, 100 μL of sample was mixed with 2 mL of 2% aqueous sodium carbonate solution. After 3 min, 100 μL of 50% Folin-Ciocalteu phenol reagent was added to the mixture. After 30 min of incubation at room temperature, absorbance was measured at 750 nm against a blank. Total phenolic content was calculated on the basis of the standard curve of gallic acid.

**DPPH radical scavenging activity and Total reducing power**

*The details of the methodologies have been described in materials and methods of chapter 1*

**Reducing sugars, total sugars and total protein content**

Reducing sugars and total sugars were determined by using the method as described by Ranganna [2001]. The total protein content was determined by the Bradford method [1976], using bovine serum albumin [BSA; Sigma Chemical, St. Louis, USA] as a standard protein.

**pH, titrable acidity and total soluble solids**

pH of the fresh juice was measured using Control Dynamics pH meter calibrated with standard buffer pH 7. Titrable acidity was determined by AOAC [1990] method. The total soluble solids [TSS] were determined by a digital refractometer [ATAGO RX-5000, ATAGO, Japan] calibrated with distilled water. Mango ginger juice was passed through Whatman No.1 filter paper using vacuum before analysis.

**Statistical analysis**

The data was subjected to Duncan’s Multiple Range Test [DMRT] to determine significant differences \( P < 0.05 \).
DEVELOPMENTAL STAGES AND YIELD OF MANGO GINGER

Synthesis and accumulation of bioactive compounds along with other soluble and storage components were investigated during developmental stages of mango ginger rhizome from 60 to 240 days. Four distinct phases of growth and development in mango ginger plant were defined, namely; [1] vegetative growth phase; [2] rhizome initiation and growth phase; [3] maturation phase; and [4] senescence phase [Fig. 3.1]. Active vegetative growth extends up to 60 days from planting. The events were evident by the formation of six to eight pairs of green leaves. Initiation of rhizomes was observed little earlier than 60 days from planting.

The yield of rhizomes increased rapidly after 90 days and highest yield was recorded on 180 days after planting. A conspicuous drying of leaves after 150 days and falling of leaves after 180 days gives a visual marker for maturity of mango ginger rhizomes. The maturation of rhizomes was characterized by the increase in size, weight and yield that may be due to rapid accumulation of bioactive and storage components like starch, proteins and phenolics. In contrast, total sugar and reducing sugar contents decreased. The decline in all these components after 180 days heralds the onset of the senescence phase.
Differentiation of rhizome

The internal tissue of rhizome remained undistinguished as white, juicy mass of cells until 90 days of planting [Fig. 3.2]. Differentiation of central, circular, yellow colored pith that was demarked from the surrounding white cortex tissue was observed after 120 days. The ratio between pith and cortex during growth period remained constant and measured [1:1, w/w]. With the onset of maturity, a significant increase in pith [2:1, w/w] was observed. In addition, change in color of pith from yellow to greenish yellow was exhibited. Dispersion of yellow color to cortex region was also visible [Fig. 3.2]. Coincidently, these changes were in accordance with physiological maturity of rhizomes. Thus, they could be identified as visual maturity indices.
Accumulation of difurocumenonol

Difurocumenonol [Fig. 3.2] - a terpenoid compound, has been reported to be an antimicrobial compound against a wide range of microbes [Policegoudra et al., 2007a]. Accumulation of difurocumenonol in mango ginger rhizomes during developmental stages [60 to 240 days] was carried out using HPLC. Newly initiated rhizomes contained no difurocumenonol and it was first observed in 120 days old rhizomes. The highest concentration of difurocumenonol [34 mg/100 g] was noticed in 180 days old rhizomes [Fig. 3.2]. The synthesis and accumulation of this compound in the developing rhizome during growth and maturation of rhizome is essential to counteract bacteria and fungi. The terpenoids play a major role in contributing to flavor components and defense intermediates in plants [Aharoni, et al., 2004]. Participation of difurocumenonol in synthesis of flavor components in mango ginger may be responsible for its decrease in concentration during senescence. The tissue-specific biosynthesis and accumulation of difurocumenonol has yet to be identified. It is interesting to note that the pattern of synthesis, accumulation and degradation of difurocumenonol is in accordance with growth, maturation and senescence of the rhizome. Hence, it has been identified as nutraceutical marker to determine the physiological maturity of rhizome and as a harvest index.

