Biochemical and Molecular Characterization of a Volatile Component of Mango (*Mangifera indica* L. *cv*. Alphonso) Flavor

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By
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Certified that the work in the Ph.D. thesis entitled **'Biochemical and Molecular Characterization of a Volatile Component of Mango (*Mangifera indica* L. *cv.* Alphonso) Flavor'** submitted by **Ms. Hemangi G. Chidley** was carried out by the candidate under my supervision. The material obtained from other sources has been duly acknowledged in the thesis.

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DECLARATION

I hereby declare that the thesis entitled **'Biochemical and Molecular Characterization of a Volatile Component of Mango (*Mangifera indica* L. *cv*. Alphonso) Flavor'** submitted for Ph.D. degree to the Savitribai Phule Pune University has not been submitted by me for a degree at any other university.

Date: Hemangi G. Chidley

Pune

Dedicated to my parents,
Anuj and in-laws for
their untiring support

CONTENT

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LIST OF ABBREVIATIONS

°C	Degree celcius
AAT	Alcohol acyl transferase
ACC	1-Aminocyclopropane-1-carboxylic acid
ACS	1-Aminocyclopropane-1-carboxylic acid synthase
ACO	1-Aminocyclopropane-1-carboxylic acid oxidase
ADH	Alcohol dehydrogenase
ANOVA	Analysis of variance
Aox	Alternative oxidase
AVG	Aminoethoxyvinylglycine
BR	Breaker
BSA	Bovine serum albumin
bp, kb	Base pairs, kilo base pair
cDNA	Complementary DNA
CaC_2	Calcium carbide
CFB	Corrugated fibre board
β-D-Glu	β-D-Glucosidase
DAH	Days after harvest
DNA	Deoxyribonucleic acid
DNSA	Dinitrosalisylic acid
dNTPs	Deoxyribonucleotide triphosphate
EDTA	Ethylenediaminetetraacetic acid
EFE	Ethylene forming enzyme
EF1α	Elongation factor 1α
EGase	Endo-1,4-β-glucanase
EIC	Extracted ion chromatogram
EIN	Ethylene-insensitive
EIL	Ethylene-insensitive like
ERE	Ethylene response element

EO	Enone oxidoreductase
FaOMTS	*Fragaria*X*Annanasa O*-methyltransferase
FID	Flame ionization detector
FPPS	Farnesyl pyrophosphate synthase
GAF	c**G**MP-specific phosphodiesterases, **a**denylyl cyclases and **F**hlA
β-Gal	β-Galactosidase
GC	Gas chromatography
g	Relative centrifugal force
g, mg, µg, kg	Gram, milligram, microgram, kilogram
GPPS	Geranyl pyrophosphate stynthase
GST	Glutathione -S-transferase
HESI	Heated electrospray ionization source
HMMF	4-hydroxy-5-methyl-2-methylene-3(2H)-furanone
HPL	Hydroperoxide lyase
β-Hex	β-D-N-acetylhexosaminidase
L, mL, µL	Liter, milliliter, microliter
LC	Liquid chromatography
LSD	Least significant difference
LOX	Lipoxygenase
α-Man	α-mannosidase
MAP	Modified Atmosphere Packaging
1-MCP	1-Methylcyclopropene
MS	Mass spectrometer
mRNA	Messenger ribonucleic acid
MMT	Million metric tons
NCBI	National Centre for Biotechnology Information
NCL	National Chemical Laboratory
NIST	National Institute of Standards and Technology
ODT	Odor detection threshold

O-MTS	O-methyltransferase
ORF	Open reading frame
PFA	Prevention of food adulteration
PG	Polygalacturonase
PL	Pectate lyase
PME	Pectin methylesterase
*p*Np	*p*-Nitrophenol
ppb	Parts per billion
PCA	Principal component analysis
PCR	Polymerase chain reaction
RACE	Rapid amplification of cDNA ends
RIN	Ripening inhibitor
RT	Room temperature
RNA	Ribionucleic acid
RNase	Ribonuclease
SAM	S-adenosyl-L-methionine
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TBG	Tomato β-galactosidase
TE	Tris-EDTA
TAE	Tris-acetate-EDTA
TSS	Total soluble solid
Ucp	Uncoupling protein
UHPLC	Ultra high-performance liquid chromatography
UTR	Untranslated region
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

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THESIS SUMMARY

Alphonso is one of the highly important mango cultivar due to its generous success at the national and international trading market. It generates sizeable amount of revenue and aids in the agro-rural development in India. However, the industry also witnesses considerable postharvest losses during handling, packaging and transport. To overcome these, various postharvest treatments have been employed by farmers as well as traders. Amongst these, pre-climacteric ethylene treatment has gained popularity recently due to the obvious reasons such as; it does not have any harmful effects and reduces ripening period without affecting the cosmetic features of the fruits.

Chapter 2: Pre-climacteric ethylene treatment modulates the ripening related glycosidase activities, their transcript abundance and sugar content in Alphonso mango fruit

To analyze the effect of such pre-climacteric ethylene treatment on the softening related cell wall hydrolysis process, ethylene treated and control fruits were studied for the changes in the activity profile and transcript abundance of three glycosyl hydrolases *viz*. β-D-galactosidase, α-D-mannosidase and β-D-glucosidase. Mature green fruits collected from mango orchards in Dapoli were given ethylene treatment. Another set of untreated fruits was considered as control. The assays performed for the activity profile of all the three enzymes during ripening of ethylene treated and control fruits displayed early induction of the activities of all the three enzymes upon ethylene treatment. The activity chart of β-D-galactosidase showed the highest share to the glycosidase activity in both, control and ethylene treated fruits, while the lowest share has been displayed by β-D-glucosidase activity. The findings suggested significant role of former and minimal involvement of later in the cell wall hydrolysis process of Alphonso mango. This was supported by the histochemical assay of β-D-galactosidase activity in ethylene treated and control ripe fruits using X-gal as a chromogenic substrate. To get insight in the regulation of their activities at molecular level, the transcript abundance of all three glycosidases was studied which showed early and enhanced transcript accumulation of β-D-galactosidase and three transcript variants of α-D-mannosidase in ethylene treated fruits. Moreover, β-D-

galactosidase transcripts showed significantly increased accumulation in ethylene treated fruits compared to control. However, β-D-glucosidase transcripts were not detected throughout the ripening stages of pulp of control and ethylene treated fruits pointing towards probable involvement of other transcript variant/variants for the effective glucosidase activity during ripening. Analysis of reducing and non-reducing sugar content upon ethylene treatment revealed substantial alteration in the pool of reducing sugars than non-reducing pointing towards variation in the sugar metabolism of Alphonso mango upon exogenous ethylene treatment. The study exemplified the alteration of cell wall hydrolysis process and sugar metabolism upon pre-climacteric ethylene treatment reducing the shelf-life of Alphonso mango.

Chapter 3: Spatial and temporal changes in the volatile profile of Alphonso mango upon exogenous ethylene treatment

Effect of pre-climacteric ethylene treatment on the volatile accumulation of Alphonso mango during ripening was studied wherein the volatile content of skin and pulp were separately analyzed by gas-chromatography. The analysis showed accelerated ripening in terms of early appearance of ripening specific compounds *viz*. lactones and furanone. However, quantitatively lactones remain unaffected in ethylene treated fruits. Alphonso pulp was found to be lactone rich while skin has high terpene content. This was clearly evident by the separate clustering of pulp and skin ripening stages across PC1 in the Principal Component Analysis score plot. The volatile profile of both pulp and skin showed significantly enhanced synthesis of mesifuran in ethylene treated fruits contributing high sweet fruity caramel like character to the treated fruits as compared to control thus altering the flavor blend of Alphonso mango fruits. The study demonstrated the role of ethylene as a vital modulator of the biosynthesis of ripening specific volatile compounds in Alphonso mango. It has also suggested the need for the standardization of ethylene doses with suitable ripening parameters for the better trade of Alphonso mango.

Chapter 4: Molecular cloning and expression of *O*-methyltransferase catalysing the synthesis of mesifuran, a key aroma volatile in Alphonso mango fruit

The biosynthesis of important flavour volatile, mesifuran was studied by isolation and characterization of *O*-methyltransferase gene (*MiOMTS*) and the encoded product. *O*-methyltransferase enzyme uses furaneol as a substrate and S-adenosyl-L-methionine (SAM) as methyl group donor producing mesifuran which is an important flavouring compound of ripe Alphonso fruits. After obtaining the complete open reading frame of *MiOMTS*, it was cloned in pGEX-4T expression vector and heterologously expressed in *E. coli* cells to obtain recombinant protein. The recombinant protein was assayed for its activity using furaneol, protocatechuic aldehyde, caffeic acid and catechol as substrates. Of these, *MiOMTS* didn't use caffeic acid and catechol but successfully converted furaneol to mesifuran and protocatechuic aldehyde to vanillin in an *in vitro* assay reaction. The optimum activity parameter for *MiO*MTS was found to be pH 7.0 and temperature was at 25°C. To evaluate the transcriptional regulation of mesifuran synthesis the relative transcript abundance of *MiOMTS* throughout the ripening stages of skin and pulp of control and ethylene treated fruits was studied. Early and significantly enhanced accumulation of its transcripts was evident in ethylene treated fruits while the high correlation of mesifuran synthesis and its transcript abundance was displayed by control fruits. The observation strongly suggested the regulation of mesifuran synthesis at transcriptional level by ethylene trigger. The study was only second after strawberry demonstrating the isolation and characterization of furaneol *O*-methyltransferase from Alphonso mango and carries immense importance for the flavour production industries.

General Introduction and Review of Literature

Fruit Ripening: A Role of Two Carbon Molecule, Ethylene

GENERAL INTRODUCTION AND REVIEW OF LITERATURE

Fruits are an indispensible part of human diet since ancient times. They are consumed in various ways and at various stages of their development and ripening process. In most of the cases, fully ripen fruit is preferred for fresh consumptions as well as processed food products. This is due to the obvious fact that ripe fruits acquire attractive color, alluring taste, distinctive blend of volatiles and high nutrition quotient during the process of ripening (Lizada, 1993). Consequently, the process of fruit ripening has been explored at biochemical and molecular level pertaining to various aspects of fruit physiology. Initially, ripening has been largely perceived as catabolic and later termed as a process of tissue differentiation. Moreover, it has been related to the senescence process as both share some common features. However, senescence essentially leads to the death of the tissue while the process of ripening involves a set of events leading to a suitably ripened fruit appropriate for consumption. Various efforts put in to understand the ripening process in fruits lead to the identification of distinct mechanisms of ripening, based on which the fruits are broadly classified as climacteric and non-climacteric.

1.1 . Ripening in climacteric and non-climacteric fruits

The studies on fruit ripening has a long history which can be traced back to a report by Blackman and Parija (1928) wherein the ripening of apples was studied in terms of their respiration pattern. Their observations suggested that there is acceleration in the respiration rate which is accompanied by the hydrolysis of vital substances. These maintain the cellular integrity thereby leading to the breakdown of the 'organizational resistance'. This has been considered as an onset of ripening events. This theory remained acceptable for considerably longer period until researchers pursued the quest to understand the correlation between the respiratory climacterics and ripening of fruits with the advent of modern tools of molecular biology. Earlier an observation stating increased protein content and enzymatic activity during fruit ripening has been reported (Hulme, 1958) followed by a study on increased incorporation of nucleotides in nucleic acids at climacteric stage during ripening of fruits (Richmond and Biale, 1976). These

have imputed the climacteric rise in respiration to the surge of protein synthesis. However, various reports thereafter in banana, tomato and pear depicted that ripening is independent of climacteric respiration and increased protein synthesis is a consequence of the varied cellular activities leading to the ripening events which are closely followed by senescence (Sacher, 1973). Although, the theories and findings have largely differentiated climacteric and non-climacteric fruits on the basis of their respiratory pattern (Biale 1960), a clearer explanation was needed to distinguish the varied ripening behaviour of fruits between these two classes. A systematic study of respiration and endogenous ethylene production in banana and citrus (lemons and oranges) fruits carried out by McMurchie et al. (1972) was a major breakthrough in this regard. Presence of two distinct systems of ethylene production responsible for different ripening related changes in climacteric and non-climacteric fruits was proposed by them. These were system 1 and system 2 wherein system 1 was suggested to be active in both, climacteric and non-climacteric fruits while continue to produce ethylene upto a level sufficient to trigger system 2 of ethylene production only in climacteric fruits. This brings about large increase in endogenous ethylene and thus, the complete incorporation of ripening related changes. Nonetheless, the system 2 was depicted to be absent in non-climacteric fruits. The system 2 of ethylene biogenesis can, therefore, be considered as a characteristic of climacteric fruits which leads to autocatalytic ethylene production and irreversible ripening events. This proposition of the presence of two systems for ethylene production was more appropriate, pragmatic and held sway till today. As it has been strongly established that both, systems 1 and 2 are present in climacteric fruits, majority of efforts directed towards understanding the components of the two systems, their regulation, cross-talk and transition from system 1 to 2 were focused on climacteric fruits. Nevertheless, non-climacteric fruits such as, grapes, citrus and strawberry showed contribution of ethylene in some of the important ripening processes such as color development, volatile production and pectin metabolism (Botondi et al., 2011; Merchante et al., 2013) pointing towards the mandatory requirement of ethylene in the ripening of various fruits irrespective of their climacteric or non-climacteric nature.

1.2 . Ethylene synthesis and its perception

Ethylene biosynthesis has been a subject of great curiosity even before the concept of system 1 and 2 of ethylene biogenesis came into picture. Although it is now well-known that ethylene is synthesized from methionine, earlier a range of precursors have been proposed for ethylene biogenesis in higher plants. They included linolenic acid, β-alanine, propanal, ethanol, organic acids, acrylic acid, thiomalic acid, glycerol, sucrose, glucose, and acetic acid (Abeles, 1972). However, all the efforts using above molecules could not assign them as a potential precursor for ethylene.

1.2.1. Synthesis of simple olefin hormone: Ethylene

A successful effort by Lieberman and Mapson (1964) displayed conversion of methionine to ethylene in the presence of Cu^{+} and ascorbic acid model system and proposed a scheme for synthesis of ethylene from C-3,4 of methionine. Later they showed the similar conversion using lebeled methionine in apple slices and projected a strong *in vivo* confirmation for methionine as an ethylene precursor (Lieberman et al., 1965). After this, majority of the research was directed towards revealing the intermediates and the enzymes involved in methionine to ethylene bioconversion. Synthesis of S-adenosyl methionine (SAM) from methionine at the expense of one ATP molecule was established as the first step by Adams and Yang (1977) closely followed by their another report, that identified 1-aminocyclopropane-1-carboxylic acid (ACC) as the next intermediate in the pathway (Adams and Yang, 1979). The enzyme responsible for conversion of SAM to ACC was found to be pyridoxal phosphate dependant and was later named as ACC synthase (ACS) (Yu et al., 1979). A number of attempts to further confirm role of ACC as ethylene precursor revealed significant ethylene production in various plant tissues upon ACC application suggesting a conversion of SAM to ACC as a rate limiting step in the ethylene biosynthesis (Yang and Hoffman, 1984). The synthesis of ethylene from ACC was shown to be oxygen dependant and the enzyme catalyzing this process was initially referred as ethylene forming enzyme (EFE). By then, much was not known about the size or the sequence of EFE. A work reported by Hamilton et al. (1990) identified mRNA encoding a 35kDa protein having ripening related

accumulation that catalysed the conversion of ACC to ethylene in the presence of oxygen. This enzyme was identified as EFE and later renamed as ACC oxidase (ACO). During ethylene biosynthesis process the utilized methionine was shown to be replenished by Yang cycle to maintain the endogenous methionine levels during high ethylene synthesis (Miyazaki and Yang, 1987). Action of ACO on ACC produces CO_2 and cyanide apart from ethylene of which cyanide gets detoxified to *β*-cyanoalanine by the action of *β*-cyanoalanine synthase preventing plants from cyanide toxicity (Argueso et al., 2007). With this finding, all the intermediates and the enzymes involved in the ethylene biosynthesis pathway along with the fate of other compounds produced were revealed (Figure 1.1).

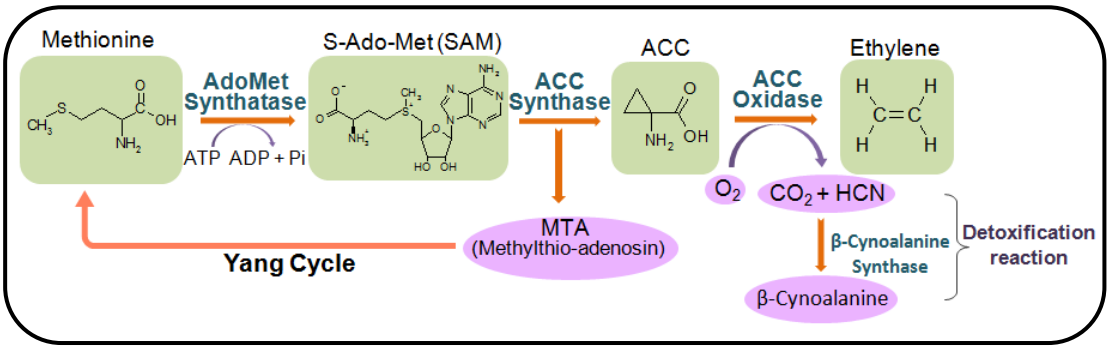


Figure 1.1: Ethylene biosynthesis pathway

1.2.2. Perceiving the signal

Ethylene thus produced is involved in many diverse processes during life cycle of a plant such as seed germination, triple response, growth, fruit ripening, senescence, abscission, response to various stresses and gravitropism (Mattoo and Suttle, 1991). Ethylene synthesised in one region of particular organ diffuses freely from cell to cell and incorporates the implied responses throughout the organ. This is typically achieved by various membrane bound ethylene receptors and a downstream signal transduction pathway leading to the expression of various ethylene response genes (Alexander and Grierson, 2002). As for most of the other receptors which are present on plasma membrane, ethylene receptors are localized on the membranes of endoplasmic reticulum (Chen et al., 2005). They form a big family consisting of various isoforms which vary from species to species. Five distinct receptor isoforms are reported from *Arabidopsis thaliana* while six are depicted from tomato (O'Malley et al., 2005). They show homology

to bacterial two component receptors having a sensor and a response regions functioning together (Chang and Stewart, 1998). A sensor region of ethylene receptor composed of a transmembrane domain, a GAF domain and a histidine kinase domain while a response region consists of receiver domain (Binder, 2008). Based on the sequence comparisons of ethylene binding domains, they are broadly classified in to sub-family I and sub-family II. Receptors from sub-family II possessed additional signal sequence at their N-terminal for their localization (Binder, 2008). The receptors form a homodimer by two disulphide bonds at their N-termini and the binding of ethylene is achieved by copper ion cofactor (Rodriguez et al., 1999). Immediately next to the receptor is a CTR1 protein kinase which acts as a negative regulator of ethylene response pathway. It has a homology to Raf family of mitogen activated protein kinase kinase kinase (MAP3K) (Kieber et al., 1993). In air, CTR1 (constitutive triple response) interacts with the receptors, gets activated and its kinase domain brings about the inhibition of downstream pathway restricting the ethylene responses. In the presence of ethylene bound to the receptors, CTR1 undergoes conformational change altering its kinase activity thereby releasing the downstream components in the ethylene response pathway. A positive regulator of ethylene signalling pathway, EIN2 (ethylene-insensitive2) thus gets activated and EIN3 (ethylene-insensitive3) and EIN3 like (EIL) protein family of transcription factors initiate transcription cascade. They bind to the ethylene response element (ERE) of *ERF1*, an ethylene inducible gene. ERF1 protein binds to the GCC box of the ethylene responsive genes and brings about their transcription (Chen et al., 2005). These include a range of genes that are required for distinct physiological as well as biochemical processes in a plant (Figure 1.2).

Although there are number of ethylene receptors in different species with overlapping functions, their functions are not fully redundant. Moreover, sub-family I receptors have important role in ethylene signalling than the other interacting more efficiently with CTR1, while *Le*ETR4 and *Le*ETR6, a sub-family II receptors are shown to modulate ripening related responses in tomato (Binder, 2008). Further, the sensitivity of ethylene receptors to the broad range of ethylene concentration in plants was reported as a consequence of receptor clustering. Here a physical interaction between the ethylene bound receptor with other

unbound receptors in the cluster changes the signalling state of the unbound receptors. This leads to a large receptor output generating amplified response to low ethylene levels (Bray et al., 1998).

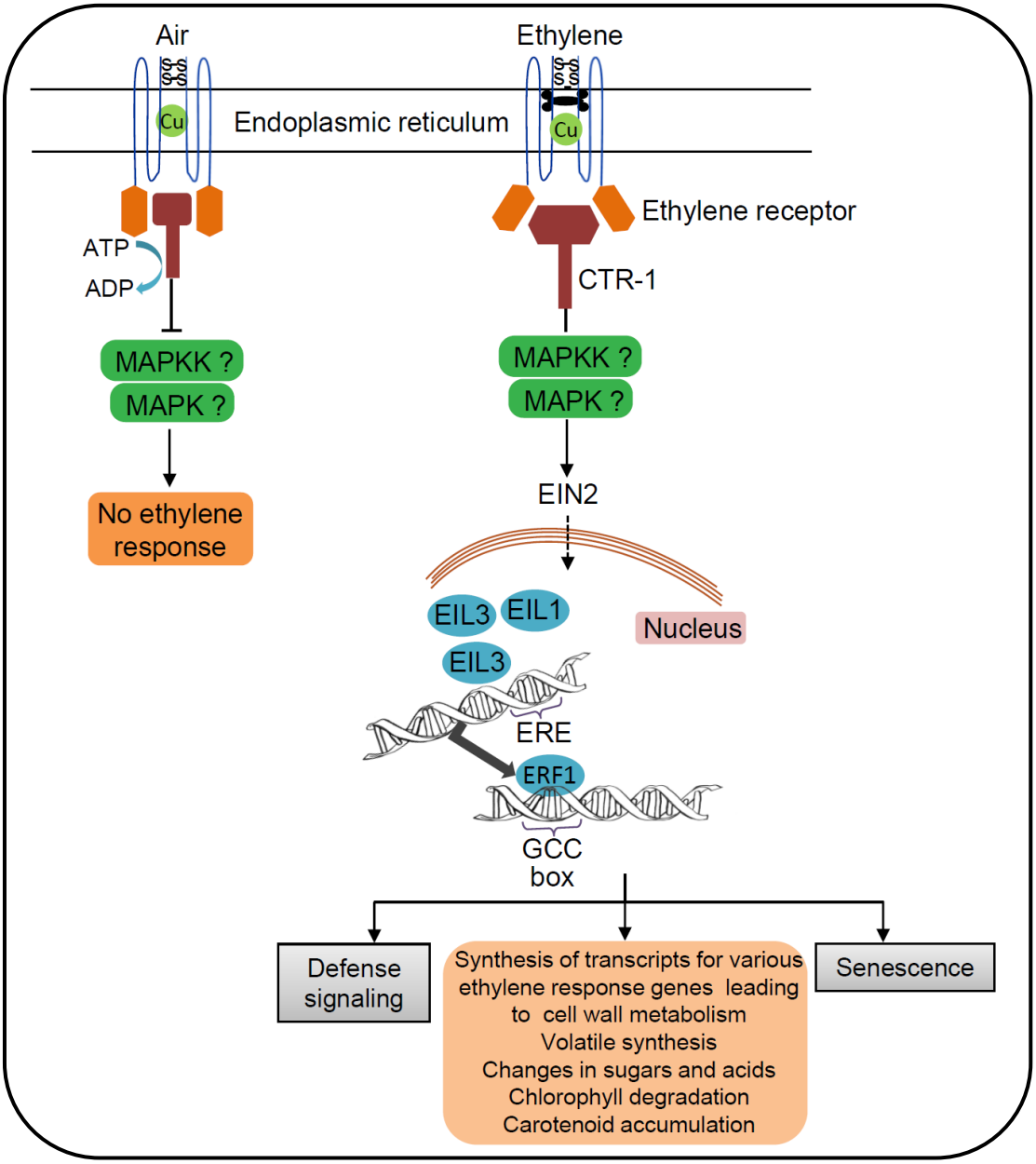


Figure 1.2: A model representing ethylene signal transduction pathway

1.3. System 1 to system 2: a function of *ACS* genes

Ethylene biosynthesis pathway from methionine to ethylene involves two enzymatic steps catalyzed by ACS and ACO. So far, eight and five distinct isoforms of *ACS* and *ACO*, respectively have been isolated from tomato (*Lycopersicon esculantum*). Out of these, five *ACS* and three *ACO* isoforms have

been expressed regulating ethylene biosynthesis in tomato (Barry et al., 2000). Although, a signalling pathway coordinating the expression of members of these gene families largely remained unknown, enough evidence is available suggesting crucial role of ethylene and certain developmental cues in their regulation. As the synthesis of ACC by the action of ACS was thought to be a rate limiting step in the ethylene biosynthesis pathway, the regulation of transition from system 1 to 2 was more focused on the members of ACS gene family. Consequently, four members of this gene family viz. *LeACS1A*, *LeACS2*, *LeACS4*, and *LeACS6* were identified and analyzed for their expression patterns in wild-type tomato fruits along with *rin* and *Nr* tomato mutant lines (Barry et al., 2000). The ripening inhibitor (*rin*) tomato fruits lacked autocatalytic ethylene production (system 2) due to mutation in RIN transcription factor crucial in the signal transduction (Herner and Sink, 1973). While never ripe (*Nr*) tomato fruits showed dominant mutation in one of the ethylene receptors leading to impaired ethylene perception (Lanahan et al., 1994).