Physiological role of difurocumenonol

It may be a biological inevitability for mango ginger rhizome to develop compounds of multifunctional activity to counteract the diversified underground abiotic and biotic challenges. Difurocumenonol proved to be one such compound with multifunctional properties found in mango ginger. Antimicrobial nature of difurocumenonol [Policegoudra et al., 2007a] can effectively thwart the constant challenges posed by underground pathogens. In addition, it possesses a wide range of antioxidant activities and other bioactive properties. High antioxidant property provides stability against auto oxidation. Thus difurocumenonol ensures prolonged antimicrobial activity during growth, senescence and dormancy period of rhizome. High lipid peroxidation inhibitory activity and metal chelating activity of difurocumenonol may act as competitive inhibitor for sprouting, due to its high oxygen demand. Thus, it may offer physiological protection to tide over dormancy. The terpenoids play a major role in contributing to flavor components and defense intermediates in plants [Aharoni, et al., 2004]. Difurocumenonol being a terpenoid compound, its derivatives may participate as
metabolic intermediaries in synthesis of flavour during distress storage conditions and during senescence depending upon the physiological needs. Thus, it may be responsible for the persistence of mango flavour in rhizome even after shriveling beyond the level of commercial acceptability. The role of difurocumenonol derivatives in various other physiological activities is interesting and worth investigating.

**Phenolic content**

The phenolic content was quantified in mango ginger at different developmental phases. Accumulation of phenolics occurred immediately after the initiation of rhizome formation, with an initial concentration of 122 mg/100 g and 26 mg/100 g in pulp and juice respectively [Fig. 3.3]. The abundance of phenolics on the 120th day in juice and 180th day in pulp is attributed to increase in weight of rhizomes during growth and maturation phase of the rhizome. The concentration of phenolics in pulp was ten times higher than that of phenolics in juice, at all the stages of development of rhizome. High content of phenolics may be an essential component for defense against various pathogens that are constantly challenging the underground rhizome, as evident from the antimicrobial activity of mango ginger extracts containing phenolics with other bioactive compounds such as difurocumenonol and amadannulen that were isolated from mango ginger rhizome [Policegoudra et al., 2007a, 2007b]. Phenolics from various plant sources and their contribution to antimicrobial and other biochemical responses are well documented [Benner, 1993; Bennett and Wallsgrove, 1994]. It appears that peak accumulation of phenolics in pulp on the 180th day may herald the onset of senescence in mango ginger rhizome. Decrease in phenolics may be attributed to strengthening of the plant cell walls by polymerization into lignans and lignins [Randhir and Shetty, 2005]. Therefore, the synthesis and accumulation
pattern of phenolics may be used as an indicator to differentiate the physiological maturity and quality of rhizome in mango ginger.

**ANTIOXIDANT ACTIVITY**

**DPPH radical scavenging activity**

Mango ginger juice exhibited a gradual decrease in DPPH radical scavenging activity until 240 days during developmental stages. In contrast, mango ginger pulp showed a gradual increase in DPPH radical scavenging activity, which was highest on 180 days of development and decreased thereafter until 240 days [Fig. 3.4]. DPPH radical scavenging activity of pulp has been attributed to the concentration of difurocumenonol and total phenolics. Antioxidant activity of amadannulen and mango ginger extracts and phenolics were reported in mango ginger and also in other vegetables [Chen and Ho, 1995; Nenadis et al., 2003; Policegoudra et al., 2007b]. In addition, terpenoids also have medicinal properties such as anti-carcinogenic, antimalarial, anti-ulcer, hepatoprotective, antioxidant and antimicrobial activity [Rodriguez-Concepcion, 2004; Policegoudra et al., 2007a]. An increase of antioxidant activity associated with accumulation of bioactive compounds like phenolics and difurocumenonol at 180 days could be a better method to determine the optimum physiological maturity to harvest mango ginger rhizomes, rather than conventional harvest from 200 to 240 days after planting.
Total reducing power

The reducing power of pulp increased with rate of growth and development and recorded the highest concentration after 180 days. There was an initial spurt in reducing power of mango ginger juice up to 90 days and decrease thereafter [Fig. 3.5]. The reducing capacity of samples from ferricyanide complex to the ferrous form may serve as a significant indicator of its antioxidant capacity [Meir, et al., 1995]. The reducing power of mango ginger pulp was almost ten times higher than that of juice. This may be attributed to the presence of high concentration of difurocumenol, amadannulen and phenolics in the pulp of mango ginger as reported earlier [Policegoudra et al., 2007a and 2007b].

Total sugars and reducing sugars

Total sugars and reducing sugars decreased gradually as the rhizome attained maturity [Fig. 3.6]. There was a steep decline in sugar during the growth phase of rhizome, because it acts as an active source for supply of energy. Reduction of total and reducing sugars was very significant on 180th day of harvest. The decrease during the maturation phase of rhizome may be attributed to formation of storage components such as starch and its derivatives. Structure, biochemical components and functional properties of mango
ginger starch is well documented [Policegoudra and Aradhya, 2007]. The starch is essential to protect the energy resource of rhizome during the dormant stage. It may acts as a store house of sugars that are necessary for the development of shoot, as in other tubers [Lewis, 1994].