Barry et al. (2000) also proposed a relay model for the expression pattern of *ACS* gene isoforms regulating the transition of system 1 to 2 of ethylene production. In green mature fruits and vegetative tissue, some unknown component from the developmental pathways regulated system 1 ethylene biosynthesis via *LeASC1A* and *LeACS6* expression. This continues until a capability to ripe was achieved and at this point a transition occurred. A RIN (ripening inhibitor) transcription factor played a vital role directing increased expression of *LeACS1A* which culminated in enhanced ethylene synthesis and induction of *LeACS4*. Up-regulation of *LeACS1A* and *LeACS4* caused high ethylene synthesis and induction of *LeACS2* thereby initiating ethylene production system 2. High level of ethylene generated by autocatalytic synthesis posed negative feed-back on the system 1, down-regulating the expression of *LeASC1A* and *LeACS6*. Thus, the transition of two systems of ethylene synthesis and their regulation was largely governed by differential expression of a family of *ACS* genes. Nevertheless, *ACO* was the terminal gene in the pathway synthesizing ethylene from ACC. Three distinct *ACO* genes *viz*. *LeACO1*, *LeACO3* and *LeACO4* also showed temporally distinct patterns of their accumulation throughout tomato fruit ripening. Transcripts of *LeACO1* and *LeACO4* accumulated in mature

green fruits with remarkable increase in their expression at climacteric burst which remained perpetual during ripening. The expression of *LeACO3* was transient at breaker stage and declined thereafter (Cara and Giovannoni, 2008). The expression of *LeACO1* and *LeACO4* was shown to be ethylene dependant (Nakatsuka et al., 1998). It has thus been proposed that initiation of catalytic ethylene production commences with the *de novo* synthesis of ACO1 which produces ethylene that triggers ACS expression producing more ACC (Figure 1.3).

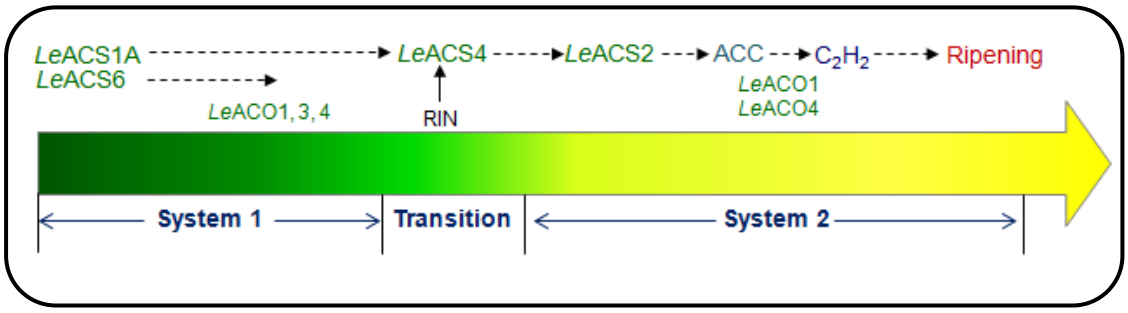


Figure 1.3: Regulation of ethylene biosynthesis during tomato fruit ripening

1.4. Role of ethylene in fruit ripening

With the initiation of ripening process fruit undergo sizeable modifications relating to softening of fruit, aroma volatile synthesis, color development, changes in sugar/acid ratios etc. Expression of some of the genes in these processes is either ethylene dependant or ethylene independent (Alexander and Grierson, 2002). Also, different genes exhibit differential sensitivity towards varying ethylene concentration and their activities are temporally regulated during fruit ripening (Hayama et al., 2006; Johnston et al., 2009). A number of attempts have been made to unravel the role of ethylene in all such ripening related processes.

1.4.1. Fruit softening

The task of fruit softening has essentially been accomplished by co-ordinated action of several cell-wall solubilising enzymes which includes polygalacturonase (PG), pectate lyase (PL), pectin methyl esterase (PME), β-galactosidase and endo-1,4-β-glucanase (EGase) and some other proteins such as expansins. Of these, PG, PL and PME are largely related to the depolymerisation of pectin

polymers loosening mainly the middle lamella and to some extent primary cell wall structure while β-galactosidase has been implied in the removal of galactose residues from cell wall polymers containing β(1→4)-D-galactan. The expansins are believed to disrupt the hydrogen bonds between the cellulose microfibrils and cross-linking polymers (Brummell and Harpster, 2001). Ethylene plays a major role in regulating the expression and activities of these ripening associated cell wall hydrolysing enzymes and proteins.

The activity of PG has shown strong regulation by ethylene with high sensitivity to low concentration of ethylene in tomato fruit (Sitrit and Bennett, 1998). Earlier, three isoforms of PG enzyme were isolated from strawberry fruit, of which one isoform (PG2) characterized as exo-PG did not display ripening associated increase in activity suggesting limited role of this enzyme in strawberry softening (Nogata et al., 1993). However, later Villarreal et al. (2008) and Quesada et al. (2009) isolated few more cDNAs corresponding to PGs in strawberry fruit providing enough evidence for role of PG in ripening associated softening of strawberry fruit. Polygalacturonase gene isolated from papaya showed induced expression during ripening which was highly ethylene dependant (Fabi et al., 2009). Such ethylene dependant expression of PGs was also reported from kiwifruit (Wang et al., 2000) and prickly pear (Rosas-Cardenas et al., 2007). Despite this, the antisense suppression of PG in tomato displayed only slightly firmer fruits suggesting that PG was not the sole determinant of tomato fruit firmness (Grierson and Schuch, 1993). PME is an enzyme that de-esterifies the methyl-esterified polygalacturonans making them susceptible to the action of PGs. Although one of the fruit specific isoforms, *PME1* showed peak of expression at breaker stage of tomato ripening, its transgenic suppression lines did not show altered fruit softening during ripening indicating indirect role of this enzyme in the process (Tieman et al., 1992). Pectate lyase (PL) is another member of pectin solubilisation machinery that cleaves glycosidic linkages between galacturonosyl residues by β-elimination mechanism (Rao et al., 1996). Its ripening associated activity has been reported from banana (Marin-Rodriguez et al., 2003), grapeberry (Nunan et al., 2001) and strawberry (Medina Escobar et al., 1997). Another study on mango (*cv*. Dashahari) depicted fruit specific and ripening related accumulation of PL transcripts with delayed expression in 1-

MCP (ethylene antagonists) treated fruits hinting its regulation by ethylene signal (Chourasia et al., 2006).

Apart from these, Pressey (1983) reported role of β-galactosidase in tomato fruit galactan degradation. It displayed ripening related synthesis of isoform II having exclusive property of hydrolysing galactan. This was later supported by the ripening related transcript accumulation of its corresponding cDNA (Smith et al., 1998). This cDNA was later denoted as *TBG4* and was shown to be actively involved in the cell wall modification leading to fruit softening in tomato (Smith et al., 2002). Moreover, a decade of 90's witnessed various attempts of purification and biochemical characterization of β-galactosidase from fruits such as muskmelon (Ranwala et al., 1992), kiwifruit (Ross et al., 1993), avocado (Deveau et al., 1993) and apple (Ross et al., 1994). All of these exhibited ripening related increase in β-galactosidase activity. Expression analysis of β-galactosidase gene isoform *JP-GAL* from Japnese Pear also showed fruit specific and ripening related accumulation of its transcripts (Tateishi et al., 2001). All these reports collectively suggest possible involvement of ethylene in the regulation of β-galactosidase activity contributing to the cell wall modifications associated with fruit ripening.

Expansin is a group of extracellular proteins that are shown to be actively involved in the cell enlargement and cell wall disassembly during fruit ripening. Ripening related transcript accumulation of one of its isoforms *LeExp1* was reported from tomato fruit with precocious mRNA synthesis upon exogenous ethylene treatment suggesting regulation of its expression by ethylene (Rose et al., 1997). This was later supported by demonstrating firmer fruits in transgenic *LeExp1* suppressed lines and softer fruits in transgenic *LeExp1* over-expressed lines, respectively as compared to the wild type tomato (Brummell et al., 1999). Similarly, a *MiExpA1* isolated from mango (*Mangifera indica cv*. Dashehari) and *MaExp1* isolated from banana (*Musa acuminata*) showed ripening associated expression in respective fruits treated with ethylene while significant reduction in transcripts level in 1-MCP treated fruits (Sane et al., 2005; Trivedi and Nath, 2004). Another member of the cell wall hydrolysis machinery, an EGase homologue *MiCel1* isolated from ripening mango (*Mangifera indica* var.

Dashehari) showed fruit specific and ripening related expression of its mRNA. Reduced accumulation of *MiCel1* transcripts in 1-MCP treated fruits was also correlated to the delayed EGase activity and ripening associated softening of these fruits (Chourasia et al., 2008). Thus, the advanced biochemical and molecular biology tools have enabled researchers to explore the correlation between ethylene and fruit softening during ripening.

1.4.2. Volatile synthesis

During ripening synthesis of various volatile compounds that impart distinctive aroma to the ripe fruits has been a crucial event. Separation and identification of the volatiles using gas-chromatography and mass spectrometry (GC-MS) have revealed complex mixture of these compounds accountable for the unique flavor of fruits. The possible involvement of ethylene in the biogenesis of these flavor volatiles has been evaluated either by exposing the fruits to ethylene or ethylene antagonists. Recently, development of the transgenic suppressed lines for ethylene biosynthesis and, its action has evolved as an important tool to understand the role of ethylene in aroma volatile biosynthesis.

The volatile composition of banana treated with 1-MCP after initiation of ripening with propylene revealed increase in the concentration of alcohols with concomitant decrease in the concentration of corresponding esters (Golding et al., 1999). Another investigation on banana ripening reported up-regulation of the genes involved in volatile biosynthesis upon ethylene treatment suggestive of its transcriptional regulation by ethylene (Yang et al., 2011). In apples, alcohol acyl-CoA transferase (AAT), an enzyme catalysing rate limiting step in ester biosynthesis found to be regulated by ethylene signal. Transgenic apple lines suppressed for ethylene synthesis and the lines treated with 1-MCP showed significant reduction in ester content signifying regulation of ester biosynthesis by ethylene. However, expression of alcohol dehydrogenase (*ADH*) involved in the interconversion of alcohol and aldehyde flavor volatiles remained largely unaffected resulting in little variation in their content (Defilippi et al., 2005). Zhu et al. (2008) showed differential expression of four apple *AAT* gene isoforms during ripening with climacteric associated increase in the expression of *MdAAT1* and *2* isoforms and thereby rise in the ester biosynthesis.

Aminoethoxyvinylglycine (AVG) is a strong inhibitor of ACC synthase enzyme in ethylene biosynthesis pathway. AVG treated Golden delicious apples showed delayed maturation with significant reduction in the esters and alcohol content compared to the control fruit (Salas et al., 2011). A report by Johnston et al. (2009) stated that volatile biosynthesis in apples had high dependency for ethylene but was less sensitive to low concentrations of ethylene. During melon ripening, two isoforms of *AAT* genes *CM-AAT1* and *CM-AAT2* were monitored for their expression pattern in antisense *ACO* suppressed (AS) fruits and wild type (WT) fruits. The data displayed strong reduction in the expression of both the isoforms in AS as well as WT fruits treated with 1-MCP. However, AS fruits retained the expression levels of these genes upon ethylene treatment implying the role of ethylene in ester biosynthesis during melon ripening (Yahyaoui et al., 2002). Similarly, in oriental sweet melon early onset of ripening with increased synthesis of characteristic volatile compounds in ethephon treated fruits while strong inhibition of volatile synthesis in 1-MCP treated fruits were evident. The study also depicted positive regulation of *LOX*, *ADH* and *AAT* genes expression by ethylene signal (Li et al., 2011). Lypoxygenase (*LOX*) has been involved in the formation of important flavor volatiles in tomato fruit i. e. hexanal, hexenal, hexenol by the hydroperoxidation of polyunsaturated fatty acids. In tomato fruits, the expression patterns of five different *LOX* genes were monitored of which *TomLoxB* isoform displayed ripening associated increase in its expression which was regulated by ethylene (Griffiths et al., 1999).

Apart from these, evaluation of volatile compounds was also performed upon exogenous ethylene or 1-MCP or AVG treatment in Kensington Pride mango (Lalel et al., 2003c). This study revealed enhanced production of aroma volatiles such as terpenes, total esters, aldehydes and alcohols in ethephon treated fruits while 1-MCP and AVG treatment diminished their synthesis. In 'Golden' and 'Golden delicious' apple cultivars, ethephon treated and control fruits showed increased production of aroma volatiles compared to 1-MCP treated fruits throughout the ripening period (Kondo et al., 2005). Although the contribution of ethylene in the regulation of terpene biosynthesis is yet to be clearly understood at molecular level, the findings suggested direct or indirect association of ethylene. Grossly, ethylene modulates the synthesis of key aroma

volatiles in the fruits during ripening and thus opens up new area of research to unravel the ripening associated synthesis of these flavorant chemicals.

1.4.3. Color development

Ripening of fruits has typically been accompanied by chlorophyll degradation and carotenoid and anthocyanin biosynthesis regardless of climacteric or non-climacteric nature of fruits. Amongst the non-climacteric fruits 'Pinot noir' grapes treated exogenously with ethephon, an ethylene releasing compound, improved color formation in dose dependant manner (Powers et al., 1980). Exogenous treatment of ethephon to grape berries elevated the levels of endogenous ethylene by 6 fold within 24h of the treatment and up-regulated the transcripts involved in anthocyanin biosynthesis causing enhanced coloration of grape skin (El-Kereamy et al., 2003). In citrus fruit, exposure to ethylene hastened the degreening process without any adverse effect on taste, aroma and nutritional quality of fruits (Mayuoni et al., 2011). Change in the color of banana fruit skin from green to yellow followed ethylene peak during ripening (Elitzur et al., 2010). In *ACO* suppressed lines of Cantaloupe Charentais melons (*Cucumis melo*), the degreening of rind was completely ethylene dependant with positive correlation between concentration of exogenous ethylene dose and the extent of degreening (Flores et al., 2001). In strawberry fruits, analysis of transgenic *etr1-1* mutant lines with diminished ethylene sensitivity showed less colored achenes compared to the control fruits. Here it was shown that ethylene regulated the expression of important transcription factors which in turn regulated the synthesis of flavonoids (Merchante et al., 2013). In tomatoes, the antisense suppression of *ACC* synthase gene resulted in fruits with delayed chlorophyll degradation and carotenoid synthesis resulting in yellowing of the fruits which never turned red as control fruits. However, exposure of these fruits to propylene initiated ethylene like action reversing the antisense effect and forming fruits which are undistinguishable from the control fruits (Oeller et al., 1991). Significant enhancement in the color index of Kensington pride mango treated with exogenous ethephon was reported by Singh and Janes (2001). All these findings suggest the role of ethylene in regulating color development in both climacteric and non-climacteric fruits during ripening. This also hints existence of common

regulatory mechanism for color formation in these two types of fruits and demands further investigation.

Starch degradation, one of the early ripening events, showed less dependency on ethylene but was highly sensitive to its low concentration in apple fruits (Johnston et al., 2009). It was earlier demonstrated that in transgenic apple fruits with impaired ethylene synthesis, sugar accumulation and loss of acidity were ethylene independent processes (Dandekar et al., 2004). However, improved total sugar content in ethephon treated Kensington pride mango was reported by Singh and Janes (2001). Thus, the exact role of ethylene in starch degradation or in maintaining the sugar/acid balance still remains enigma and insists more elaborate research.

1.5. Modulation of fruit ripening: a role of ethylene antagonists

Postharvest ripening of fruits many a times witnesses excessive softening and development of unpleasant aroma during storage which poses serious impact on the economic value of the fruits. Extensive research to understand the ripening process of various fruits has revealed the central role of ethylene in most but not all transformations. Thus, use of various techniques to minimize the endogenous ethylene production to appropriate level and to maintain the desired quality of fruits for longer duration has been a major thrust area in the postharvest research (Figure 1.4). The discovery of 1-methylcyclopropene (1-MCP) and aminoethoxyvinylglycine (AVG) as ethylene inhibitors was a major breakthrough in the field. The former act by binding to the ethylene receptors with higher affinity than ethylene itself and the later by strongly inhibiting the activity of ACC synthase. This has helped not only to modulate the ripening process of fruits but also to understand their regulation by ethylene.

1.5.1. Spray them for better quality!

Majority of the efforts targeting this objective included the exogenous treatment of these chemicals either alone or in combination or along with controlled atmosphere conditions to the fruits and monitoring the efficacy thereof. Mature green bananas when treated with 1-MCP at pre-climacteric stage resulted in delayed degreening of the skin and significant reduction in total aroma volatiles

(Golding et al., 1998) which was later reported with increase in alcohols content and reduction in their corresponding esters (Golding et al., 1999). Four apple cultivars when treated with 1-MCP and 1-MCP+controled atmosphere conditions, the former treatment showed improved quality parameters such as better firmness and acidity and relatively minimal loss of volatiles (Bai et al., 2005). Also, when tested for their consumer preference displayed similar liking as non-treated fruits without affecting their sale (Marin et al., 2009). In case of strawberry, a broad range of 1-MCP concentration used for the treatment displayed no significant effect on the acceptability of fruits however; the treatment had little or no effect on the shelf life of the fruit (Bower et al., 2003). 1-MCP treatment of kiwifruit cultivar 'Hayward' resulted in delayed softening, color development and incidence of rot decay during ripening (Koukounaras and Sfakiotakis, 2007). Harvesting of tomato at different ripening stages followed by 1-MCP treatment presented better quality, color and shelf life of treated breaker stage (BR) fruits compared to the other stages (Baldwin et al., 2011). In pears, 1-MCP treatment followed by cold storage of fruits proved more suitable with better texture and aroma character of fruits as evaluated organoleptically (Moya-Leon et al., 2006). Although the work has collectively put forth the usefulness of the technique in maintaining keeping quality of fruits, the parameters for 1-MCP treatment varies for each fruit and majority of the times results in significant losses of aroma volatiles such as esters. AVG has also been used, although less popularly, for postharvest maintenance of fruit quality. In peach cultivars 'Redhaven' (Byers, 1997), and 'Biscoe' and 'Encore' (Belding and Lokaj, 2002) pre-harvest sprays of AVG delayed harvest maturity of fruits and retained them longer on trees. The treatment also improved flesh firmness and shelf life of peaches. Combined postharvest treatment of AVG and 1-MCP to 'Melting Flesh' peach cultivar displayed effect of 1-MCP on firmness character during early stages of ripening while AVG reduced the softening during later stages, both collectively maintaining the ground color of the peaches (Hayama et al., 2008). Pre-harvest AVG treatment of 'Golden delicious' apples delayed maturity, maintained color and minimized changes in acidity and total soluble solids. However, the treatment resulted in significant loss of aroma volatiles (Salas et al.,

2011). Thus, similar to 1-MCP, AVG also substantially maintains the postharvest quality of fruits albeit with loss of aroma volatiles to certain extent.

1.5.2. Coat them and preserve them!

Taking into consideration the major concern of prolonging the shelf-life without losing the desirable properties of fruits, postharvest biologists identified the usefulness of applying edible coating and films to the fresh produce. These coatings mainly include 1) hydrocolloids having water based mixture of either proteins and/or polysaccharides, 2) lipids comprising waxes, fatty acids and shellac and 3) composites that are combinations of above two coatings combining the advantages and minimizing the disadvantages of individual coatings. The utility of these coatings has been screened depending on their ability to minimize moisture loss, modify gas exchange, prevent microbial spoilage and maintain the aroma volatile composition and color character. When the polysaccharide based and lipid based coatings were compared for orange (Baldwin et al., 1995) and mango fruits (Baldwin et al., 1999), both imparted attractive shine and color to the fruits. However, polysaccharide based coatings were less active against water lose in both the fruits albeit it delayed decay and enhanced aroma volatile concentration in mango fruits than those in oranges. The starch based composite coatings to strawberry (Garcia et al., 2001) and chitosan based composite coatings to banana (Kittur et al., 2001) showed reduced water loss, minimized microbial growth, selective gas permeability along with better appearance of fruits thereby prolonging their shelf-life. The storage period of cherries was improved by the application of composite coatings although no benefits on color and titratable acidity retentions were achieved (Rojas-Argudo et al., 2005). Use of *Aloe vera* gel as edible coating to Crimson seedless grapes displayed the reduced microbial counts, delayed dehydration and had better appearance without any adverse effects on flavor character (Valverde et al., 2005). This technique also carried the added advantage of medicinal properties of *Aloe vera* gel (He et al., 2005). Recently, a report on effect of aqueous mango carnauba, Semperfresh and *Aloe vera* gel coating on various ripening parameters of Kensington pride mango showed high efficacy of mango carnauba in retarding ripening, improving aroma and fatty acid content compared to the other coatings (Dang et al., 2008).

All these edible coatings create modified atmosphere within fruits however, the rate of respiration, temperature and percent humidity in the surrounding atmosphere are the major limiting factors in the maintenance of modified atmospheres (Baldwin et al., 1995). Besides, there are certain sociological elements involved in the use of coatings such as food allergies or dietary preferences and thus have limited utility. Nevertheless, the industry is ever growing to find more suitable options for variety of fruits to minimize postharvest losses incurred during storage and transport.

1.5.3. Molecular advances for delayed ripening

The progressive advancement in the molecular biological techniques and substantial knowledge of various genes and the pathways associated with distinct ripening related processes have aided in developing the strategies for prolonged shelf-life of vital commodities. For majority of these efforts, tomato has been a plant of choice. Earlier reports describing antisense suppression of ACC synthase, (Oeller et al., 1991) followed by a report on transgenic tomato plant expressing ACC deaminase, an enzyme responsible for degradation of ACC (Klee et al., 1991), showed greater fruit firmness and delayed incidence of ripening extending the shelf-life of fruits. These studies suggested the possibility of manipulating the ripening process of fruits by up- or down-regulation of enzymes involved in ethylene biosynthesis using transgenic approaches. RNAi mediated silencing of a gene encoding ACC oxidase (ACO) resulted in significantly long shelf-life of tomato fruits without any adverse effects on levels of total soluble sugars and solids, titratable acid and amino acids (Xiong et al., 2005). Similarly, cosuppression of *ACO* in papaya fruits resulted in extended shelf-life however, transgenic fruits did not display color change and softening initiation even after 21 days on tree resulting in pathogen attack (Lopez-Gomez et al., 2009). Such studies need further investigation to obtain precisely ripened transgenic papaya fruits. Antisense suppression of various cell wall hydrolysis related genes that bring about softening of fruits has emerged as another beneficial approach. β-galactosidase II (*TBG4*) suppressed lines of tomato and pectate lyase (*PL*) suppressed lines of strawberry produced firmer fruits at ripe stage compared to the respective controls. Moreover, transgenic strawberry showed delayed

postharvest softening with no detectable differences in color, size, shape and weight of transgenic and control fruits (Jimenez-Bermudez et al., 2002; Smith et al., 2002). Expression of two *N*-glycoprotein modifying enzymes, α-mannosidase (α-man) and β-D-N-acetylhexosaminidase (β-Hex) was suppressed by RNAi mediated silencing in tomato fruits. These two enzymes bring about the cleavage of glycosidic linkages of carbohydrates and between carbohydrate and non-carbohydrate, thereby enhancing the pool of free *N*-glycans. The analysis of transgenic tomatoes revealed significantly firmer fruits and approx. 30 days enhanced shelf-life (Meli et al., 2010). All these and few other efforts have provided a number of candidates for genetic manipulation of ripening process in fleshy fruits to obtain better quality and long shelf-life.

1.5.4. Advancing ripening

Hastening of the ripening process, in case of many fruits, has mainly been done to satisfy high market demand and fruit supply. For this purpose, the fruits are usually harvested and transported at mature green stage and are ripened at market places. In such a scenario, early ripening is intended for better economic value of fruits. To solve the purpose, fruits are exposed to ethylene or to compounds that produce or mimic the action of ethylene such as ethrel, ethephon, acetylene or propylene. Of these, direct application of ethylene or ethrel and ethephon treatment are commercially more popular techniques for advancing the ripening. Fast and uniform ripening of avocado fruits has been achieved upon postharvest ethylene treatment after 4 days of cold storage (Kohne, 1985). Preconditioning of 'd'Anjou' pears immediately after harvest or after cold storage with ethylene was found efficient to induce ripening (Chen et al., 1996). Post harvest ethylene application of Bartlett pears at colder temperature during transport yielded better quality fruits with slower rate of ripening reducing the losses during transport (Elizabeth Mitcham, 2002). Postharvest ethephon application to Kensington pride mango (Lalel et al., 2003c) accelerated ripening, increased aroma volatile biosynthesis and fatty acid content while in Langra mango (Gill, 2014) ethephon treatment initiated early softening with increased TSS/acid ratio and improved pulp color. Ethephon treatment of melon fruits displayed advanced climacteric peak with positive influence on the

accumulation of vital aroma volatile such as esters giving more flavored melons (Li et al., 2011). Being natural ripening hormone, meticulous standardization of postharvest ethylene application has no detrimental effects on fruit quality although it results in reduction of fruit shelf-life.

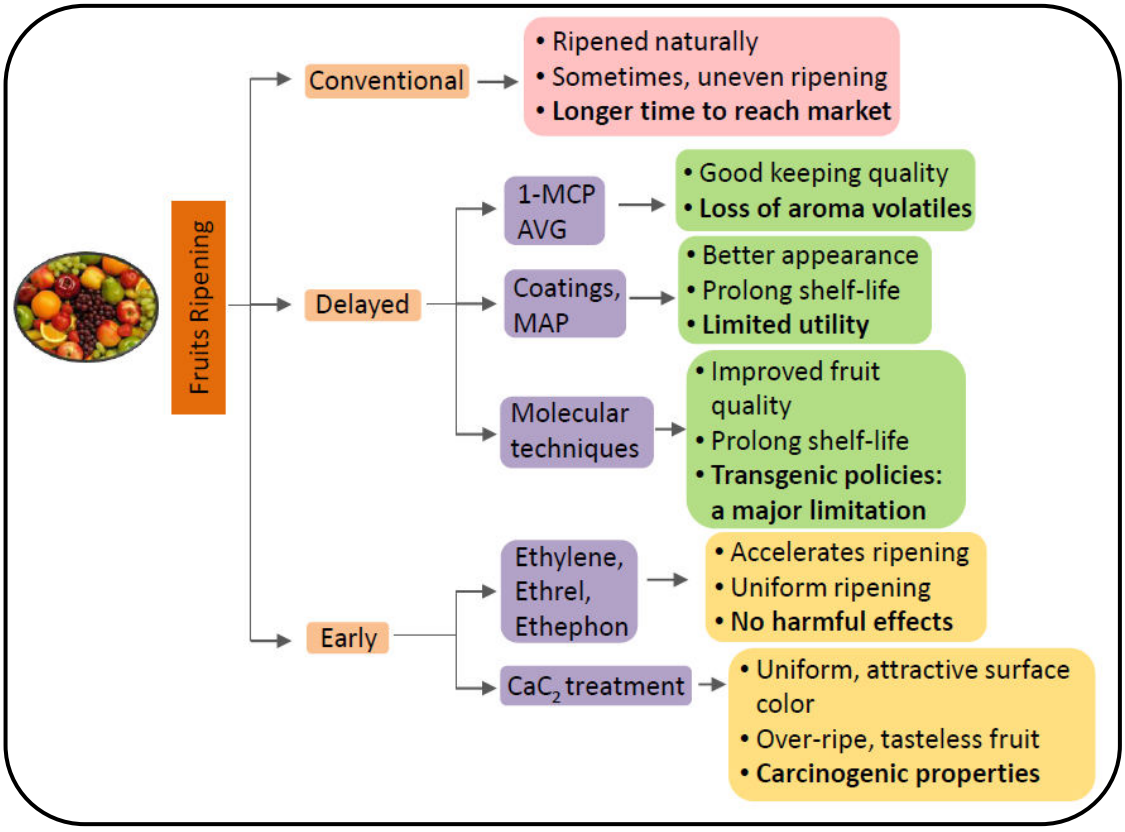


Figure 1.4: Schematic representation of commonly used postharvest treatments for modulation of ripening process

Despite of these advantages, treating fruits with ethylene requires building and maintenance of ethylene chambers which is expensive and requires expertise. Hence, the use of less expensive and easily available chemical alternative, calcium carbide (CaC_2) has increasingly become popular among the local traders. Calcium carbide reacts with moisture in the air and releases acetylene which mimics the action of ethylene. Fruits ripened by this method develop uniform attractive color but inside it remains tasteless and raw with inferior quality and nutritive value (Talat, 2013). Besides, CaC_2 used for the purpose usually has traces of arsenic and phosphorus hydride having carcinogenic properties and the acetylene gas produced has harmful effects on human health. Considering the high risk involved in the use of this chemical, it has been banned under PFA rules

1955 and Food Safety and Standards Regulations (Prohibitions and Restrictions on Sale), 2011 therein (http://www.fssai.gov.in/Portals/0/Pdf/Article_on_fruits.pdf). Continuing work by the postharvest biologists to fetch more suitable and non-hazardous alternatives for ripening of fruits will facilitate the supply of fruits with better cosmetic as well as nutritive value at consumer end.

1.6. Mango: the king of fruits

Mango (*Mangifera indica* L.), is a dicotyledonous fruit of *Anacardiaceae* family. It is one of the most important tropical fruits originated in Indo-Burma region. Its cultivation in India is as old as Indian civilization (DE Candolle, 1884) and the country possesses huge diversity of this fruit. As per the recent statistics, India has 2.46 million ha area for mango plantation which is the highest amongst mango growing countries (Sekhar, 2013). Moreover, India is the top most mango producing country accounting for 14.8 MMT yield contributing 51% of the total world production (28.8 MMT). However, India stands at fourth position amongst various mango exporting countries in the world. Among the various fruits exported by India, mango stands second after grapes with 12.4% share of fruits export to various countries (http://apeda.gov.in/apedawebsite/six_head_product/FFV.htm). Within India, mango plantation covers the highest (35%) of the total fruit cultivation area; while, in terms of production, mango contributes 21% to the total fruit yield of the country, closely following citrus fruits and banana (http://nhb.gov.in/area%20_production.html). All this statistics clearly display importance of this fruit in foreign exchange and agro-rural developments in India (Figure 1.5). Among the vast diversity of mango cultivars, Alphonso is highly favoured and most exported mango. This is mainly due to its captivating attributes such as attractive color, sweet aroma, excellent sugar/acid ratio, thin skin, soft and low fibre containing pulp and good keeping quality. Nevertheless, the quality of ripe Alphonso fruits is largely influenced by the cultivation location, harvest maturity, tree nutrition and postharvest handling and storage conditions. Development of spongy tissue during ripening of Alphonso is one of such physiological disorders which acquires majority of losses. Besides, the

contamination of fruits by fruit flies and other quarantine pests drew ban on export of Alphonso mangoes by EU countries last year. This has brought serious apprehension and losses at the international trade.

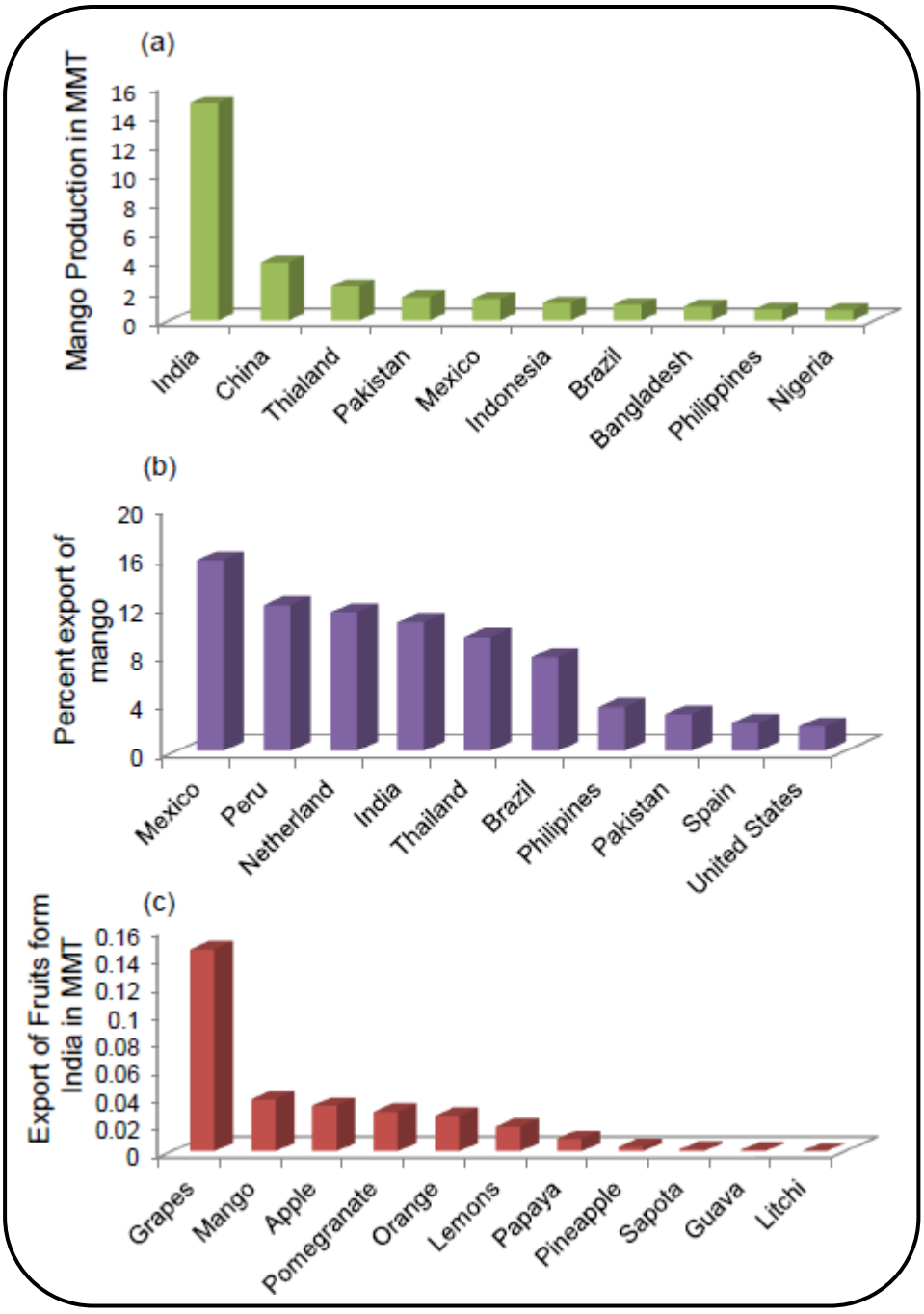


Figure 1.5: Production (a) and export (b) of mango by few top most countries in the year 2013 and export of various fruits from India in the year 2013 (c).

In the light of this knowledge, postharvest researchers are constantly into finding the techniques for precise ripening and minimum deterioration of the fruits to deliver best quality to the consumer at national as well as international market.

1.6.1. Mango postharvest management

A conventional way of mango ripening includes harvesting of fruits at complete mature stage and ripening them at ambient temperature in a stack of hay (usually paddy straw) either open or in corrugated fibre board (CFB) boxes having holes for aeration. Ripening period of mangoes varies from 6-15 days after harvest depending on cultivar and usually gets over-ripe and excessive soft beyond this (Tharanathan, 2006). When mangoes are destined for long distance transport, minimal losses and early ripening with better appearance and eating quality are intended. In this view various attempts have been made using different post harvest treatments to mango fruit. Exogenous ethylene treatment at low temperature and high humidity conditions followed by transport at low temperature (Barmore, 1977), cold storage of harvested mangoes after ethylene treatment (Lam and Wong, 1988; Montalvo et al., 2007) and vacuum treatment for short time (20 min) followed by ethylene exposure (Tovar, 2011) resulted in uniform ripening with better color and eating quality and reduction of ripening periods. Controlled atmosphere storage at colder temperature (13°C) with appropriate ratio of O_2/CO_2 was suitable for longer storage (38 days) of 'Delta R2E2' cultivar without alteration in vital aroma volatile composition (Lalel and Singh, 2006). Modified atmosphere packaging of ethephon treated Kensigton pride mango resulted in long shelf-life and improved fruit quality after 25 days of storage at colder temperature (Singh and Janes, 2001). Apart from these various postharvest treatments utilizing mainly exogenous ethylene application, utility of certain edible coatings was also evaluated for storage of various mango cultivars. These included polysaccharide based, lipid based or composite coatings. The technique has grabbed a considerable success in retarding ripening process while retaining the important attributes such as fruit firmness, aroma volatiles and color index (Baldwin et al., 1999; Carrillo-Lopez et al., 2000; Dang et al., 2008; Hoa et al., 2002; Kittur et al., 2001). Recently, a utility of gamma irradiation and colder temperature storage for long shelf-life and better quality of Alphonso mango has been reported by Yadav (2014). Although, all these techniques satisfy the goal of receiving better quality mangoes at appropriate time to certain extent, they are burdened to pass the quarantine to reach the international consumer. In this view, concerted efforts by postharvest biologists, mango growers, transporters

and exporters can assist to enhance the quality of mango produce and facilitate the betterment of mango trade.

1.6.2. Mango ripening: biochemical and molecular perspective

Apart from being economically important commodity, mango fruits are excellent dietary supplement owing to their high nutritive value and unprecedented organoleptic properties. During ripening, mango fruit undergoes well coordinated and extensive biochemical and molecular changes imparting the desired attributes to the ripe fruit. Considerable efforts have been put in to understand various aspects of mango ripening. Color change as a consequence of chlorophyll degradation and analysis of pigment composition of various mango cultivars was reported by a number of researchers (Lizada, 1993 and references therein). Significant transition in the taste of mango fruits during ripening was analyzed in terms of starch degradation (Gonicalves Peroni et al., 2008; Simao et al., 2008), sugar metabolism (Castrillo et al., 1992; Fuchs et al., 1980) and changes in the sugar acid ratio (Medlicott and Thompson, 1985). Evaluation of fatty acid composition during ripening and its correlation to the flavor characteristic of mango reported by (Bandyopadhyay and Gholap, 1973a) was one of the pioneering studies on flavor biochemistry of mango. Pectic compositions of cell wall and changes during ripening were analysed using state-of-art techniques (Prasanna et al., 2004; Yashoda et al., 2005). Activities of various enzymes such as carbohydrases (Abusarra and Abugoukh, 1992; Roe and Bruemmer, 1981; Yashoda et al., 2007), β-galactosidase (Ali et al., 1995; Prasanna et al., 2005) and polygalactouronase (Tharanathan et al., 2006) were studied with respect to fruit softening during ripening. As volatile biosynthesis is an imperative part of mango fruit ripening, changes in its composition as influenced by ripening (Lalel et al., 2003a), maturity stage at harvest (Lebrun et al., 2008), on-tree ripening and conventional ripening (Shivashankara et al., 2006), fungal infection (Moalemiyan et al., 2006), storage temperature (Lalel and Singh, 2004; Nair et al., 2003) and within fruit variation (Lalel et al., 2003b) were reported. Diversity of volatile compounds in 27 mango cultivars depicting sesquiterpene and monoterpene dominant cultivars (Pandit et al., 2009a) followed by a study on dynamics of volatile compounds during development and ripening of Alphonso mango

showing the *de novo* appearance of lactones and furanones at late ripening stages (Pandit et al., 2009b) were reported. Also, cultivation locality based variation in flavor character of Alphonso mango was also studied with respect to variation in the volatile content from three distinct cultivation localities (Kulkarni et al., 2012a). Moreover, fruit drop in Alphonso mango causing considerable losses was also related to the changes in ethylene production and ACC content (Murti et al., 2008).

Molecular studies on various aspects of mango ripening have fortified our knowledge of the process to considerable extent. Being climacteric fruit, ethylene is central to various ripening processes. Gene encoding *ACC oxidase*1 involved in ethylene biosynthesis (Zainal et al., 1999) and ethylene receptor ETR1-homologue (Martinez et al., 2001) were isolated from mango. Saiprasad et al. (2004) isolated five ripening related cDNAs from Alphonso and Totapuri mango cultivars however; their involvement as regulators of ripening rather than direct modulators was anticipated. Isolation and characterization of few genes differentially expressed during spongy tissue development in Alphonso mango were reported by Vasanthaiah et al. (2006). Ripening associated increase in the message and protein levels was observed for alternative oxidase (Aox) and uncoupling protein (Ucp) during mango ripening suggesting their role in post-climacteric senescence process (Considine et al., 2001). Besides, ripening associated expression of expansin gene *MiExp1* (Trivedi and Nath, 2004), pectate lyase *PL* (Chourasia et al., 2006) and endo-β-1,4-glucanase (EGase) homologue, *MiCel1* (Chourasia et al., 2008) from Dashahari mango gave insight in the softening of mango fruits. Recently, three distinct isoforms of alcohol dehydrogenase cDNA (*ADH*) and their differential expression during development and ripening of mango fruits has also been reported (Singh et al., 2010). Moreover, isolation and characterization of three prenyl transferases *viz*. *MiGPPS1*, *MiGPPS2* and *MiFPPS* from Alphonso mango (Kulkarni et al., 2013b) followed by isolation and characterization of *MiEO* involved in furaneol biosynthesis have been reported (Kulkarni et al., 2013a). This collectively points towards growing interest of the molecular biologists to get insight in the unique ripening process of this King of fruits.

1.7. Genesis of thesis and its organization

High economic importance of this fruit drives the tireless efforts by post harvest researchers and mango growers to meet the quality requirements at consumer end and to generate a better trade. Here at the Plant Biochemistry and Molecular Biology Group, NCL, we initiated work to unravel the molecular and biochemical basis of flavor biogenesis and few other aspects of ripening of one of the highly important indigenous mango cultivar, Alphonso. The number of studies carried out till date by our group have collectively enlightened our perception of mango ripening which included, cultivar based variation in the volatile composition of mango (Pandit et al., 2009a), dynamics of volatile composition during development and ripening of Alphonso (Pandit et al., 2009b), chemical basis for cultivation locality dependant variation in the flavor of Alphonso mango (Kulkarni et al., 2012a), expression profiling (Pandit et al., 2010) and characterization (Kulkarni et al., 2013a; Kulkarni et al., 2013b) of genes involved in flavor biosynthesis in Alphonso mango.

A step ahead on the similar lines, the present thesis mainly addresses the influence of popular postharvest technology, 'pre-climacteric ethylene treatment', on vital ripening attributes of Alphonso mango at biochemical and molecular level, which primarily decides the market success of the cultivar.

The thesis has been organized in the following manner

Chapter 1: General Introduction and Review of Literature
Fruit ripening: A role of two carbon molecule, Ethylene

Chapter 2: Pre-climacteric ethylene treatment modulates the ripening related glycosidase activities, their transcript abundance and sugar content in Alphonso mango fruit

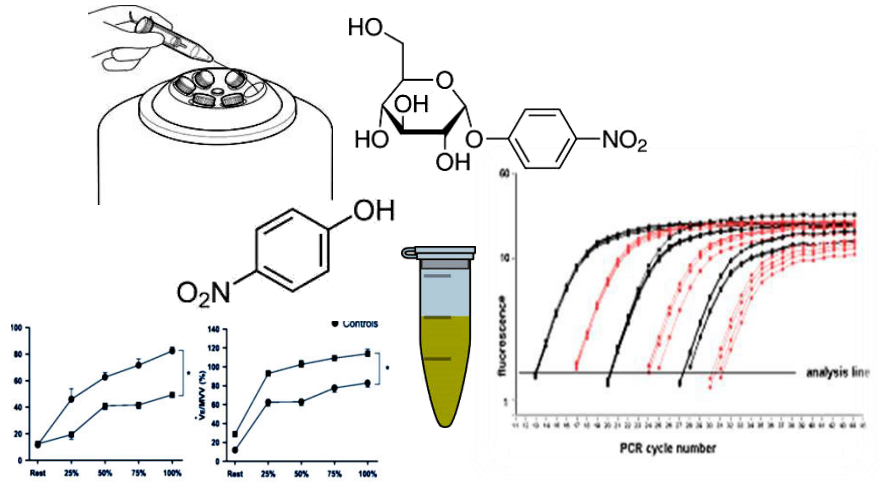
Chapter 3: Spatial and temporal changes in the volatile profile of Alphonso mango upon exogenous ethylene treatment

Chapter 4: Molecular cloning and expression of *O*-methyltransferase catalysing the synthesis of mesifuran, a key aroma volatile in Alphonso mango fruits

Summary and future directions

Bibliography

Chapter 2



Pre-climacteric Ethylene Treatment Modulates The Ripening Related Glycosidase Activities, Their Transcript Abundance and Sugar Content in Alphonso Mango Fruit

2.1 Introduction

Fruit ripening, a well coordinated phenomenon, has gained considerable attention among the researchers owing to many desirable traits that are acquired by the fruit during this process (Giovannoni, 2001). Fruits, based on the presence of system I or system II of ethylene production, are categorised as non-climacteric and climacteric, respectively (McMurchie et al., 1972). Lot of efforts have been put in to decipher the role of ethylene in the fruit ripening process through exposure of peach and apple fruits to varying concentrations of ethylene (Hayama et al., 2006; Johnston et al., 2009) or by application of ethylene antagonists 1-MCP and AVG to peach fruits (Belding and Lokaj, 2002) or by analysis of a series of tomato mutant lines varying in ripening stages related to ethylene perception (Cara and Giovannoni, 2008). Direct or indirect involvement of ethylene signal in controlling the biosynthesis of enzymes active in cell wall solubilisation, pigments synthesis, volatile production and starch degradation has been evident from large number of investigations targeting these aspects of ripening (Bapat et al., 2010). All these reports have collectively fortified our perception of various aspects of climacteric fruit ripening.

Mango being a climacteric fruit, its ripening is also governed by system II of ethylene production which triggers irreversible ripening events (Lizada, 1991). The ripening process of mango fruits has been explored with major focus on changes in sugar content (Castrillo et al., 1992), cell wall composition (Prasanna et al., 2004), pulp softening (Yashoda et al., 2006), volatile biosynthesis (Pandit et al., 2009b), gene expression (Pandit et al., 2010) and switch from chloroplast to chromoplast (Medlicott et al., 1986). Moreover, two enzymes from the glycosyl hydrolase family, β-D-Galactosidase (EC 3.2.1.23) and α-D-mannosidase (EC 3.2.1.24) having proven role in tomato fruit softening (Meli et al., 2010; Smith et al., 2002) have been reported as major glycosidases in the ripening of Alphonso mango fruit (Prasanna et al., 2005; Yashoda et al., 2007). Moreover, Ali et al. (1995) have purified and characterized β-galactosidase from 'Harumanis' mango suggesting its key role in pectin modification during ripening. Ethylene synthesis being crucial step of climacteric fruit ripening process, its involvement in tissue softening and aroma volatile synthesis has been a major area of focus. These factors pose direct influence on the consumer acceptability of the fruit (Bapat et

al., 2010; Barry and Giovannoni, 2007). Effect of exogenous application of ethylene on the volatile accumulation in Kensington pride mango (Lalel et al., 2003c), eating quality in Harumanis mango (Lam and Wong, 1988) and expression pattern of pectate lyase in Dashehari mango has been investigated so far (Chourasia et al., 2006). Exogenous application of ethylene has also been shown to stimulate catalase and peroxidase activity in Alphonso fruit slices (Mattoo and Modi, 1969). Activity profiling of various cell wall hydrolysing enzymes during the ripening stages of Alphonso mango has depicted peak at the climacteric stage (except pectin methyl esterase) suggesting correlation between the ethylene evolution and initiation of softening process in mango (Yashoda et al., 2007). However, the effect of exogenous ethylene application on the ripening associated softening of mango has not been investigated so far. The present work was carried out to analyze the enzyme activity profile and transcript abundance of three major glycosyl hydrolases *viz*. β-D-galactosidase, α-D-mannosidase and β-D-glucosidase (EC 3.2.1.21) in various ripening stages of Alphonso fruits upon exogenous ethylene application.