**Total protein content**

The protein concentration increased gradually during growth and maturation period. The concentration ranged from 6.5 to 11.1 mg/100g, with a peak accumulation period at 180 days [**Fig. 3.7**]. Increase in concentration of protein is in accordance with increase in phenolics and difurocumenonol. The accumulation of storage proteins were reported in tubers like potato, sweet potato, yam, taro, cassava, where the major role was to act as stores of nitrogen, sulfur and carbon, that are required to survive periods of adverse conditions and to provide nutrients for shoot formation [Shewry, 2003]. The storage proteins also exhibit biological activities that are consistent with a role in protecting the tubers against pests, pathogens and also abiotic stresses as antioxidants and enzyme inhibitors [Shewry, 2003]. Protein abundance decreased during the senescence phase [**Fig. 3.7**]. Protein synthesis and accumulation is complex phenomenon governed by the physiological and abiotic factors during developmental stages of the rhizome.

![Fig. 3.7: Changes in total protein content in mango ginger rhizome during development](image-url)

*Each value is a mean of three different observations. Values showed by different letters for each line are significantly different at p < 0.05.*
**pH, titrable acidity and total soluble solids**

The pH of mango ginger juice increased gradually with advance in maturity and was highest on the 120th day. Later it remained constant throughout with a pH range of 6.2 to 6.4 [Fig. 3.8].

A gradual increase in percentage of total solubles with increase in growth and advancement of maturity of mango ginger rhizome was noticed until 120 days. The trend was reversed recording a decrease up to 240 days because the solubles, which mainly contains sugars, form a source of energy for various physiological functions that varied with different developmental stages of mango ginger rhizome.

In contrast, titrable acidity of mango ginger gradually increased throughout the growth and developmental phases of mango ginger rhizome [Fig. 3.9]. The increased or decreased concentration of pH and titrable acidity along with TSS appears to be governed by the variation in composition of cellular metabolites and their functions. It is interesting to note that the point of intersection between total soluble solids and acidity coincides with 150 days [Fig. 3.9] of developmental stages of mango ginger. This point of may terminate the growth phase and initiate the onset of maturation.
Maturity markers for mango ginger

The present study clearly indicated that the synthesis and accumulation pattern of difurocumenonol, phenolics and protein concentrations served as bioactive markers to determine the physiological maturity for harvest of the mango ginger rhizomes. Difurocumenonol was first observed in 120 day old rhizomes after planting, while phenolics and protein accumulation were detected after 60 days after planting. However, they follow a similar pattern of accumulation as a function of growth and maturation of the rhizome. During the growth phase there was a gradual increase in concentration of phenolics, difurocumenonol and total proteins, while their accumulation was maximum after 180 days. High concentration of soluble and storage components along with bioactive compounds is of paramount important, since their concentrations depleted with delay in harvest after 180 days, which indicates the onset of senescence phase. The distinct patterns of these biochemical markers were associated with conspicuous display of drying and detachment of leaves from the rhizome. This provides a visual clue for maturation of rhizome in mango ginger plant. The various maturity indices displayed on 180 days [Table 3.1] from planting were found to be optimum for harvest, compared with the conventional harvest ranges from 200 to 240 days.

Table 3.1 Maturity standards for harvest of mango ginger rhizome

<table>
<thead>
<tr>
<th>Sl No.</th>
<th>Maturity markers</th>
<th>Index</th>
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<tr>
<td></td>
<td><strong>Biochemical</strong></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>✓ Phenolic content [mg/ 100 g]</td>
<td>380</td>
</tr>
<tr>
<td>2</td>
<td>✓ Difurocumenonol concentration [mg/ 100 g]</td>
<td>34</td>
</tr>
<tr>
<td>3</td>
<td>✓ Protein content [mg/ 100 g]</td>
<td>12</td>
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<tr>
<td></td>
<td><strong>Morphological</strong></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>✓ Drying and detachment of leaves [days]</td>
<td>180</td>
</tr>
<tr>
<td></td>
<td><strong>Characteristics of rhizome</strong></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>✓ Size of rhizome; a] Length [cm]</td>
<td>12-13</td>
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<tr>
<td></td>
<td>b] Diameter [cm]</td>
<td>3-4</td>
</tr>
<tr>
<td>6</td>
<td>✓ Lemon yellow pigmentation of pith region [days]</td>
<td>180</td>
</tr>
<tr>
<td>7</td>
<td>✓ Pith and cortex ratio of rhizome</td>
<td>2:1</td>
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</table>
For the first time, a new set of optimum maturity indices for harvest of mango ginger rhizome has been established, based on the synthesis and accumulation pattern of difurocumenonol and phenolics during development of mango ginger rhizome. Its maturation is also directly influenced by coordinated alterations of several biochemical factors. Interestingly, they demonstrated a distinct pattern of accumulation governed by the physiological maturity of the rhizome during development. The importance of these patterns highlighted in the present investigation which I believe has commercial application.