2.2. **Materials and methods**

2.2.1. Plant material sampling, treatment and storage

Mature raw fruits of Alphonso mango were collected from mango orchard of Dr. Balasaheb Sawant Konkan Agriculture University at Dapoli, Maharashtra, India (N17°45′ E73°11′). Three trees were randomly chosen and two sets of at least 20 fruits devoid of visual symptoms of any disease or abnormality were collected from each of these plants and used for the analysis to generate statistically valid data. These randomly chosen trees from the orchard were distantly located (at least 30 m away) from each other and the fruits were harvested from the branches at the periphery and at the height range of 2–5 m to have the fruits exposed to the similar intensity of sunlight. Simultaneously, mature leaves and open flowers were also sampled. This practice was followed for all the trees. Immediately after harvest, one of these two sets of fruits was kept in plastic crates, and further kept inside a closed chamber (3 m X 3 m X 3 m). Ethylene gas was sprayed inside the closed chamber to a final concentration of 100 ppm. The chamber was closed for 24 hr and maintained at 30±2°C and 85–90% humidity. The other set was kept

untreated and was referred to as the control. Fruits from the both sets were then put in hay-packed boxes, carried to the laboratory and allowed to ripen at ambient temperature. Freshly harvested fruits were immediately cut and frozen in liquid nitrogen and this stage was considered as 'mature raw' which was denoted as 0 DAH (days after harvest) stage of control fruits. In case of ethylene treated fruits, 1 DAH (mature raw) corresponds to 0 DAH of control fruits owing to the 24 hr ethylene treatment given to this set of mangoes after harvest. Subsequent ripening stages of control and ethylene treated fruits were frozen according to the ripening indices as described by Yashoda et. al. (2007) as stated below,

Mature raw: dark green, stony hard ⟶ 0 DAH (control), 1 DAH (ethylene treated)

Pre-climacteric: light green, hard ⟶5 DAH (control), 5 DAH (ethylene treated)

Mid-ripe: yellowish green, intermediate ⟶10 DAH (control), 7 DAH (ethylene treated)

Ripe: yellow, soft⟶ 15 DAH (control), 9 DAH (ethylene treated)

Over-ripe: Dark yellow, excessive soft ⟶20 DAH (control), 11 DAH (ethylene treated).

In this way, five ripening stages each for control and ethylene treated fruits along with mature leaf and open flowers were stored at -80°C until use. To clearly depict the effect of pre-climacteric exogenous ethylene treatment on the ripening of Alphonso mango, all the sampling stages were referred in terms of days after harvest (DAH) for control and ethylene treated fruits in the present thesis. For the present analysis only pulp tissue was taken in to consideration.

2.2.2 Enzyme extraction and assays

To obtain crude enzyme extract from all the ripening stages under study, 1g mesocarp tissue from each stage was ground to a fine power in liquid N_2 and extracted with 5ml of extraction buffer containing 50mM HEPES-NaOH (pH 7.4), 5mM $MgCl_2$, 2% (v/v) glycerol and 2% (w/v) polyvinylpyrolidone. The homogenate was centrifuged at 15,000g for 30min at 4°C. The supernatant thus obtained was used as a crude enzyme preparation for the following enzyme assays. The protein content of the crude extract was estimated using Bradford's method (Bradford, 1976) employing BSA as a protein standard.

The enzyme assays for β-D-glucosidase and α-D-mannosidase were performed in 0.1M sodium acetate buffer (pH 5.6) having 100µL enzyme and 8mM *p*-nitrophenyl-β-D-glucopyranoside and *p*-nitrophenyl-α-D-mannopyranoside, respectively in a total volume of 0.5mL. The reaction mixture was incubated at 37°C for 15min and liberated *p*-nitrophenol was measured on spectrophotometer (BioRad xMark™ Microplate Spectrophotometer) at 420 nm after the addition of 0.5M Na_2CO_3. The assay for β-D-galactosidase was performed in the similar way in a 0.1M phosphate buffer (pH 6.6) with *p*-nitrophenyl-β-D-galactopyranoside as a substrate. The enzyme activities for all the three enzymes were reported in terms of mM of *p*NP released per minute in a standard assay condition.

2.2.3 Reducing and non-reducing sugar estimation

To determine the amount of reducing and non-reducing sugars in the ethylene treated and control fruits, 250mg tissue was ground to fine powder in liquid N_2 and added to 1mL extraction buffer containing 50mM HEPES-NaOH (pH 7.4), 5mM $MgCl_2$, 1mM EDTA, 2% (v/v) glycerol and 2% (w/v) polyvinylpolypyrrolidone. The homogenate was centrifuged (Eppendorf, Centrifge 5424R) at 15,000g for 30 min at 25°C. The supernatant thus obtained was used for the estimation of reducing sugars using DNSA reagent wherein 50µL of supernatant was added to 500µL of DNSA reagent and the mixture was kept in boiling water bath for 5 min. The sugars were estimated spectrophotometrically at 540 nm. The total sugar content was obtained by acidic hydrolysis of the obtained supernatant. For hydrolysis 10µL of supernatant and 10µL of 1N H_2SO_4 were added to 80µL of buffer and reaction was kept in boiling water bath for 15min. Mixture was then neutralized with 40µL of 1N NaOH. To the reaction mixture 500µL of DNSA reagent was added and total sugars were estimated spectrophotometrically at 540nm. Quantitation of sugars was done using maltose standard curve. Quantity of non-reducing sugars was obtained by subtracting the values of reducing sugar from that of total sugars. All the sugars were represented in terms of mg of sugar per gram of tissue.

2.2.4 RNA isolation and cDNA synthesis

Isolation of total RNA from 1g of all the tissues individually was performed using RNeasy Plus mini kit (Quiagen, Venlo, Netherlands). Reverse transcription of the isolated RNA was carried out over 2µg of total RNA using Applied Biosystem High Capacity reverse transcription kit (Applied Biosystems, Waltham, Massachusetts, USA). cDNA obtained this way was used to isolate partial fragments of β-D-galactosidase, α-D-mannosidase and β-D-glucosidase genes and also for measurement of transcript abundance using quantitative real time PCR.

2.2.5 Isolation of partial genes and qRT-PCR

Degenerate primers were designed based on the conserved region in the nucleotide sequences of the β-D-glucosidase and α-D-mannosidase genes from the other plants reported in the NCBI database (http://www.ncbi.nlm.nih.gov/). For β-D-galactosidase gene fragment, gene specific primers were designed directly based on the partial sequence of the gene reported in the NCBI data base from *Mangifera indica cv.* Tommy Atkins (NCBI accession no. AM040280). These primers (Table 2.1) were used to amplify partial gene sequences using above synthesized ripe fruit cDNA (1 µL). PCR was performed in 20 µL reaction volume with 1X buffer, 1.5 mM $MgCl_2$, 200 µM dNTPs, 0.6U *Pfu* DNA polymerase (Promega, Madison, WI, USA)) and 0.5 µM each of forward and reverse primers. The thermal cycler reaction conditions were as follows – initial denaturation of 94°C for 3 min, 35 cycles of 94°C for 45 sec, annealing temperature (dependent on the primer pair) for 45 sec min, extension at 72°C for 2 min and final extention for 5 min at 72°C. The fragments obtained were resolved on 1-1.5% agarose gel in 0.5X Tris-acetate EDTA (TAE) (Sambrook and Russell, 2001) with a 1kb ladder (Promega) as the size marker and the amplicons were visualized by GelRed (Biotium Inc. Hayward, CA). Amplicons of desired size were gel eluted using GenElute™ Gel Extraction Kit (Sigma Chemical Co., USA) and cloned in pGEM-T Easy vector as per the manufactures' protocol (Promega, Madison, WI, USA) and the ligation reactions were transformed in *E. coli* cells (Top10, Invitrogen, USA). Positive recombinant colonies were identified by colony PCR and the presence of desired insert was confirmed by sequencing. Based on these partial gene sequences primers for quantitative real time PCR

were designed (Table 2.1). Quantitative real-time PCR was performed using FastStart Universal SYBR Green master mix (Roche Inc. Indianapolis, Indiana, USA) and elongation factor 1α (*EF 1α*) as an endogenous control (Pandit et al., 2010) employing primers mentioned in Table 2.1.

Table 2.1 Primers used for the isolation of partial genes from Alphonso mango and for their transcript quantification with qRT-PCR

Primer ID	Gene	Group[a]	Sequence (5' to 3')
GluDe F	β-D-Glucosidase	A	GCRCCDGGNMGVTGYTC
GluDe R	β-D-Glucosidase	A	TRRTTBANBCCDAYRWARTC
ManDe F1	α-D-Mannosidase	A	ATGCWCAYACATGGTTYCG
ManDe R1	α-D-Mannosidase	A	GRCGRAASAYATAWGCYCCAG
ManDe F2	α-D-Mannosidase	A	TATGMATGAYGAGGCWAMYY C
ManDe R2	α-D-Mannosidase	A	WYAMTYCYTSMCCAASACCWC
MiβGal F	β-D-Galactosidase	B	CCACCTTGGTCCATCAGCAT
*Mi*βGal R	β-D-Galactosidase	B	CGCCGGTGCATTGAAAGTAG
*Mi*βGal RT-F	β-D-Galactosidase	C	TGCCCAAAGCTCCCTGAAAC
*Mi*βGal RT-R	β-D- Galactosidase	C	CCACAGCCCATCAGTGGTAA
*Mi*αMan RT-F1	α-D-Mannosidase	C^{I}	CCTGGAAGTGACCGAACCAA
*Mi*αMan RT-R1	α-D-Mannosidase	C^{I}	CACCGAGCGATCCACTAACA
*Mi*αMan RT-F2	α-D-Mannosidase	C^{II}	TCTCCTCCGTCGTATGGGTT
*Mi*αMan RT-R2	α-D-Mannosidase	C^{II}	CAGCATCCCAAGTTTGACGC
*Mi*αMan RT-F3	α-D-Mannosidase	C^{III}	CGGACCGTGCAAATGCTTAC
*Mi*αMan RT-R3	α-D-Mannosidase	C^{III}	TGCCTTGCCGCCAAATAGTA
EF1α F	Elongation factor 1α	C	AATACGACTCACTATAGGGCA AGCAG
EF1α R	Elongation factor 1α	C	ATACGACTCACTATAGGGCTCC TTCTC

[a] Group A primers were degenerate and used to isolate the partial β-D-Glucosidase and α-D-Mannosidase gene sequences; Group B primers were gene specific (NCBI accession no. AM040280) and used for isolation of partial β-D-Galactosidase gene sequences; Group C were gene specific primers used for the transcript quantification with qRT-PCR. $^{I, II, III}$ represents primers for α-D-mannosidase transcript variant 1, 2, and 3 respectively.

Abundance of β-D-galactosidase and α-D-mannosidase transcripts was quantified on ViiA™ 7 Real-Time PCR System (Applied Biosystems, CA, USA) with thermal cycle programme consisting of initial denaturation at 95°C for 10min with subsequent 40 cycles of 95°C for 3sec and 60°C for 30sec followed by a dissociation curve analysis of transcripts. Considering the transcript abundance at 0DAH (control fruit) as 1, the fold difference in the transcript levels of β-D-galactosidase and α-D-mannosidase genes in rest of the tissues was calculated. This complete process for both the genes was repeated for three independent biological replicates, each of which was represented by at least three technical replicates.

β-D-glucosidase gene transcripts were analyzed by semi-quantitative PCR in raw as well as ripe pulp, skin, leaf and flower cDNAs. Two microliters of each of these cDNAs were used in the 20µL PCR reaction containing 1X *Pfu* buffer (Promega, Madison, WI, USA), 2.0mM $MgCl_2$, 0.5mM dNTPs, 0.5µM of each gene specific primer and 1 unit *Pfu* DNA polymerase (Promega, Madison, WI, USA). The thermal cycling programme consisted of initial denaturation at 95°C for 3 min with subsequent 35 cycles of 95°C, annealing temperature and extension at 72°C each for 30 sec. *EF 1α* gene was used as an endogenous control to monitor the uniformity of expression across the tissues. Equal amount of loading dye (6X) with 1X final concentration of GelRed (Biotium Inc. Hayward, CA) was added to the PCR products for visualisation and the PCR products were separated through 2% agarose gel.

2.2.6 β-D-galactosidase histochemical assay

To assess the β-galactosidase activity *in vivo*, Alphonso fruits at 15 DAH (ripe-control) and 9 DAH (ripe-ethylene treated) were sectioned longitudinally and incubated in staining solution containing 50 mM sodium phosphate pH 7; 10mM EDTA; 0.1% (w/v) Triton X 100; 20% (v/v) methanol and 1mM X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) for 4 hr at 25°C in dark (Figueiredo et al., 2011). The staining solution was discarded after incubation and the fruits were decolorized in methanol. Blue color developed due to the activity of β-galactosidase was monitored visually. Color pixels in the fruit were calculated using Windows Photoshop CS6 software wherein complete fruit was

selected and total number of pixels was recorded. Then yellow color from the fruit was selected and number of pixels for that was recorded for the same fruit. Thus, the number of blue color pixels was calculated by subtracting number for yellow pixels from the total number of pixels. This analysis was performed for both the ethylene treated and control fruits for all the three biological replicates individually.

2.2.7 Statistical analysis

ANOVA by fisher's LSD (least significant difference) test was carried out ($p \leq 0.05$) using StatView software, version 5.0 (SAS Institute Inc., Cary, NC, USA) to compare the enzyme activities of β-D-glucosidase, β-D-galactosidase and α-D-mannosidase between the ripening stages of control and ethylene treated fruits seperately. To compare the relative transcript abundance of β-D-galactosidase and three distinct α-D-mannosidase transcript variants independently amongst the entire tissue set, ANOVA by fisher's LSD test was performed ($p \leq 0.05$) as mentioned previously.

2.3. **Results**

2.3.1 Changes in the enzyme activity profile of three distinct glycosidases upon ethylene treatment

The total protein content (10.64 ±0.35 mg mL^{-1}) of the extracts as evaluated by Bradford's assay was found to be uniform (without any significant difference) among all the stages of control and ethylene treated fruits. From the ripening profile it was clearly evident that ethylene treated fruits attained ripe stage six days prior to control fruits (conventionally ripened) suggesting the accelerated ripening upon ethylene treatment. Thus, the sampling of five ripening stages was represented by different days after harvest for control and ethylene treated fruits covering the entire process of ripening with or without ethylene treatment. The activity profiling of three glycosidases i.e. β-D-galactosidase, α-D-mannosidase and β-D-glucosidase during ripening of Alphonso mango revealed progressive increase in the activity of these enzymes as fruit attained ripe stage. However, upon pre-climacteric ethylene treatment there was early onset of activities of all the three enzymes with significantly enhanced activity in 5 DAH (pre-climacteric)

stage of ethylene treated fruit. Such surge of activity was the highest for β-D-galactosidase which was followed by activity of α-D-mannosidase with the least increase in the activity of β-D-glucosidase (Figure 2.1). Among the three glycosidases, β-D-galactosidase showed the highest activity in control as well as in ethylene treated fruits. In the control fruits, no significant difference in the activity of this enzyme was observed between 10 (mid-ripe), 15 (ripe) and 20 DAH (over-ripe) stages, but was significantly higher from that of 0 (mature-raw) and 5 DAH (pre-climacteric) stage which depicted the lowest activity. Nonetheless, in ethylene treated fruit, 35 fold increase in the activity was observed from 1(mature-raw) to 5 DAH (pre-climacteric) stage with no significant variation among the 5 DAH (pre-climacteric) and the stages thereafter. As clearly evident from the activity profile, control fruit displayed steady activity once it reached to its maxima at the 10 DAH (mid-ripe) while in case of ethylene treatment, even after surge in the activity at 5 DAH (pre-climacteric), a consistent increase in the activity was observed till the 11 DAH (over-ripe) stage (Figure 2.1I). The activity sketch of α-D-mannosidase presented similar activity profile as that of β-D-galactosidase for control fruits. However, on ethylene treatment a steady increase in the activity was seen from 1 to 9 DAH (mature-raw to ripe) with significant lowering at 11 DAH (over-ripe) stage.

Further, the activity of α-D-mannosidase in the 5 DAH (pre-climacteric) ripening stage of ethylene treated fruits was 19 fold higher than that at the 5 DAH (pre-climacteric) stage of control fruits. Collectively, amongst all the ripening stages of control and ethylene treated fruits, the 10 (mid-ripe), 15 (ripe) and 20 DAH (over-ripe) pulp of control fruits and the 7 (mid-ripe) and 9 DAH (ripe) ethylene treated fruit did not vary significantly for their α-D-mannosidase activity (Figure 2.1II). The activity pattern of β-D-glucosidase revealed comparable profile in the control and ethylene treated fruits except the 5 DAH (pre-climacteric) stage of ethylene treated ones. In the control fruits, increase in the activity of β-D-glucosidase was evident from the 5 to 10 DAH (pre-climacteric to ripe) stage with no significant change till the 15 DAH (ripe) stage followed by significant decline in the activity on the 20 DAH (over-ripe). However, in ethylene treated fruits, this enzyme reached to the peak of its activity at the 7 DAH (mid-ripe) after which it exhibited decreased activity till the 11 DAH (over-ripe). Even so, the 5 DAH (pre-

climacteric) ethylene treated fruits varied significantly from the 5 DAH (pre-climacteric) control fruits with 5.6 fold enhanced activity than the later (Figure 2.1III).

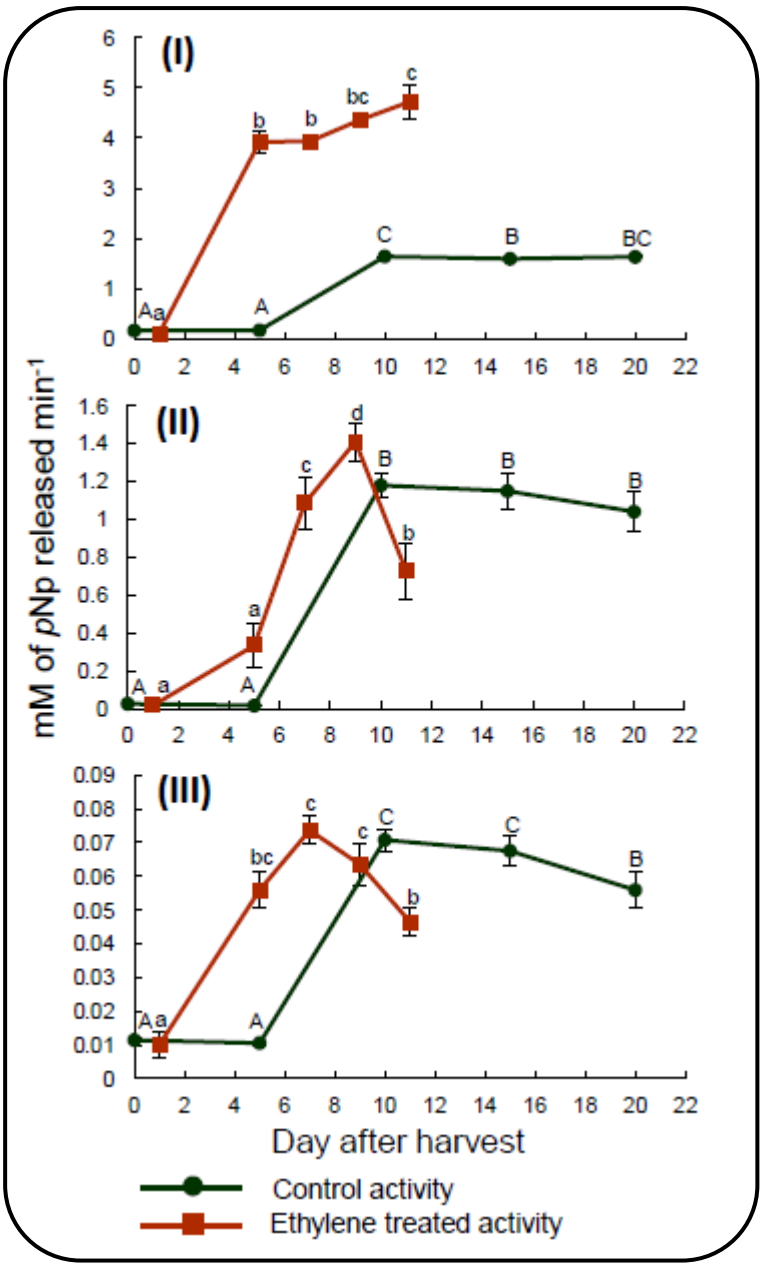


Figure 2.1: Activity (mM of *p*NP released min^{-1}) of β-D-galactosidase (I), α-D-mannosidase (II) and β-D-glucosidase (III) detected in the ripening stages of ethylene-treated and control Alphonso mango. Stages with different letters above the trend line for the ethylene treatment (a, b, c, . . ., etc.) and control (A, B, C, . . .,etc.) show values significantly different at $p \leq 0.05$. Vertical bars at each point represent standard error of measurement among the biological replicates.

Taken together, the activity profiles of all the three enzymes followed a very consistent pattern in the control fruits wherein the 0 (mature raw) and 5

DAH (pre-climacteric) stages showed 'near zero' activity of all the three enzymes with concomitant increase from the 5 (pre-climacteric) to 10 DAH (mid-ripe) stage, presenting the 10 DAH (mid-ripe) as a point of the highest activity for all of them. Although β-D-galactosidase did not experience a significant decline in the activity post 10 DAH (mid-ripe) maxima, other two enzymes displayed lowered activity profile suggesting their reduced activity as fruit progressed towards over-ripe stage. In case of ethylene treated fruits, β-D-galactosidase, α-D-mannosidase and β-D-glucosidase showed peak activity at the 11 DAH (over-ripe), 9 DAH (ripe) and 7 DAH (mid-ripe), respectively. Also, α-D-mannosidase and β-D-glucosidase demonstrated declined activity post optima while no such decline was observed for β-D-galactosidase.

2.3.1.1 Activity contribution of three glycosidases to ripe and over-ripe stages and *in vivo* β-galactosidase activity assay

As the enzyme activities of all the three glycosidases showed considerable variation amid various ripening stages of control and ethylene treated fruits, their activities at ripe and over-ripe stages of the control (15 and 20 DAH) and ethylene treated fruits (9 and 11 DAH) were compared on radar plot (Figure 2.2).

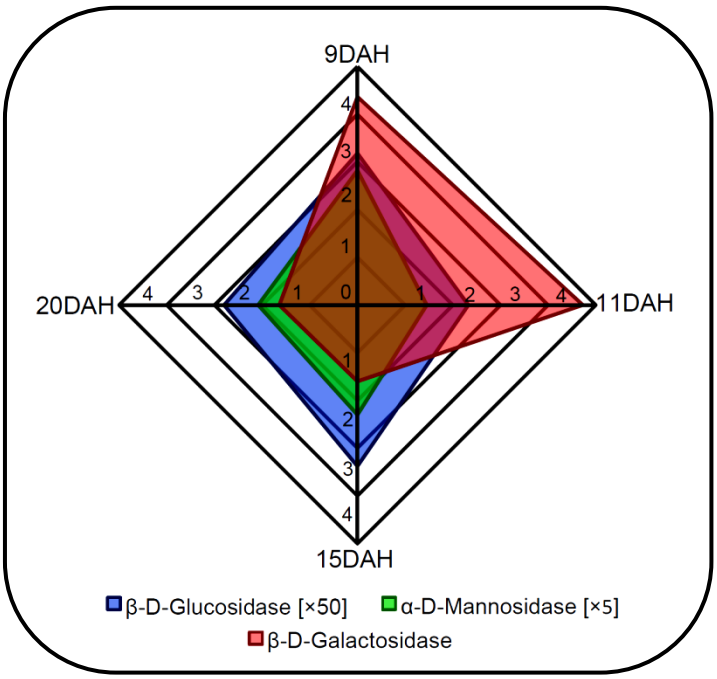


Figure 2.2: Radar plot showing the contribution of β-D-glucosidase, α-D-mannosidase and β-D-galactosidase activities (mM min^{-1}) to ripe and over-ripe stages of ethylene treated and control Alphonso mango. For better visualization, the scale for the activities has been magnified. Values in the bracket represent the scale for respective legend.

The analysis clearly showed the highest activity of β-D-galactosidase among all the stages with maximum contribution to the 9 (ripe) and 11 DAH (over-ripe) stages. Moreover, in case of control fruits, activities of β-D-galactosidase and α-D-mannosidase displayed comparable contributions with the least share of β-D-glucosidase for both, the 15 (ripe) and 20 DAH (over-ripe) stage. In ethylene treated fruits, however, there was exceptionally high share of β-D-galactosidase activity while the activity chart of the other two enzymes did not experience remarkable variations at these stages. This observation suggested selective trigger in the β-D-galactosidase activity upon ethylene treatment.

To support the above mentioned findings, a histochemical assay of β-D-galactosidase activity was performed using X-gal as a chromogenic substrate. Green colored areas in the longitudinal section of the 15 DAH (ripe) control and the 9 DAH (ripe) ethylene treated fruit indicated the β-galactosidase activity, as degradation of X-gal by β-galactosidase generates 5,5'-dibromo- 4,4'-dichloro-indigo which is an insoluble blue color product (Sambrook and Russell, 2001). This on mixing with the yellow color of carotenoides appeared green (Figure 2.3I).

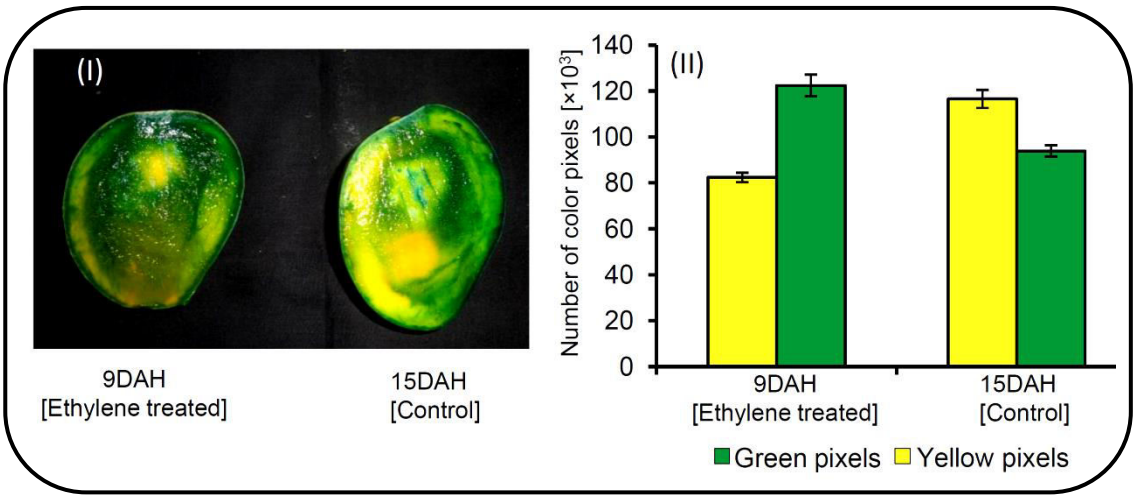


Figure 2.3: Representative picture showing longitudinal section of ripe stage of Alphonso mango fruit of 9 DAH (ethylene treated) and 15 DAH (control) stage with green color region corresponding to β-galactosidase histochemical activity (I). Histogram presenting the number of green and yellow color pixels in ripe ethylene treated and control mango fruit. Vertical lines on each bar represent standard error of measurement among the biological replicates (II).

Thus, the number of green and yellow color pixels from the 15 (ripe) and the 9 DAH (ripe) fruits were calculated which clearly showed high *in vivo* activity of β-

galactosidase in the ethylene treated fruits than the control ones (Figure 2.3II). Nevertheless, the probable changes in the activity of β-galactosidase could not be analyzed in over-ripe fruits i.e. the 20 DAH (control) and 11 DAH (ethylene treated) as fruits turned excessive soft and juicy at this stage releasing majority of the enzyme into the buffer after sectioning. Due to this, almost all the substrate molecules got degraded imparting blue color to the buffer making substrate unavailable for *in vivo* activity.

2.3.2 Variation in the total sugar content upon exogenous ethylene treatment

In the total sugar content graph of Alphonso mango, share of non-reducing sugars was comparatively higher than the reducing sugars (Figure 2.4). Even so, both reducing and non-reducing sugars followed analogous pattern of their content during ripening of control fruits. Reducing and non-reducing sugar content did not vary significantly between the 0 (mature-raw) and 5 DAH (pre-climacteric) stage of control fruits. However an increase in their content was observed from the 5 (pre-climacteric) to 10 DAH (mid-ripe) stage with no significant variation post 10 DAH (mid-ripe) for reducing sugars (Figure 2.4I) while 20 DAH (over-ripe) stage experienced significant decline in its non-reducing sugar content (Figure 2.4II).

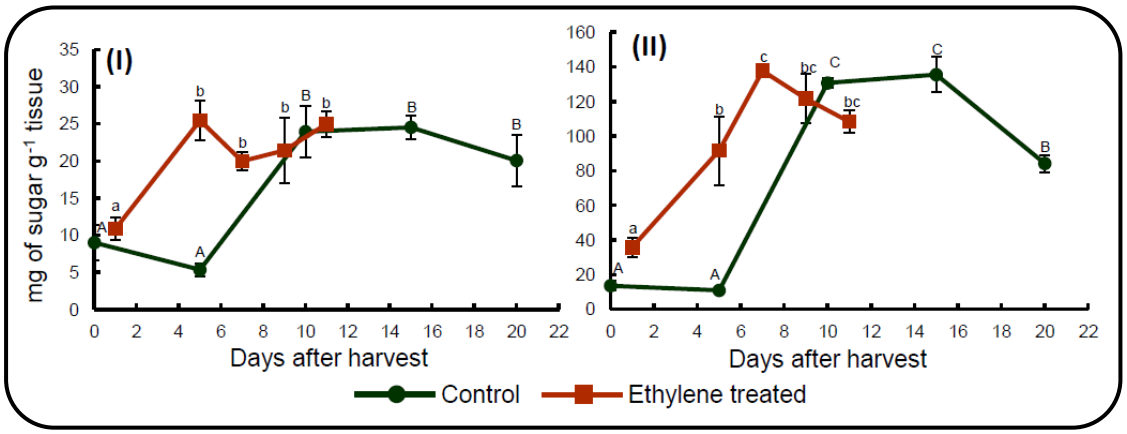


Figure 2.4: Reducing (I) and non-reducing (II) sugar levels (mg g^{-1} tissue) during ripening stages of ethylene-treated and control Alphonso mango. Stages with different letters above the trend line for the ethylene treatment (a, b, c, . . ., etc.) and control (A, B, C, . . .,etc.) represent values significantly different at $p \leq 0.05$. Vertical bars at each point represent standard error of measurement among the biological replicates.

On the other hand, a remarkable variation in the pattern of their content was evident on ethylene treatment. A significant increase in the reducing sugar

content was observed from the 1 (mature-raw) to 5 DAH (pre-climacteric) stage for the ethylene treated fruit with no significant variation from the 5 (pre-climacteric) to 11 DAH (over-ripe) stage (Figure 2.4I). In case of non-reducing sugars, a continuous and significant increase was observed from the 1 (mature-raw) to 7 DAH (mid-ripe) stage followed by steady decline till the 11 DAH stage with no significant change in their content (Figure 2.4II). Collectively, there was 4.7 and 8.3 fold increase in the content of reducing and non-reducing sugars upon ethylene treatment at the 5 DAH (pre-climacteric) stage compared to the 5 DAH (pre-climacteric) of control fruits, respectively.

2.3.3 Isolation of partial gene sequences and quantitative real-time PCR analysis

Partial fragments of the genes encoding α-D-mannosidase, β-D-galactosidase and β-D-glucosidase were isolated from the cDNA of ripe fruit pulp of Alphonso mango employing degenerate primer approach (Table 2.1). PCR amplification, cloning and sequencing of the obtained amplicons gave clear match for their respective genes upon BLAST against the NCBI data base. Moreover, the exercise yielded three distinct transcript variants of α-D-mannosidase. All these obtained sequences have been deposited to the NCBI data base with the accession numbers KM100127, KM100128, KM100129, KM100130 and KM100131 for α-D-mannosidase transcript variant 1, transcript variant 2, transcript variant 3, β-D-galactosidase and β-D-glucosidase, respectively (APPENDIX I). In a view to evaluate the expression pattern of these genes during the conventional ripening of Alphonso mango and after ethylene treatment, transcript abundance of each of these genes was quantified through all the tissues under study (Figure 2.5).

2.3.3.1 β-D-Galactosidase

During ripening of the control fruits, transcript abundance at the 0 (mature-raw) and 5 DAH (pre-climacteric) remained at lower level with no significant variation. However, a significant increase in the abundance was seen from the 5 (pre-climacteric) to 10 DAH (mid-ripe) stage followed by no significant changes thereafter. On ethylene treatment, a significant increase (5.6 fold) in the transcript level from the 1 (mature-raw) to 5 DAH (pre-climacteric) stage of ethylene treated fruits was observed with the later as the highest point of transcript abundance

among all the tissues analysed. Post 5 DAH (pre-climacteric) a significant decline in the transcript level was noticed till the 9 DAH (ripe) followed by minor and non-significant rise at the 11 DAH (over-ripe) stage. Ethylene treatment caused 20 fold increase in the transcript level at 5 DAH (pre-climacteric) stage (Figure 2.5I).

2.3.3.2 α-D-Mannosidase

Three transcript variants of α-D-Mannosidase displayed varied patterns of their expression in control as well as in ethylene treated fruits. For transcript variant1 the transcript levels at the 0 (mature-raw) and 5 DAH (pre-climacteric) stages of control fruits varied significantly from the 10 (mid-ripe) and 15 DAH (ripe) stage with no variation amongst them. However, the 20 DAH (over-ripe) showed the highest transcript abundance which was significantly different from all the earlier stages. On ethylene treatment, a significant boost in the expression of transcript variant1 was observed from the 1 (mature-raw) to 5 DAH (pre-climacteric) stage which was followed by steady and significant decline till the 11 DAH (over-ripe). The relative transcript abundance at the 5 DAH (pre-climacteric) stage of ethylene treated fruit was 2.6 fold higher than the 5 DAH (pre-climacteric) stage of control fruit (Figure 2.5II). For transcript variant 2, a continuous increase in the transcript abundance was observed from the 0 (mature-raw) to 20 DAH (over-ripe) stage with no significant variation at the 0 (mature-raw), 5 (pre-climacteric) and 10 DAH (mid-ripe) stage. However, in case of ethylene treated fruits, a steep high in the transcript level was seen from the 1 (mid-ripe) to 5 DAH (pre-climacteric) with no significant variation thereafter. When compared collectively, transcript abundance at the 5 DAH (pre-climacteric) stage of ethylene treated fruits was 4 fold higher than the 5 DAH (pre-climacteric) control fruit (Figure 2.5III). In case of transcript variant 3, significantly high transcript abundance was seen at the 0 DAH (mature-raw) stage of control fruits which was followed by significant drop till the 5 DAH (pre-climacteric). Post this stage there was an increase in the transcripts till the 20 DAH stage wherein the abundance at the 0 DAH (mature-raw) and 20 DAH (over-ripe) displayed no significant change. In ethylene treated fruits, although the 1 (mature-raw) and 5 DAH (pre-climacteric) stage showed high transcript level followed by steady fall till the 9 DAH (ripe) with again

increased transcripts at the 11 DAH (over-ripe) stage, none of the stages exhibited significant variation in their transcript levels (Figure 2.5IV).

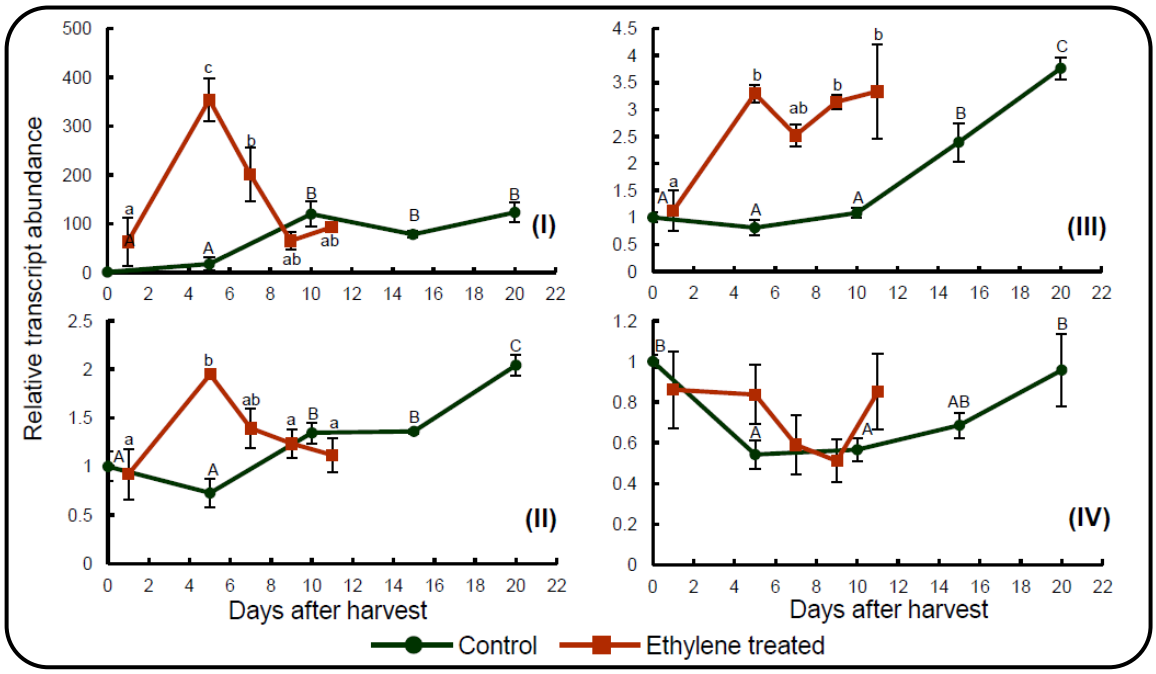


Figure 2.5: Transcript abundance profiles of β-D-galactosidase (I), α-D-mannosidase transcript variant 1 (II), α-D-mannosidase transcript variant 2(III) and α-D-mannosidase transcript variant 3 (IV) relative to the transcripts of elongation factor 1α (*EF 1α*) in the ripening stages of pulp of ethylene-treated and control Alphonso mango. Values presented are average of three independent biological replicates each of which was represented by at least three technical replicates. The stages indicated by different letters for ethylene treated (a, b, c, . . ., etc.) and control (A, B, C, . . .,etc.) are significantly different from each other at $p \leq 0.05$. Vertical bars at each point represent standard error of measurement among the biological replicates.

When all the tissues were compared collectively for the transcript variant 3, transcript level at the 0 (mature-raw) and 20 DAH (over-ripe) remained significantly higher than the rest of the stages.

2.3.3.3 β-D-glucosidase

Transcript abundance of β-D-glucosidase gene could not be observed amongst the ripening stages of Alphonso pulp. Thus, to evaluate the possibility of spatial variation of β-D-glucosidase transcript, gene specific primers of β-D-glucosidase were amplified in leaf, flower, the 0 (mature-raw) and 15 DAH (ripe) pulp and the 0 (mature-raw) and 15 DAH (ripe) skin cDNA templates.

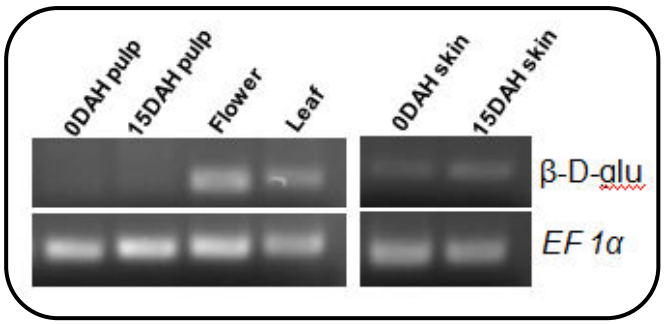


Figure 2.6: Expression pattern of β-D-glucosidase transcript among the six different tissues (0 and 15 DAH pulp and skin, flower and leaf) as evaluated by semi-quantification PCR.

Expression of β-D-glucosidase was not detected in the 0 (mature-raw) and 15 DAH (ripe) pulp. However, flower showed the highest expression level followed by leaf and skin. Thus, although skin showed the expression of β-D-glucosidase, its transcripts could not be detected in the pulp of fruits (Figure 2.6).

2.4. Discussion

In climacteric fruits, involvement of ethylene is instrumental in softening wherein its varying concentration triggers, inhibits or synchronizes the activities of diverse softening related enzymes leading to optimal softness at ripe stage. Key biochemical processes such as aroma volatile biosynthesis, softening of pulp and polysaccharides hydrolysis which largely determine three vital components of flavor *viz*. aroma, texture and taste are crucial part of the ripening process. It is thus essential to study the effect of exogenous ethylene application on the overall ripening process of mango.

The present work was aimed at analysing the effect of pre-climacteric ethylene treatment in terms of changes in the activity profiles of three important glycosyl hydrolases viz. β-D-galactosidase, α-D-mannosidase and β-D-glucosidase during 20 days long ripening period of Alphonso mango. Further, their expression profiles were also evaluated to get an insight into the influence of exogenous ethylene at transcriptional level. This study showed accelerated ripening in ethylene treated fruits which led to variation of sampling days for ripening stages of control and ethylene treated fruits. Such variation of sampling days was previously reported for control and ethrel treated tomato fruits. The study reported accelerated maturation of treated tomato fruits by seven days than

control fruits leading to the completion of ripening process of treated fruits seven days prior to control (Iwahori and Lyons, 1969). Also, tissue of varied sampling days was analysed for physicochemical changes of three distinct banana cultivars depending upon the time period required by them from shooting to harvest maturity (Newilah et al., 2010). In the present study complete ripening duration of Alphonso mango, either conventionally ripened or upon ethylene treatment was considered as 0-20 DAH (mature raw to over-ripe) or 1-11 DAH (mature raw to over-ripe), respectively.

2.4.1 Pre-climacteric ethylene treatment and activity profiles of three distinct glycosidases

A climacteric trigger in the activities of varied cell wall hydrolysing enzymes leading to softening of pulp has largely been correlated to the ethylene burst (Yashoda et al., 2007). In the present study, enzyme activity profile of three glycosidases showed significant changes upon ethylene treatment (Figure 2.1). Early and increased activity of all the three enzymes and remarkable increase in β-D-galactosidase activity in the ethylene treated fruits strongly suggested that even in the conventionally ripened fruits, the activity of these enzymes might be triggered by endogenous ethylene produced during climacteric stage. This is in congruence with the earlier report on Alphonso mango wherein all enzymes showed their optimal activity at post climacteric stage (Yashoda et al., 2007). Also, a study on purification and detailed characterization of β-D-galactosidase from Alphonso mango revealed similar observation except the decline in the activity at the final ripe stage which contradicted the present observation. This might be the consequence of varied enzyme extraction protocols employed in these studies (Prasanna et al., 2005). Ali et al. (1995) showed a continuous increase in the activity of β-D-galactosidase in the crude enzyme preparation of 'Harumanis mango' during its ripening. This contrasted the present finding which displayed no significant variation in the activity of β-D-galactosidase once it reached to the optima at the 10 DAH (mid-ripe) suggesting varying activity chart of β-D-galactosidase in these two mango cultivars. Conversely, the effect of ethylene treatment on the activity profile of β-D-galactosidase has not been analysed till date in mango fruit. The present report has elaborately analysed the

effect of exogenous ethylene treatment that displayed exceptionally high activity of this enzyme in the ethylene treated fruits at the 5 DAH (pre-climacteric) stage with consistent increase thereafter. This observation confirmed regulation of β-D-galactosidase activity by ethylene trigger. This is in agreement with the report on exogenous ethylene treatment of Aleatico red grapes (a non-climacteric fruit) which showed increased activity of β-D-galactosidase to aid cell wall degradation promoting better water loss and berry dehydration for wine making (Lodola and Mencarelli, 2010). Also, Jeong and Huber (2004) reported significantly reduced β-D-galactosidase activity upon inhibition of ethylene signalling in avocado fruits which remained unaffected even after delayed ethylene treatment suggesting definitive role of ethylene in induction and maintenance of β-D-galactosidase activity during ripening.

The activity pattern of α-D-mannosidase in the control Alphonso mango displayed peak at the 10 DAH (mid-ripe) followed by non-significant decline till the 20 DAH (over-ripe). This is supported by the earlier finding in Alphonso mango wherein a steady increase in the activity of this enzyme was encountered from mature-unripe to post-climacteric stage followed by a decline in the activity at ripe stage (Yashoda et al., 2007). The highest ripening related activity of α-D-mannosidase has also been reported from *Capsicum annuun* and banana fruits compared to other glycosidases which pointed towards relatively more contribution of this enzyme in ripening related softening of these fruits (Prabha and Bhagyalakshmi, 1998; Sethu et al., 1996). Despite the immense significance of α-D-mannosidase in softening related cell wall modifications and its regulation by ethylene (Meli et al., 2010), alterations in its activity profile on ethylene treatment remained unattended in mango. The present study revealed an early trigger in its activity on ethylene treatment (5 DAH, pre-climacteric) followed by no prominent changes thereafter in control and ethylene treated fruits suggesting partial dependence or varied sensitivity of this enzyme to ethylene (Johnston et al., 2009; Nishiyama et al., 2007). Also, in the light of the fact that mannose content in the polymeric fraction of ripening Alphonso fruit is relatively less (Yashoda et al., 2005), the activity of α-D-mannosidase could be more indicative of its possible involvement in *N*-glycan processing (Meli et al., 2010; Strasser et al., 2006) rather than hydrolysis of mannose containing side-chains linked to

other polysaccharides. However, lack of knowledge about presence, distribution and processing of *N*-glycans in mango fruits pose limitations on such hypothesis.

The lowest activity of β-D-glucosidase evident in the present study was in congruence with the earlier report on Alphonso mango glycosidases (Yashoda et al., 2007). β-D-glucosidase is one of the members of cellulase multienzyme system that is associated with the hydrolysis of terminal non-reducing residues of β-D-glucosides and in the hydrolysis of cellobiose releasing glucose molecules. Low activity profile with comparable quantitative pattern in both, control and ethylene treated fruits after initial trigger at the 5 DAH (pre-climacteric) after ethylene treatment suggested its less sensitivity to ethylene trigger and comparatively less contribution in the cell wall solubilisation process.

Considering the involvement of the three glycosidases in the softening of Alphonso fruit, their activity contribution to the ripe and over-ripe stages of control (15 and 20 DAH) and the ethylene treated (9 and 11 DAH) fruits were compared on radar plot (Figure 2.2). The analysis hinted strong correlation between ethylene induction and β-D-galactosidase activity while the activity of other two glycosidases remained relatively less sensitive to ethylene treatment in the quantitative terms. The observation was also supported by histochemical assay of β-D-galactosidase which displayed enhanced activity of β-D-galactosidase in the ethylene treated fruit rather than the controls (Figure 2.3). These findings justified the earlier reports which stated uniform ripening and softening of fruits upon exogenous ethylene treatment (Montalvo et al., 2007).

2.4.2 Alterations in the total sugar content

Enzyme activities of three glycosyl hydrolases under study are mainly implied in the pectin dissolution wherein they bring about the deglycosylation of pectin side chains. Further, such side chains are also attached to hemicellulosic polysaccharides through α-linkages which are potential targets of the present glycosidases (Yashoda et al., 2007). Thus, considering the early and enhanced activity of glycosidases under study in ethylene treated fruits, changes in the reducing and non-reducing sugar content on ethylene treatment during ripening was analysed. Comparison revealed considerably high share of non-reducing

sugars than the reducing to total sugar content during ripening (Figure 2.4). This observation was supported by the earlier reports which showed increased activity of starch hydrolysing enzymes (amylase) during ripening of mango fruits that culminated in generation of sucrose (non-reducing sugar), fructose and glucose (reducing sugar) as the ripening progressed (Yashoda et al., 2005). Moreover, the efficient distribution of ^{14}C-starch into sucrose, fructose and glucose to aid sufficient depolymerisation and sugar interconversions during Alphonso mango ripening was also demonstrated (Yashoda et al., 2006). Further, a study on sugar metabolism in Haden mango showed very high content of sucrose followed by fructose and glucose during the ripening period (Castrillo et al., 1992). This supported the present findings that portrayed comparable quantities of reducing and non-reducing sugar at the 0 (mature-raw) and 5 DAH (pre-climacteric) with significantly high amount of non-reducing sugar at the 10 DAH (mid-ripe) compared to the reducing sugars. Decline in the content of the reducing as well as the non-reducing sugars at the over ripe stage (20 DAH) was likely due to the complete degradation of starch which could be seen as the main reservoir of these sugars and also due to their utilization in other energy consumption processes during ripening (Fuchs et al., 1980). Further, in ethylene treated fruits, early acquired highest levels for both reducing and non-reducing sugars implied early onset of hydrolysis of starch and other cell wall polysaccharides, releasing constituent sugars on ethylene trigger. The finding collectively pointed towards altered sugar metabolism upon exogenous ethylene treatment with considerable variation in the pool of reducing sugars than that of non-reducing which could be the consequence of increased activity of glycosidases upon ethylene treatment.

2.4.3 Expression profiling of glycosidases as influenced by exogenous ethylene treatment

The study displayed exceptionally high relative transcript abundance for β-D-galactosidase. Moreover, transcript abundance during the ripening of control fruits showed paralleled progression with the activity profile of β-D-galactosidase implying positive correlation between them (Figure 2.5). However, early and significantly enhanced transcript levels in the ethylene treated fruits at pre-climacteric stage (5 DAH) suggested transcriptional regulation of its activity by

ethylene signal. This was in agreement with the earlier reports on tomato fruits which showed suppression of β-D-galactosidase transcripts in its *rin*, *nor* and *nr* mutant lines which had impaired functions of ethylene synthesis and perception (Smith and Gross, 2000). Enhanced β-D-galactosidase transcript in immature and ripe watermelon fruits on exogenous ethylene treatment further confirmed the ethylene mediated regulation of this gene (Karakurt and Huber, 2004). Also, multiple isoforms of this gene from tomato (Smith and Gross, 2000), Japanese pear (Tateishi et al., 2001) and strawberry (Trainotti et al., 2001) displayed varied patterns of expression during ripening. Prasanna et al. (2004) purified and characterized three isoforms of β-D-galactosidase from Alphonso mango pulp which exhibited maximum activity towards arabinogalactan, a pectic polymer. However, scarcity of the efforts targeted to decipher their molecular structure or to evaluate the possibility for presence of even more isoforms in mango restricted further discussion.

Three transcript variants of α-D-mannosidase exhibited varied transcript profile during ripening of the control as well as the ethylene treated fruits. Transcript variant 1 and 2 displayed more ripening related appearance suggesting their significant role during ripening. Nonetheless, sequence encoding transcript variant 2 upon BLAST against NCBI data base showed high identity (80%) to golgi alpha mannosidase II which are largely implied in *N*-glycan processing in plants (Strasser et al., 2006). In addition, Meli et al. (2010) demonstrated vital role of α-D-mannosidase in *N*-glycan processing by virtue of α-mannosidase RNAi lines of tomato that showed reduced fruit softening and enhanced shelf-life. Further, the study also confirmed that the expression of α-mannosidase is induced by ethylene. These findings supported the present data which displayed significantly enhanced transcript levels for all the three transcript variants on ethylene treatment. Furthermore, transcript variant 2 maintained high level of transcripts till the over ripe stage suggesting its consistent expression pattern post optima in ethylene treated fruits.

In case of β-D-glucosidase transcript, high expression was seen in the flower than any other tissue with no detectable expression in the pulp. The finding implied the probable involvement of other transcript variant/variants in

the pulp to bring about the effective glucosidase activity detected during ripening. It also suggested the wide distribution of substrates for enzyme encoded by this transcript among various parts of plant (Smith and Gross, 2000). However, lack of detail molecular studies on β-D-glucosidases posed limitation on further discussion.

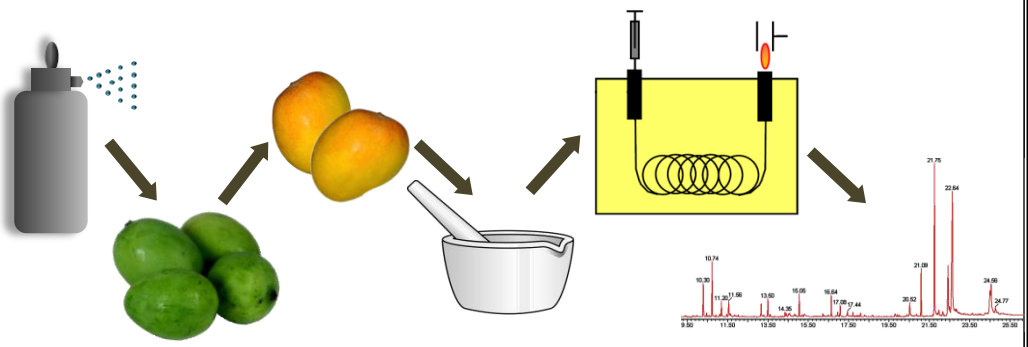
All these findings collectively pointed towards regulation of β-D-galactosidase and α-D-mannosidase expression by ethylene at transcriptional level. Exceptionally high transcript level of β-D-galactosidase in control and ethylene treated fruits than all the three transcript variants of α-D-mannosidase implied vital role of this gene in ripening related softening of Alphonso mango.

2.4.4 Ripening related softening of fleshy fruits and implications of exogenous ethylene

Softening of fleshy fruits is a concerted consequence of assorted activities by diverse group of enzymes. As softening exacerbates majority of damage incurred during handling and thereby poses serious impact on market acceptability of fruits, its modulation by ethylene has been a major area of interest amongst post harvest biologists (Bapat et al., 2010). These efforts have collectively put forth two glycosidases: β-D-galactosidase and α-D-mannosidase as vital contributors in ripening related softening of fleshy fruits (Bapat et al., 2010; Meli et al., 2010). In the present study, increased activity of β-D-galactosidase in the control as well as the ethylene treated fruits during ripening and noticeable induction in its expression upon ethylene treatment depicted β-D-galactosidase as a potential member of 'cell wall solubilisation machinery' in Alphonso mango. Purification and biochemical characterization of these enzymes from Alphonso mango reported presence of more than one isoforms (Prasanna et al., 2005; Yashoda et al., 2007). However, detail molecular characterization to understand their regulation mechanism and utilization the information for RNAi experiments aimed at delayed softening of Alphonso mango is essential. The present study demonstrated accelerated ripening upon ethylene treatment reducing its ripening period by almost 5 days. Although, early ripening might encourage early arrival of mango to the market thereby promoting greater monetary gains to mango growers, such mangoes might remain restricted only to local market hampering

their export. Considering this fact, it will be ideal to find the appropriate period after harvest along with optimum concentration of ethylene for exogenous treatment to obtain suitably softened Alphonso fruit with perfect ratio of its major volatile flavorants.

Chapter 3



Spatial and Temporal Changes in The Volatile Profile of Alphonso Mango Upon Exogenous Ethylene Treatment

3.1 Introduction

Mango, one of the most delicious tropical fruits is also categorised as climacteric fruit. India, the country of origin of mango, possesses vast diversity of this national fruit (Salvi and Gunjate, 1988). Among thousands of cultivars, Alphonso is the most popular mainly because of its deeply attractive blend of flavor volatiles. As synthesis of aroma volatiles is a key feature of ripening, in our previous study, we mapped volatiles through development and ripening of Alphonso mango. This study revealed mono and sesquiterpenes as quantitatively dominant volatiles but lactones and furanones as ripening specific compounds (Pandit et al., 2009b). Further, to study the chemical basis of cultivation locality dependant variation in Alphonso flavor, ripening fruits of Alphonso from three cultivation localities in Konkan were probed for the volatile content. This work showed that Alphonso fruits from one locality (Dapoli, Maharashtra, India) contained higher concentration of terpenes as compared to those from the other two localities (Deogad and Vengurle, Maharashtra, India), whereas those from Deogad had relatively higher quantity of lactones and furanones (Kulkarni et al., 2012a).

It is well known that ethylene plays a decisive role in fruit ripening. Further, to evaluate its involvement in ripening process of fruits, effect of external application of ethylene on few key ripening processes has been analyzed by researchers. Pre-climacteric application of ethylene to Alphonso slices was shown to stimulate catalase and peroxidise activity (Mattoo and Modi, 1969). Reports on post- harvest ethrel treatment of Alphonso and Langra mango cultivars (Chikkasubbanna and Huddar, 1982; Lakshminarayana, 1980) were more focused on some other aspects of ripening such as softening and color development. Exogenous applications of ethephon was also reported to improve the flavor of `Keitt' (Sergent et al., 1993) and `Kensingston Pride' mangoes (Singh and Janes, 2001), as evaluated organoleptically; however, this study did not address the chemical analysis of volatile blend. A detailed study on volatiles after ethephon treatment of Kensington Pride mango demonstrated an increase in the total aroma volatiles (Lalel et al., 2003c). Alphonso mango being highly favoured and commercially most valued mango cultivar of India; the need of studying the

effect of exogenous application of ethylene on the accumulation of volatiles in this fruit was realized. Thus, present work illustrates spatial (with respect to skin and pulp) and temporal (with respect to days after harvest) variation in the volatile content of Alphonso mango upon exogenous ethylene application.

3.2 **Materials and methods**

3.2.1 Plant material

Mature raw fruits of Alphonso mango were collected from the mango orchards at Dr. Balasaheb Sawant Konkan Agriculture University at Dapoli, Maharashtra, India (N17°45' E73°11'). The collection and ripening of fruits was carried out as detailed in 'Materials and Methods' of Chapter 2. Fruits from control and ethylene treated sets were then put in the hay packed boxes, carried to the laboratory and allowed to ripen at ambient temperature. As practiced conventionally as well as according to the well discussed ripening indices (Bandyopadhyay and Gholap, 1973b) Alphonso fruit takes 15 days after harvest (15 DAH) for complete ripening and 20 DAH is considered as an over-ripe stage (Pandit et al., 2009b). To span this whole period of ripening, control fruits of Alphonso were cut at 0, 2, 5, 10, 15 and 20 DAH, and ethylene treated fruits at 1, 3, 5, 7, 9 and 11 DAH. Skin and pulp were separated for each fruit separately, immediately frozen in the liquid nitrogen and stored at -80°C until use leading to six stages of ripening for skin and pulp of each plant. In this study 2 DAH of control and 3 DAH of ethylene treated fruit were taken as additional 'table green' stage as compared to the stages detailed in 'Materials and Methods' section of Chapter 2.

3.2.2 Extraction of volatiles

Extraction of volatiles was carried out from the skin and pulp at various ripening stages of ethylene treated and control fruits using dichloromethane as a solvent according to standardized method employed in our laboratory (Kulkarni et al., 2012a; Pandit et al., 2009a). Concisely, extraction was carried out from 2 g of skin and pulp tissue separately for 30 min in 10 mL dichloromethane having 40 µg nonyl acetate as an internal standard. Dehydration of decanted supernatant was done over anhydrous sodium sulphate. After concentrating the extracts to 1

mL in a rotary evaporator at ambient temperature (ca. 25°C), they were stored at -20°C overnight to precipitate the high molecular weight lipids. These precipitated lipids were removed the next day by centrifugation at 16,000 g at 4°C for 10 min. These extracts were again concentrated in a similar way to 50 µL and stored at -20°C until use.

3.2.3 GC-MS and GC-FID analysis

Gas chromatographic analysis was carried out on Clarus 500 (Perkin Elmer, Waltham, MA) instrument with GsBP-5MS (General Separation Technologies, USA) capillary column (30 m X 0.32 mm i.d. X 0.25 µm film thickness). Column temperatures were programmed from 40°C for 5 min, raised to 180°C at 5°C/min followed by an increase till 280°C at the rate of 20°C/min and held at 280°C for 5 min. Injector and detector temperatures were 200 and 250°C, respectively. Helium was used as carrier gas at a flow rate 1 mL/min. Mass spectra were obtained using Clarus 500 (Perkin–Elmer) gas chromatograph–mass spectrometer at 70 eV with a scan time of 0.2 sec for m/z 30–300 under the GC conditions same as those applied for GC–FID analyses. Using a series of n-paraffins (C5–C22), retention indices were determined for all the peaks. Identification of all the compounds was done by comparing acquired mass spectra with those stored in Wiley registry of mass spectral data (8th edition). Identification of some of the compounds was also confirmed by comparing the obtained mass spectra with mass spectra and retention indices of authentic external standards and with those reported in the literature [the external standards namely α-pinene (CAS no. 7785-70-8), β-pinene (CAS no. 18172-67-3), β-myrcene (CAS no. 123-35-3), β-caryophyllene (CAS no. 87-44-5) and humulene (CAS no. 6753-98-6) were procured from Fluka; whereas, α-methylbutyrolactone (CAS no. 1679-47-6), γ-hexalactone (CAS no. 695-06-7), mesifuran (CAS no. 3658-77-3), δ-hexalactone (CAS no. 823-22-3), γ-octalactone (CAS no. 104-50-7), isolongifolene (CAS no. 1135-66-6), longifolene (CAS no. 475-20-7) and γ-decalactone (CAS no. 706-14-9) were procured from Sigma Aldrich (Germany)]. Quantification of compounds was carried out by internal standard method, wherein concentrations of the volatiles were normalised with that of nonyl acetate.

3.2.4 Statistical analysis

Minimum two fruits from each of the three plants (biological replicates) were used for each of the ripening stage of ethylene treated and control fruits. At least two solvent extractions from each tissue (technical replicates) followed by at least two independent GC analyses per extracts (analytical replicates) were carried out to minimize the experimental error. For the present dataset, ANOVA and principal component analysis (PCA), the Systat statistical software (Version 11, Richmond, CA, USA) was used. Fisher's LSD test was carried out ($p \leq 0.05$) to compare the quantity of each compound and each class of the compounds in skin and pulp of ethylene treated and control fruits independently, for various ripening stages.

3.3. Results

3.3.1. Overall volatile profile of Alphonso mango

A total of thirty six volatile compounds were detected from the pulp and skin of ethylene treated and control fruits. Of these, 24 compounds from pulp and 20 compounds from skin were quantified (Table 3.1A and 3.1B, respectively) based on their above-detection level (> 0.02μg g^{-1}) and consistent appearance among all the biological replicates during Alphonso fruit ripening stages. Irrespective of the ethylene treatment, pulp tissue comprised seven monoterpenes, six sesquiterpenes, eight lactones, one furanone and two unidentified compounds whereas skin tissue consisted of eight monoterpenes, six sesquiterpenes, three lactones, one furanone and two unidentified compounds. Monoterpene hydrocarbon was found to be quantitatively as well as qualitatively (in terms of number of compounds) dominant class of volatiles in the present study with concentration ranging from 10.6 μg g^{-1} to 844 μg g^{-1}. Pulp and skin of control fruits at the 15 DAH (ripe) stage showed the highest volatile content (203 μg g^{-1} and 882 μg g^{-1}) among all the pulp and skin tissues analysed. Of the seven monoterpenes from the pulp, two oxygenated monoterpenes *viz*. (*Z*) 2,6-dimethyl-3,5,7-octatriene-2-ol and (*E*) 2,6-dimethyl-3,5,7-octatriene-2-ol were present only in the pulp of ethylene treated fruits; whereas, skin showed their ubiquitous presence. β-Pinene was detected only in the skin of control fruits. Amid all the volatiles studied (including unidentified), (*Z*)-ocimene was detected as the highest

contributor to the total volatile content across all the tissues (except 11 DAH pulp of ethylene treated fruits) while α-pinene and β-pinene were the least contributor to pulp and skin, respectively. Among sesquiterpenes, longicyclene was observed only in the pulp while α-copaene was detected in all except in the pulp of control fruits. Besides, the presence of germacrene D was limited to the skin of Alphonso fruits. Alphonso pulp was seen as the main reservoir of lactones wherein out of total 8 lactones identified, 5 were exclusive to pulp. Of these, α-methylbutyrolactone and δ-valerolactone were detected only in the pulp of ethylene treated fruits. Skin had a share of three lactones, of which γ-hexalactone was present only at the 20 DAH (over-ripe) stage of control fruits. Apart from this, limonene, δ-3-carene, α-pinene epoxide, *trans*-pinane, *epoxy* ocimene, caryophyllene oxide, β-cyclocitral and α-longipinene from pulp and camphene and linalool from skin were detected inconsistently at trace levels and hence were not quantified. Two ripening specific but unidentifiable compounds, same as those reported earlier (Kulkarni et al., 2012a), were also detected and quantified in the present study. Out of these two, unidentified 1 was not detected in the pulp of control fruits whereas unidentified 2 showed ripening related appearance in all the tissues analysed. In congruence with our previous report (Kulkarni et al., 2012a), furaneol could not be quantified in the present study due to its inconsistent detection.

3.3.2. Spatio-temporal changes in the volatile content upon ethylene treatment

There was an early appearance of ripening related compounds i.e. lactones and furanones in the ethylene treated fruits as compared to the naturally ripened fruits. Grossly, the skin had qualitative and quantitative abundance of terpene volatiles while the pulp was seen as relatively lactone-rich (Figure 3.1).

3.3.2.1. Furanones

Out of the two important furanones commonly found in Alphonso mango (Pandit et al., 2009a and 2009b) viz. furaneol and its methyl derivative mesifuran, only mesifuran was quantified in the present study. The pulp and the skin of ethylene treated and control fruits at various ripening stages revealed similar pattern of mesifuran content at corresponding stages (Figure 3.1A and B).

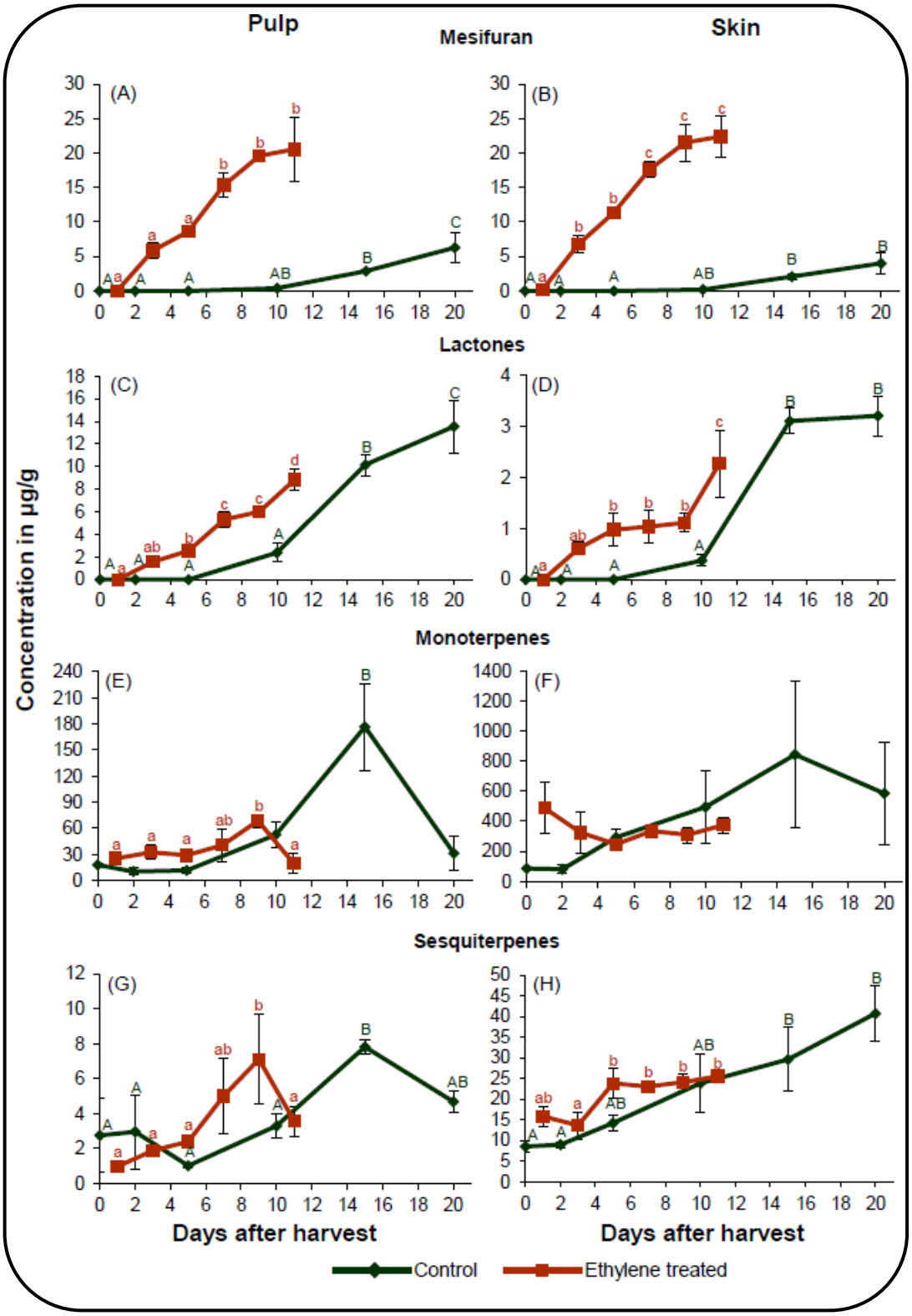


Figure 3.1: Concentration (µg g^{-1} tissue) of four classes of volatiles detected among the ripening stages of the pulp and the skin of ethylene-treated and control Alphonso mango. Points with different letters above the trend line for the ethylene treatment (a, b, c, . . ., etc.) and control (A, B, C, . . .,etc.) show values significantly different at $p \leq 0.05$. Vertical bars at each point represent standard error of measurement of average of quantities of each compound class.

Concentration of mesifuran in the pulp ranged between 0.05 μg g^{-1} to 20.6 μg g^{-1} while skin showed a range of 0.18 μg g^{-1} to 22.4 μg g^{-1}. For both, skin and pulp of the control fruits, mesifuran was absent in 0 (mature-raw), 2 (table green) and 5 DAH (pre-climacteric) fruits and was detected at increasing concentration from 10 (mid-ripe), 15 (ripe) and 20 DAH (over-ripe) stages (Figure 3.1A and B). In case of the pulp of ethylene treated fruits, presence of mesifuran could be noted even at 1 DAH (mature-raw) and there was a gradual increase in its concentration till 11 DAH (over-ripe) stage. Pulp at this stage contained 3.3 folds higher mesifuran content than 20 DAH (over-ripe) pulp of control fruits which had the highest mesifuran content among all the control stages. In case of the pulp of ethylene treated fruits, all the ripening stages could be divided into two groups, group 1 consisted of 1(mature-raw), 3 (table green), and 5 DAH (pre-climacteric) and group 2 of 7 (mid-ripe), 9 (ripe) and 11 DAH (over-ripe) wherein concentrations of mesifuran were statistically similar within members of each of these groups; however, group 2 members contained significantly higher concentration of mesifuran than group 1 members (Figure 3.1A). When ethylene-treated and control fruits were compared with each other, 7 (mid-ripe), 9 (ripe) and 11 DAH (over-ripe) stages of the pulp of ethylene treated fruits showed significantly higher quantities of furanones than that of any other stage of ethylene treated or control fruits (Table 3.1A).

When the skin of ethylene treated and control fruits were compared for their mesifuran content, a range of 0.18 -22.4 μg g^{-1} was seen for the skin of ethylene treated fruits while it was 0.21-4.01 μg g^{-1} for the skin of control fruits. In case of ethylene treated fruits, the skin showed a continuous increase in the amount of mesifuran till 9 DAH (ripe) ; the mesifuran content of 11 DAH (over-ripe) stage was, however, almost the same as that of 9 DAH (ripe) (Figure 3.1B). When mesifuran content of the skin of individual ripening stages of ethylene treated and control fruits were compared, 5 (pre-climacteric), 7 (mid-ripe), 9 (ripe) and 11 DAH (over-ripe) skin of ethylene treated fruit showed at least 2.8 fold higher levels of mesifuran than any control tissue (Table 3.1B).

3.3.2.2. Lactones

A total 8 lactones detected in the study quantitatively ranged between 2.38 -13.6 μg g^{-1} in the pulp of control fruits and 1.58 -8.85 μg g^{-1} in the pulp of ethylene treated fruits. Lactones appeared early in the pulp of ethylene treated fruits (3 DAH, table green) than control ones (10 DAH, mid-ripe). γ-Octalactone had the highest share in the total lactone content at 10 (mid-ripe) and 15 DAH (ripe) pulp of control fruits (0.71 μg g^{-1} and 2.82 μg g^{-1}, respectively) as well as at 3 (table green), 5 (pre-climacteric), 7 (mid-ripe), 9 (ripe) DAH pulp of ethylene treated fruits while γ-butyrolactone and δ-hexalactone contributed the highest to 20 DAH (over-ripe) pulp of control (4.51 μg g^{-1}) and 11 DAH (over-ripe) pulp of ethylene treated (1.97 μg g^{-1}) fruits, respectively (Table 3.1A). Lactones were not detected in the pulp of control fruits analyzed at 0 (mature-raw), 2 (table green) and 5 DAH (pre-climacteric); later, total lactone content showed continuous increase from 10 (mid-ripe) to 20 DAH (over-ripe). However, their concentration at 20 DAH (13.55 μg g^{-1}) pulp of control fruits remained significantly higher than that of 11 DAH (over-ripe) (8.85 μg g^{-1}) pulp of ethylene treated fruits (Table 3.1A). Similarly, in the pulp of ethylene treated fruits, continuous increase in total lactone content was observed from 3 (table green) to 11 DAH (over-ripe) and 1 DAH (mature-raw) did not exhibit the presence of lactone (Figure 3.1C).

Three lactones were present in the skin of Alphonso *viz*. γ-hexalactone, γ-octalactone, δ-octalactone. In the skin of ethylene treated fruits δ-octalactone was found only at 11 DAH (over-ripe) in low concentration (0.38 μg g^{-1}); whereas it was detected in the 15 (ripe) and 20 DAH (over-ripe) skin of non- treated fruits (0.53 μg g^{-1} and 0.39 μg g^{-1}, respectively). γ-Octalactone contributed maximum to the total lactone content of the skin at all the stages of both ethylene treated and control fruits with its highest concentration in 15 DAH (ripe) skin of non- treated fruits (2.58 μg g^{-1}) (Table 3.1B). Skin of 15 (ripe) and 20 DAH (over-ripe) control fruits varied significantly from all the other ripening stages for their lactone content. Though the 11 DAH (over-ripe) skin of ethylene treated fruits had significantly higher lactone content than that in any other stage, 5 (pre-climacteric), 7 (mid-ripe) and 9 DAH (ripe) skins did not vary significantly in their lactone content (Figure 3.1D).

3.3.2.3. Monoterpenes

A total of seven monoterpenes detected in the pulp of which five were present in the pulp of control fruits. Total monoterpene content in the pulp of control fruits ranged between 10.6-177 μg g^{-1} whereas that of the pulp of ethylene treated fruits was between 19.9 - 68.7 μg g^{-1}. Two monoterpene alcohols *viz*. (*Z*) 2,6-dimethyl-3,5,7-octatriene-2-ol and (*E*) 2,6-dimethyl-3,5,7-octatriene-2-ol which contributed less than 2% to the monoterpene content of the pulp of ethylene treated fruits at any given stage, could not be detected in the pulp of control fruits at various ripening stages (Table 3.1A). In case of the pulp among the ripening stages of control fruits, only 15 DAH (ripe) stage, which had the highest monoterpene content, differed significantly from any other stage in terms of monoterpene content. Similarly, no variation in the pulp was observed within the ripening stages of ethylene treated fruits; nonetheless, the highest monoterpene content was detected at 9 DAH (ripe) stage (Figure 3.1E).

The skin had very high monoterpene content ranging between 81.6 -844 μg g^{-1} for the skin of control and 247-489 μg g^{-1} for the skin of ethylene treated fruits, as compared to the pulp at the same stages. The skin of ethylene treated and control fruits at various ripening stages did not vary significantly for their monoterpene content (Figure 3.1F).

3.3.2.4 Sesquiterpenes

The sesquiterpene concentrations in the pulp, which ranged from 1.03-7.82 μg g^{-1} in control and 0.99-7.11 μg g^{-1} in ethylene treated fruits, were lower than the respective monoterpene concentrations. β-Caryophyllene and humulene were the highest contributors to the sesquiterpene blend in all the tissues analyzed. Among the pulp, α-copaene was detected only at 3 (table-green), 5 (pre-climacteric) and 7 (mid-ripe) DAH stages of ethylene treated fruits.

Within the pulp of control fruits, β-caryophyllene and humulene showed continuous increase in their content from 0 DAH (mature-raw) to 15 DAH (ripe) followed by a decrease at 20 DAH (over-ripe). Likewise, the pulp of ethylene treated fruits also revealed increase in the β-caryophyllene and humulene content from 1 DAH (mature-raw) to 9 DAH (ripe) and a significant decrease at 11 DAH

(over-ripe). Levels of longicyclene, isolongifolene and longifolene in the pulp of control fruits were low at 5 DAH (pre-climacteric) and there was a steady increase till over-ripe stage (20 DAH) only for isolongifolene and longifolene. Among the pulp of ethylene treated fruits, α-copaene, longicyclene, isolongifolene and longifolene exhibited varied pattern of their appearance with maximum contribution at various ripening stages (Table 1A). Quantitatively, 15 DAH (ripe) pulp of control fruit showed significant variation from early ripening stages (0, 2, 5 and 10 DAH) while 20 DAH (over-ripe) was not significantly different from any of the ripening stages. In the pulp of ethylene treated fruits, 9 DAH (ripe) revealed significant variation from 1, 3, 5 and 11 DAH pulp and 7 DAH was not significantly different from any of the ripening stages (Figure 3.1G).

The skin had six sesquiterpenes with concentration ranging between 8.54 - 40.7 μg g^{-1} for the skin of control and 13.7 -25.5 μg g^{-1} for the skin of ethylene treated fruits. In the skin of control fruits, β-caryophyllene and humulene showed steady increase in their content from 0 DAH (mature-raw) to 20 DAH (over-ripe); whereas, isolongifolene and germacrene D, seen at 2 DAH (table green) and 10 DAH (mid-ripe) stage, respectively, also showed linear increase till 20 DAH (over-ripe). Quantities of α-copaene and longifolene were comparable with each other at all the ripening stages except 0 (mature-raw) and 10 DAH (mid-ripe). In the skin of ethylene treated fruits, α-copaene, β-caryophyllene and humulene were detected at all the stages, germacrene D was detected 3 DAH (table-green) onwards; whereas, isolongifolene and longifolene showed their presence late after 7 DAH (mid-ripe) stage. Individual sesquiterpenes showed varied pattern of their quantities through the ripening stages of the skin of ethylene treated fruits (Table 3.1B). Comparing quantitatively, total sesquiterpene content of the skin of control fruits at 0 (mature-raw) and 2 DAH (table-green) was significantly different from 15 (ripe) and 20 DAH (over-ripe) while 5 (pre-climacteric) and 10 DAH (mid-ripe) did not show significant variation from any of these. In the skin of ethylene treated fruits, 5, 7, 9 and 11 DAH (pre-climacteric, mid-ripe, ripe and over-ripe) displayed significant variation from 3 DAH (table green) with no significant variation among them (Figure 3.1H).

Comparison between the pulp of ethylene treated and control fruits for the total volatile content revealed some similarities among these tissues. The pulp of conventionally ripened fruits exhibited increase in the volatile content from 2 to 15 DAH (ripe) with considerable lowering at 20 DAH (overripe) stage. Similarly, the pulp of ethylene treated fruits also showed steady increase in volatile content from 1 to 9 DAH (ripe) and a decrease at 11 DAH (overripe) stage of pulp.

3.3.3. Principal Component Analysis

Principal component analysis (PCA) was carried out to investigate the distribution patterns of various data points across the principal components as defined by their loadings on factors. For the present data set, a PCA was performed on the quantities of all the 24 compounds (except unidentified) across all the ripening stages of ethylene treated and control fruits. First five principal components could cover total 88% of variation of which the first two accounted for 65% variance (Figure 3.2A).

In the loading plot of the data set, all monoterpenes and four sesquiterpenes *viz.* β-caryophyllene, humulene, germacrene D and α-copaene showed high positive loadings for PC1 while all lactones and three sesquiterpenes *viz.* longicyclene, isolongifolene and longifolene revealed negative loadings for PC1. Although most of the monoterpenes and β-caryophyllene and humulene showed positive loadings to PC2, the values were lower as compared to lactones. Moreover, γ-octalactone, among all the lactones and mesifuran displayed very high positive loading to PC2 than PC1. Nonetheless, such distinct grouping among the compounds exemplified a correlation between the quantities of members of these groups through all the tissues analysed (Figure 3.2B). In the score plot, a separation of pulp and skin tissue across PC1 was clearly evident. Such clustering of the skin in the first and the fourth quadrant could be attributed to the positive loadings of mono- and sesquiterpenes across PC1 while clustering of the pulp in the second and the third quadrant could be attributed to the negative loadings of lactones across PC1. Mesifuran showed zero loadings for PC1 which could be because of its analogous quantities among the skin and the pulp as mentioned above (Figure 3.2B).

Table 3.1A: Volatile composition (µg g^{-1} tissue) of ripening fruits (DAH: days after harvest) of ethylene treated and control Alphonso mango pulp. Compounds have been ordered according to their retention indices. Values shown are average of three plants sampled for the study. Difference between the stages was significant ($p \leq 0.05$) if the letters (a, b, c.....) following the quantity of the compounds are different.

Compounds	**Pulp**											
	Control						**Ethylene treated**					
	0DAH	**2DAH**	**5DAH**	**10DAH**	**15DAH**	**20DAH**	**1DAH**	**3DAH**	**5DAH**	**7DAH**	**9DAH**	**11DAH**
	Mature raw	**Table green**	**Pre-climactric**	**Mid-ripe**	**Ripe**	**Over-ripe**	**Mature raw**	**Table green**	**Pre-climactric**	**Mid-ripe**	**Ripe**	**Over-ripe**
γ-Butyrolactone	n.d.^{a}	n.d.^{a}	n.d.^{a}	0.26^{ab}	1.39^{ab}	4.51^{c}	n.d.^{a}	0.08^{a}	0.21^{a}	0.53^{ab}	0.93^{ab}	1.57^{b}
α-Pinene§	n.d.^{a}	n.d.^{a}	n.d.^{a}	0.08^{b}	0.13^{c}	0.05^{ab}	n.d.^{a}	0.04^{ab}	0.03^{ab}	0.04^{ab}	0.08^{b}	0.02^{ab}
α-Methylbutyrolactone§	n.d.^{a}	n.d.^{a}	n.d.^{a}	n.d.^{a}	n.d.^{a}	n.d.^{a}	n.d.^{a}	n.d.^{a}	n.d.^{a}	n.d.^{a}	0.19^{b}	0.3^{c}
δ-Valerolactone	n.d.^{a}	n.d.^{a}	n.d.^{a}	n.d.^{a}	n.d.^{a}	n.d.^{a}	n.d.^{a}	n.d.^{a}	n.d.^{a}	0.13^{b}	0.24^{c}	0.21^{c}
β-Pinene§	n.d.^{a}	n.d.^{a}	n.d.^{a}	n.d.^{a}	n.d.^{a}	n.d.^{a}	n.d.^{a}	n.d.^{a}	n.d.^{a}	n.d.^{a}	n.d.^{a}	n.d.^{a}
β-Myrcene§	0.14^{a}	0.08 ^{a}	0.08^{a}	0.41^{a}	1.29^{b}	0.24^{ac}	0.22^{a}	0.22^{a}	0.2^{a}	0.27^{a}	0.43^{a}	0.16^{a}
(*Z*)-Ocimene	17^{ab}	9.95^{a}	11.1^{a}	49.5^{ab}	166^{c}	29.6^{ab}	23.8^{ab}	30.4^{ab}	27.1^{ab}	37.9^{ab}	63.4^{b}	17.8^{ab}
(*E*)-Ocimene	0.79^{a}	0.48^{a}	0.53^{a}	2.27^{ab}	7.95^{c}	1.26^{ab}	1.12^{ab}	1.44^{ab}	1.37^{ab}	1.85^{ab}	3.2^{b}	1.03^{ab}
^{γ}-Hexalactone§	n.d.^{a}	n.d.^{a}	n.d.^{a}	0.36^{b}	1.76^{e}	1.96^{e}	n.d.^{a}	0.45^{bc}	0.68^{c}	1^{d}	1.24^{d}	1.06 ^{d}
Mesifuran§	n.d.^{a}	n.d.^{a}	n.d.^{a}	0.39^{a}	2.89^{ab}	6.28^{b}	0.05^{a}	5.84^{b}	8.62^{b}	15.4^{c}	19.6^{cd}	20.6^{d}
Unidentified 1	n.d.^{a}	n.d.^{a}	n.d.^{a}	n.d.^{a}	n.d.^{a}	n.d.^{a}	n.d.^{a}	2.11^{b}	2.6^{bc}	3.77^{c}	3.64^{bc}	4.82^{c}

δ-Hexalactone[§]	n.d.[a]	n.d.[a]	n.d.[a]	0.52[ab]	1.73[c]	2.29[c]	n.d.[a]	0.24[ab]	0.4[ab]	0.88[b]	0.97[bc]	1.97[c]
Allo-Ocimene	0.11[a]	0.07[a]	0.06[a]	0.55[b]	0.95[c]	0.21[ab]	0.19[ab]	0.25[ab]	0.3[ab]	0.34[ab]	0.52[b]	0.56[b]
(*Z*) 2,6-Dimethyl-3,5,7-octatriene-2-ol	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]	0.12[ab]	0.13[ab]	0.2[ab]	0.35[b]	0.8[c]	0.27[ab]
(*E*) 2,6-Dimethyl-3,5,7-octatriene-2-ol	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]	0.04[a]	0.05[a]	0.06[a]	0.07[a]	0.31[b]	0.06[a]
γ-Octalactone[§]	n.d.[a]	n.d.[a]	n.d.[a]	0.71[b]	2.82[e]	2.46[e]	n.d.[a]	0.4[b]	0.77[b]	1.63[cd]	1.36[c]	1.87[d]
Unidentified 2	n.d.[a]	n.d.[a]	n.d.[a]	0.23[a]	1.46[ab]	3.06[b]	n.d.[a]	5.17[b]	8.44[c]	13.6[d]	14.7[d]	10.4[c]
δ-Octalactone	n.d.[a]	n.d.[a]	n.d.[a]	0.32[b]	1.17[e]	1.13[de]	n.d.[a]	0.29[b]	0.37[b]	0.71[c]	0.68[c]	0.95[d]
Longicyclene	0.62[a]	0.67[a]	0.05[a]	0.11[a]	0.22[a]	0.09[a]	n.d.[a]	n.d.[a]	0.05[a]	0.07[a]	0.78[a]	0.08[a]
α-Copaene	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]	0.04[a]	0.06[a]	0.86[b]	n.d.[a]	n.d.[a]
Isolongifolene[§]	0.34[ab]	0.33[ab]	0.05[a]	0.27[ab]	1.44[bc]	1.6[7c]	n.d.[a]	0.09[a]	0.37[ab]	0.89[b]	0.96[b]	1.84[c]
Longifolene[§]	1.06[a]	1.18[a]	0.02[a]	0.07[a]	0.62[a]	0.85[a]	n.d.[a]	0.04[a]	0.16[a]	1.36[a]	1.46[a]	0.5[a]
β-Caryophyllene[§]	0.42[a]	0.44[a]	0.53[a]	1.8[c]	3.52[e]	1.35[bc]	0.61[a]	1.08[b]	1.11[b]	1.13[b]	2.42[d]	0.76[a]
Humulene[§]	0.32[a]	0.36[a]	0.39 [a]	1.07[b]	2.02[d]	0.72[b]	0.38[ab]	0.64[ab]	0.69[ab]	0.7[ab]	1.5[c]	0.39[a]
γ-Decalactone[§]	n.d.[a]	n.d.[a]	n.d.[a]	0.2[ab]	1.3[d]	1.2[cd]	n.d.[a]	n.d.[a]	0.13[a]	0.39[b]	0.41[b]	0.92[c]
Germacrene D	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]

n.d.: not detected.

[§] Compounds were identified by comparing mass spectrum and Kovat's index with available of authentic external standards; remaining compounds from the list were identified by comparing their mass spectrum and Kovat's index with those reported in the literature.

Table 3.1B: Volatile composition (µg g^{-1} tissue) of ripening fruits (DAH: days after harvest) of ethylene treated and control Alphonso mango skin. Compounds have been ordered according to their retention indices. Values shown are average of three plants sampled for the study. Difference between the stages was significant ($p \leq 0.05$) if the letters (a, b, c.....) following the quantity of the compounds are different.

Compounds	**Skin**											
	Control						**Ethylene treated**					
	0DAH Mature raw	**2DAH** Table green	**5DAH** Pre-climactric	**10DAH** Mid-ripe	**15DAH** Ripe	**20DAH** Over-ripe	**1DAH** Mature raw	**3DAH** Table green	**5DAH** Pre-climactric	**7DAH** Mid-ripe	**9DAH** Ripe	**11DAH** Over-ripe
γ-Butyrolactone[§]	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]
α-Pinene[§]	0.08[a]	0.06[a]	0.1[a]	0.2[ab]	0.56[b]	0.71[b]	0.19[ab]	0.29[ab]	0.12[ab]	0.26[ab]	0.16[ab]	0.34[ab]
α-Methylbutyrolactone[§]	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]
δ-Valerolactone	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]
β-Pinene[§]	n.d.[a]	0.04[a]	0.06[a]	0.06[a]	0.21[b]	0.26[b]	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]
β-Myrcene[§]	0.6[a]	0.53[a]	1.47[a]	2.36[ab]	5.09[b]	3.78[ab]	2.84[ab]	2.43[ab]	1.29[a]	1.63[a]	1.56[a]	2.99[ab]
(*Z*)-Ocimene	81.3[a]	76.75[a]	279[ab]	469[ab]	805[b]	556[ab]	464[ab]	305[ab]	2308[a]	3163[ab]	2891[ab]	353[ab]
(*E*)-Ocimene	3.8[a]	3.47[a]	10.1[a]	18.1[ab]	29.82[b]	21.41[ab]	18.1[ab]	14.3[ab]	9.73[a]	12.2[ab]	12.4[ab]	16.3[ab]
γ-Hexalactone[§]	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]	0.58[b]	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]
Mesifuran[§]	n.d.[a]	n.d.[a]	n.d.[a]	0.21[ab]	2.08[ab]	4.01[b]	0.18[ab]	6.57[b]	11.4[c]	17.6[d]	21.5[e]	22.4[e]
Unidentified 1	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]	1.79[bc]	0.56[ab]	n.d.[a]	1.1[ab]	1.39[b]	2.84[cd]	2.69[c]	3.92[d]
δ-Hexalactone[§]	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]
Allo-Ocimene	0.32[a]	0.48[ab]	1.77[ab]	2.33[ab]	2.92[ab]	3.07[b]	2.71[ab]	2.76[ab]	1.73[ab]	2.48[ab]	2.6[ab]	2.38[ab]

(*Z*) 2,6-Dimethyl-3,5,7-octatriene-2-ol	0.13^{a}	0.15^{a}	0.46^{ab}	2^{b}	0.13^{a}	0.33^{ab}	0.28^{ab}	0.81^{ab}	1.22^{ab}	0.99^{ab}	1.85^{ab}	0.17^{a}
(*E*) 2,6-Dimethyl-3,5,7-octatriene-2-ol	0.16^{a}	0.18^{a}	0.67^{ab}	1.52^{ab}	0.12^{a}	0.3^{ab}	0.37^{ab}	0.57^{ab}	2.66^{b}	1.48^{ab}	2.34^{ab}	0.16^{a}
γ-Octalactone[§]	n.d.^{a}	n.d.^{a}	n.d.^{a}	0.37^{a}	2.58^{c}	2.24^{c}	n.d.^{a}	0.64^{ab}	0.98^{ab}	1.04^{ab}	1.11^{b}	1.9^{c}
Unidentified 2	n.d.^{a}	n.d.^{a}	n.d.^{a}	1.99^{b}	0.36^{a}	0.72^{a}	n.d.^{a}	2.45^{b}	4.42^{bc}	5.88^{c}	5.2^{c}	9.84^{d}
δ-Octalactone	n.d.^{a}	n.d.^{a}	n.d.^{a}	n.d.^{a}	0.53^{b}	0.39^{b}	n.d.^{a}	n.d.^{a}	n.d.^{a}	n.d.^{a}	n.d.^{a}	0.38^{b}
Longicyclene	n.d.^{a}	n.d.^{a}	n.d.^{a}	n.d.^{a}	n.d.^{a}	n.d.^{a}	n.d.^{a}	n.d.^{a}	n.d.^{a}	n.d.^{a}	n.d.^{a}	n.d.^{a}
α-Copaene	0.16^{a}	0.31^{a}	0.45^{ab}	0.47^{ab}	0.75^{b}	0.7^{b}	0.49^{ab}	0.27^{a}	0.51^{ab}	0.52^{ab}	0.37^{ab}	0.81^{b}
Isolongifolene[§]	n.d.^{a}	0.1^{a}	0.11^{a}	0.43^{ab}	1.45^{bc}	1.78^{c}	n.d.^{a}	n.d.^{a}	n.d.^{a}	0.85^{b}	0.7^{ab}	1.56^{bc}
Longifolene[§]	0.34^{ab}	0.31^{ab}	0.4^{b}	0.24^{ab}	0.68^{b}	0.76^{b}	n.d.^{a}	n.d.^{a}	n.d.^{a}	0.3^{ab}	0.25^{ab}	0.54^{b}
β-Caryophyllene[§]	5.32^{a}	5.56^{ab}	8.83^{ab}	14^{b}	14.4^{b}	21.3^{b}	10.1^{ab}	6.73^{ab}	13.2^{ab}	11.7^{ab}	13.4^{b}	12.3^{ab}
Humulene[§]	2.88^{a}	3.02^{a}	4.86^{ab}	7.91^{b}	8.03^{b}	11.6^{b}	5.74^{ab}	3.76^{a}	7.54^{ab}	6.78^{ab}	7.66^{b}	6.78^{ab}
γ-Decalactone[§]	n.d.^{a}	n.d.^{a}	n.d.^{a}	n.d.^{a}	n.d.^{a}	n.d.^{a}	n.d.^{a}	n.d.^{a}	n.d.^{a}	n.d.^{a}	n.d.^{a}	n.d.^{a}
Germacrene D	n.d.^{a}	n.d.^{a}	n.d.^{a}	1.3^{ab}	5.09^{c}	5.28^{c}	n.d.^{a}	1.72^{ab}	3.09^{bc}	3.39^{bc}	2.05^{b}	4.35^{c}

n.d.: not detected.

Amid various data points in the score plot, 0 (mature-raw) and 2 DAH (table-green) skins of control fruits positioned together in the third quadrant which could be credited to their low levels of terpenes and absence of lactones. Also, skins of all the ripening stages of ethylene treated fruits (except 11 DAH, over-ripe) together with 10 DAH (mid-ripe) skins of control fruits grouped together across first and fourth quadrant. This grouping could be affiliated to their low lactone content as well as high levels of terpenes. Similarly, distinct position of 15 (ripe) and 20 DAH (over-ripe) skins of control fruits in the first quadrant could be the result of high lactone as well as terpene content in them among all the skin tissue analysed. Among various ripening stages of pulp, 1, 3 and 5 DAH pulp of ethylene treated fruits along with 0, 2, 5 and 10 DAH pulp of control fruits gathered together in the third quadrant mainly due to their low terpene content. Further, 7, 9 and 11 DAH pulp of ethylene treated fruits with 15 and 20 DAH pulp of control fruits have migrated collectively in the second quadrant which could be correlated with their high lactone and mesifuran content, which were located at the similar positions in the loading plot (Figure 3.2A).

3.3.4. Influence of exogenous ethylene on flavor character of Alphonso fruits

Of the 26 compounds analysed in the present study, four compounds *viz*. (*Z*)-ocimene, mesifuran, γ-octalactone and γ-decalactone are reported to have relatively lower odour detection threshold (ODT) (55, 0.03, 7 and 11 ppb, respectively). Considering these values available in the literature along with concentration of these compounds in Alphonso mango, their odour unit contribution was calculated using formula, odour units= concentration of a compound/ its odour detection threshold. Based on these calculations, above mentioned compounds contributed the odour unit higher than that of any other compound to the ripe stages viz. 9 (ripe) and 11 DAH (over-ripe) of ethylene treated and 15 (ripe) and 20 DAH (over-ripe) of control fruits. Moreover, based on their sensory perception data available in the literature, (*Z*)-ocimene accounts for terpenic flavor while mesifuran contributes caramel like notes. Two lactones namely, γ-octalactone and γ-decalactone impart coconut and peach like flavor, respectively. Thus, considering these compounds to be the vital flavor

contributors, their odour units in the ripe stages of ethylene-treated and control tissues were compared on a radar plot (Figure 3.3).

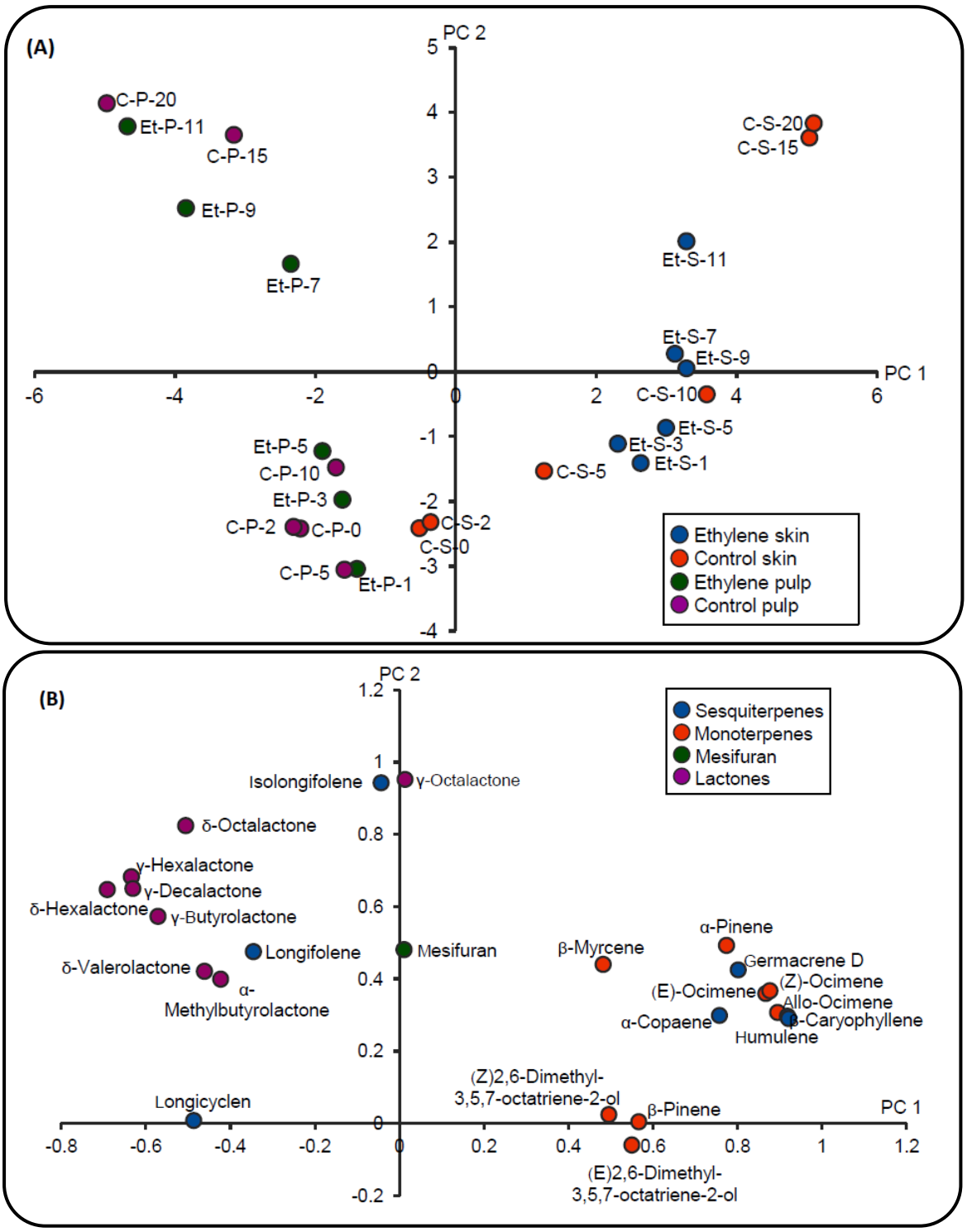


Figure 3.2: Score plot (A) and loading plot (B) of principal component analysis of the quantities of all the 24 compounds detected across the ripening stages analysed for the pulp and the skin of control and ethylene-treated Alphonso fruits. For the score plot (A), C: control; Et: ethylene treated; P: pulp; S: skin and numbers in the data labels denote days after harvest.

It was clearly evident that, 15 DAH (ripe) pulp of conventionally ripened fruit had the highest terpenic character with contribution of coconut and peach flavor maximum to this tissue than any other stage. However, 20 DAH (overripe) pulp depicted very low share of terpenic notes, slightly lower share of coconut and peach flavor and higher contribution of caramel notes than 15 DAH (ripe). In the pulp of ethylene treated fruits, both 9 (ripe) and 11 DAH (overripe) stages exhibited much higher contribution of caramel flavor as compared to the pulp of control fruits, with least share of terpenic notes. Levels of coconut flavor were comparable at both the stages while peach note contributed almost double odour units to 11 DAH (overripe) than 9 DAH (ripe) (Figure 3.3A). However, contribution of coconut and peach notes to the pulp of ethylene-treated fruits was, lower than that to the pulp of control fruits.

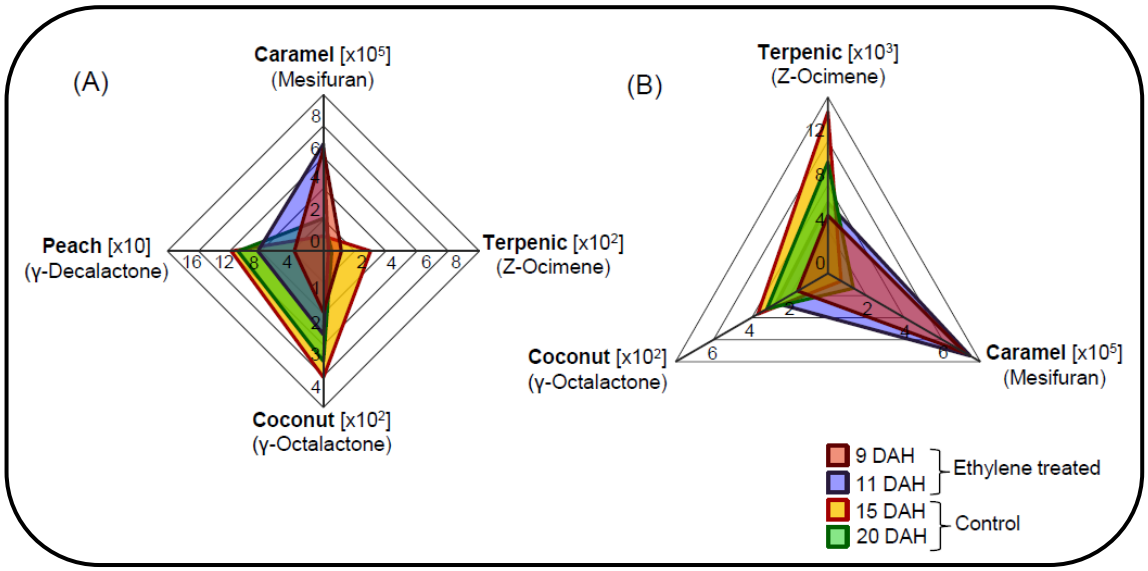


Figure 3.3: Radar plot showing the contribution of caramel, terpenic, coconut and peach flavor to the pulp (A) and caramel, terpenic and coconut flavor to the skin (B) of ethylene-treated and control Alphonso mango in terms of odour units of these compounds. Values in parentheses represent the scale for each axis.

For the skin of Alphonso a similar radar plot was drawn considering the odour unit contribution of (*Z*)-ocimene, mesifuran and γ-octalactone (γ-decalactone was absent in skin) (Figure 3.3B). Here also, the skin of ethylene treated fruits exhibited very high caramel flavor with low terpenic and coconut notes at both 9 DAH (ripe) and 11 DAH (overripe). The skin of control fruits showed high terpene content with more contribution of coconut note than that in the skin of ethylene treated fruits. However, caramel character experienced drastic

lowering in the skin of control fruits collectively at 15 (ripe) and 20 DAH (overripe) stage.

3.3. Discussion

Fruit ripening is an irreversible process involving complicated yet well co-ordinated series of physiological, biochemical and organoleptic changes. Climacteric fruits especially are marked by the concomitance of respiratory peak and ethylene production that trigger various ripening events including flavor biosynthesis. Given this, a highly crucial job of this tiny two carbon molecule in fruit ripening has popularized it as 'ripening hormone'. Taking the advantage of this knowledge, ethylene or ethylene producing or mimicking chemicals such as ethephon or propylene are used commercially to advance the ripening in the climacteric fruits, including mango (McMurchie et al., 1972; Sergent et al., 1993). As aroma volatiles form an indispensible feature of fruit flavor, they represent the first line of the features to be assessed upon exogenous application of ethylene. Moreover, Alphonso mango, which features diverse volatile profile with 15 days long ripening period (Kulkarni et al., 2012a; Pandit et al., 2009b), is an ideal system to track the dynamics of volatile patterns upon pre-climacteric application of this ripening hormone. Thus, in the present study we analysed the effect of ethylene treatment on the Alphonso ripening in terms of changes in their volatile composition.

3.4.1 Influence of exogenous ethylene on volatile profile of ripening Alphonso mango

Although involvement of ethylene in the fruit ripening is well established, most of the work in this direction is focused on other aspects of ripening than aroma volatiles (Cara and Giovannoni, 2008). For example, an exogenous ethylene treatment was employed to get homogenous and early ripening in Mexican mango wherein the ripening was monitored in terms of acidity loss, total soluble solid content and color development (Tovar, 2011). Moreover, instead of direct application of ethylene, ethephon (an ethylene source) has been popularly used to study the effect of ethylene treatment (Singh and Janes, 2001). However, flavor volatiles, which form one of the most important attributes of mango, have never

been monitored upon ethylene treatment in Indian mangos. Also, it is evident from various reports that ethylene treatment accelerates ripening process in mango (Lalel et al., 2003c; Singh and Janes, 2001).

In the present study, a gas chromatographic analysis of the skin and the pulp of mango fruits at various stages of ripening revealed significant change in the volatile profiles of these tissues upon ethylene treatment. Lactones and furanones, were detected much earlier [3 DAH (table green) onwards] in the pulp and the skin of ethylene treated fruits as compared to conventionally ripened fruits [10 DAH (mid-ripe) onwards]. Moreover, in case of mesifuran, there was a huge increase in its concentration in the ethylene treated fruits. These observations strongly suggested that in the naturally ripened fruits also; the production of lactones and mesifuran might be induced by the climacteric ethylene. The effect of ethylene on the volatiles of mango fruits has till now been studied only in Kensington Pride mango (Lalel et al., 2003c). This study showed an increase in mono- and sesquiterpene content on ethephon treatment of Kesington Pride mango. This contrasts to the present observation in Alphonso mango. Lowering of the monoterpene content in the skin and the pulp of Alphonso upon ethylene treatment whereas no significant difference in the sesquiterpene content of pulp of ethylene treated and control fruits were observed in the present study. Further, γ-octalactone, the only lactone detected in Kensington Pride mango (Lalel et al., 2003c) could be quantified only upon ethylene treatment; whereas, eight different lactones could be quantified collectively from ethylene treated and control fruits of Alphonso mango with no significant effect of ethylene treatment. These observations suggest that the differential effect of ethylene on the volatile profiles of these two cultivars might be because of their varied phytochemistries.

The high concentration of mesifuran observed in the skin of Alphonso mango might result in spread of mango odour in the surrounding environment and in the light of the fact that mesifuran has very low odour detection threshold, this might help in attracting frugivorous animals. On the other hand, in the pulp, the high concentration of lactones along with mesifuran appears to play a role in both, the ortho- and retronasal perception of mango flavor. Higher concentration

of lactones in the pulp than the skin of mango fruits was also consistent with the earlier report on Kensington Pride mango (Lalel et al., 2003b). The identical pattern exhibited by two sesquiterpenes, β-caryophyllene and humulene in all the tissues analyzed, which was consistent with our earlier report (Kulkarni et al., 2012a), suggested that they might be the products of a single terpene synthase enzyme in mango as was found in other plants (Cheng et al., 2007). In relation to ethylene treatment, these two sesquiterpenes showed totally different profile in the skin with not much variation in the pulp tissue (Table 3.1A and B) suggesting differential response of the skin and the pulp to the exogenous ethylene.

The PCA showed clear separation of the skin and the pulp across PC1 in score plot which could be clearly attributed to the presence of terpenes and lactones on either side of the PC1 in loading plot. Close appearance of all the ripening stages from the skin of ethylene treated fruits as compared to the skin from control fruits was likely to be because of narrow range of terpene content of the previous group (271-504 μg g^{-1}) as opposed to much broad range for the later group (90.6-874 μg g^{-1}). Distinct positions of all the data points on the score plot could be correlated with volatile content of each of them and distribution pattern of the volatiles on the loading plot, respectively.

3.4.2. Transition of flavor blend with respect to ethylene treatment

Considering the important contribution of (*Z*)-ocimene, mesifuran, γ-octalactone and γ-decalactone to Alphonso flavor (Idstein and Schreier, 1985; Wilson et al., 1990), odour units contributed by these compounds to the ripe and over-ripe stages of skin and pulp of ethylene treated and control fruits were calculated and were analysed on the radar plot.

Interestingly, the analysis evinced the dominance of terpenic (Z-ocimene), coconut (γ-octalactone) and peach notes (γ-decalactone) with least caramel character (mesifuran) in the ripe (15 DAH) pulp of conventionally ripened Alphonso mango while 20 DAH (overripe) pulp experienced significant lowering in the terpenic notes with slight increase in caramel character. On the contrary, pulp of ethylene treated fruits demonstrated surge of caramel odour paralleled with lowering of terpenic, coconut and peach notes. Moreover, share of caramel

notes was five to six times higher in pulp of ethylene treated fruits than the control ones. Similarly, in case of the skin, ethylene treatment imparted considerably high caramel character with lowering of terpenic and lactone notes; whereas, the skin of control fruits had higher terpene flavor with more coconut like notes than caramel. These findings pointed towards far different flavor blends of ethylene treated and control Alphonso mangoes. It also revealed the strong relationship between ethylene production and mesifuran and lactone biosynthesis in Alphonso mango fruit.

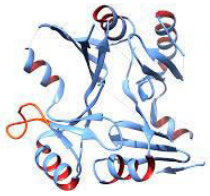
3.4.3. Modulation of ripening: An ethylene perspective

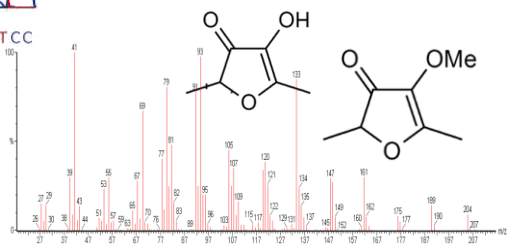
Fruits, one of the highly indispensible assets of the plants have gathered enough attention from researchers worldwide largely due to their high nutritional and aesthetic qualities. Nonetheless, most of the important features of fruits such as sweetness and flavor are acquired during the process of ripening, making completely ripened fruit the dense reservoir of many desirable traits. Interestingly, exposure of ethylene or chemicals that produce ethylene/ mimic the action of ethylene (ethephon, ethrel, propylene and acetylene) during this pre-climacteric period triggers system II of ethylene production in climacteric fruits (McMurchie et al., 1972). This leads to the onset of various ripening related events including increased production of aroma volatiles (Lalel et al., 2003c) and activity of enzymes involved in cell expansion and cell wall solubilisation machinery (Chourasia et al., 2006). In the present study also, ethylene treatment caused advanced (both mesifuran and lactones) and increased (mesifuran) production of the ripening-related volatiles. Further, as evidenced by enhanced enzymatic activities of glycosyl hydrolases involved in the cell wall hydrolysis in the previous chapter, ethylene treated mangoes experienced comparatively faster tissue softening. This led to treated fruits overripe and difficult to handle after 11 DAH reducing their shelf-life and rendering them unsuitable for long distance transport. In view of this, it would be interesting to investigate the possibility of combining the strategy of ethylene treatment with that of silencing the softening causing genes (Meli et al., 2010). In such a case, ethylene treatment would cause advancement of ripening in terms of volatiles and possibly other metabolites also. At the same time, inactivity of softening causing enzymes would enhance the

shelf life of the fruits ultimately resulting in bidirectional expansion of the time window available for the utilization of fruits. In order to explore this and other approaches to improve the desirable characters in mango, understanding the regulatory action of ethylene on ripening of mango at the molecular level would be immensely useful. Expression analysis of key genes involved in various metabolic pathways in ethylene treated fruits would aid to understand their transcriptional regulation.

In conclusion, the present study shows alteration of volatile profile upon ethylene treatment in Alphonso mango. The early appearance of ripening-related compounds not only suggests the involvement of ethylene in triggering biosynthesis of these compounds but it also highlights the usefulness of ethylene treatment in advancing the ripening. However, the fact that ethylene treatment also results in reduction of the shelf life of mango fruits, demands more research on advancing the ripening without affecting the shelf-life. In this direction, an appropriate manipulation of ethylene doses with standardization of suitable ripening parameters will aid commercialization of Alphonso mango.

Chapter 4





Molecular Cloning and Expression of *O*-methyltransferase Catalysing The Synthesis of Mesifuran, A Key Aroma Volatile in Alphonso Mango Fruit

4.1. Introduction

Synthesis and composition of aroma volatiles during mango ripening has been a major area of interest amongst the plant biologists. Various efforts aiming to decipher their volatile composition portrayed vast diversity of these compounds in mango fruits (Macleod and Pieris, 1984; Pandit et al., 2009a; Pino and Mesa, 2006). Amid these, studies on volatile composition of Alphonso mango pertaining to various aspects of development and ripening revealed the dominance of terpene hydrocarbons (Kulkarni et al., 2012b). However, their impact on the overall flavor of ripe Alphonso was found to be relatively less owing to their high odour detection threshold. On the contrary, lactones and furanones which are particularly synthesized during the late ripening stages have significantly low odour detection threshold and thus contribute majority of flavor imparting sweet fruity caramel like notes to ripe Alphonso fruits (Kulkarni et al., 2012a; Pandit et al., 2009b). In Alphonso mango, furanones comprise 4-hydroxy-2,5-dimethyl-3(2H)-furanone (furaneol) and its methyl ether, 2,5-dimethyl-4-methoxy-3(2H)-furanone (mesifuran). Both these compounds have odour detection threshold of 10 and 0.03 ppb, respectively and thus have high 'aroma value'. Moreover, the importance of furaneol and mesifuran to the flavor of Alphonso mango was first evaluated organoleptically by Wilson et al. (1990). Besides, Alphonso mango showed quantitative dominance of these compounds when compared with other cultivars (Pandit et al., 2009a). Also, apart from mango their presence has been well reported from various other fruits such as strawberry, arctic brambles, lychee, snake fruit, pineapple, raspberry, grapes, tomato and kiwi (Schwab, 1997).

High importance of these furanones for the food industry has promoted standardization of their chemical synthesis by various researchers, however; the identification of suitable biosynthetic process for the production of these compounds is a field still in infancy (Schwab, 2013). Earlier, Lavid et al. (2002) have partially purified and biochemically characterized the S-adenosyl-L-methionine (SAM) dependant *O*-methyltransferase from strawberry fruit extracts which displayed active conversion of furaneol to mesifuran in an assay reaction. This was later supported by the molecular isolation of corresponding cDNA followed by the expression of its recombinant protein (*Fa*OMT) which demonstrated a successful synthesis of mesifuran from furaneol in an *in vitro* assay

reaction (Wein et al., 2002). To study the biosynthesis of furanones in mango fruits, an enone oxidoreductase (*MiEO*) which catalysed the synthesis of furaneol, a precursor molecule of mesifuran from Alphonso mango has recently been characterized (Kulkarni et al., 2013a).

In our previous study detailed in Chapter 3, we observed that ethylene treatment results in early and exceptionally enhanced synthesis of mesifuran in Alphonso mango fruits. To understand the biosynthesis of mesifuran and its regulation by ethylene in mango fruits we further studied the isolation and characterization of cDNA encoding *O*-methyltransferase (*O-MTS*) catalysing the synthesis of mesifuran from furaneol in the ripe fruits of Alphonso mango.

4. 2. Material and methods

4.2.1 Plant material

Two sets of twenty mature green fruits of mango (*cv*. Alphonso) were collected from three distinct mango trees located at mango orchards of Dr. Balasaheb Sawant Konkan Agriculture University, Dapoli, Maharashtra, India (N17°45′ E73°11′). Harvesting of the fruits, ethylene treatment and ripening conditions were same as described previously in Materials and Methods of Chapter 2. The harvested fruits were immediately cut, skin and pulp were separated and frozen in liquid nitrogen and this stage was considered as 'mature raw' which was denoted as 0 DAH (days after harvest) stage of control fruits. The subsequent five ripening stages of control fruits *viz.* table green (2 DAH), pre-climacteric (5 DAH), mid-ripe (10 DAH), ripe (15 DAH) and over-ripe (20 DAH) were frozen to cover the entire ripening period of control fruits. Upon ethylene treatment all six ripening stages were covered within 11 days due to accelerated ripening of fruits. Thus, to span the entire period of ripening upon ethylene treatment, the fruits were frozen and stored at table green (3 DAH), pre-climacteric (5 DAH), mid-ripe (7 DAH), ripe (9 DAH) and over-ripe (11 DAH) stages. In this way, six ripening stages each for pulp and skin of control and ethylene treated fruits were sampled along with mature leaf and open flowers and stored at -80°C until use.

4.2.2 RNA isolation and cDNA synthesis

Total RNA was isolated from all the tissues under study using RNeasy Plus mini kit (Quiagen, Venlo, Netherlands). Two microgram of total RNA was subjected to reverse transcription using Applied Biosystem High Capacity reverse transcription kit (Waltham, Massachusetts, USA) employing oligo dT.

4.2.3 Isolation of full length cDNA gene encoding *O*-methyltransferase

To obtain the partial cDNA sequence of Alphonso mango *O*-methyltransferase (*O-MTS*), the degenerate primers reported by Frick and Kutchan (1999) were used. The amplification was performed over the cDNA prepared from ethylene treated ripe stage fruits (9 DAH) similarly as mentioned in Materials and Methods section of Chapter 2. Out of the two pairs (MTsI-MTasII and MTsI-MTasIV), later gave the expected size amplicon which was gel eluted using GenElute™ Gel Extraction Kit (Sigma Chemical Co., USA) and ligated in pGEM-T Easy vector as per the manufactures' protocol (Promega, Madison, WI, USA). The ligation reaction was transformed in *E. coli* cells (Top10, Invitrogen, USA) and the positive recombinants were confirmed by colony PCR followed by sequencing. Based on the sequence, gene specific primers were designed (Table 4.1) and used for the rapid amplification of cDNA ends (RACE) to acquire the complete open reading frame (ORF) of *O-MTS* using the SMART™ RACE cDNA Amplification Kit (Clontech, USA). The 5' and 3' RACE amplicon sequences thus obtained were aligned using MEGA software (Version 4.1) (Tamura et al., 2007) with the other plant *O*-methyltransferase sequences reported in the NCBI database and the primers corresponding to the terminal regions were designed (Table 4.1). These primers were again used for PCR over ethylene treated ripe stage (9 DAH) fruit pulp cDNA (1 µL) using Q5 High Fidelity Taq DNA polymerase (NEB Inc, Ipswich, MA, USA). PCR was performed in 20 µL reaction volume with 1X buffer, 200 µM dNTPs, 0.6U Q5 DNA polymerase and 0.5 µM each of forward and reverse primers. The thermal cycler reaction conditions were as follows – initial denaturation of 98°C for 3 min, 30 cycles of 98ºC for 30 sec, annealing temperature (63°C) for 30 sec min, extention at 72ºC for 30 sec and final extention for 2 min at 72ºC. The fragments obtained were resolved on 1.5% agarose gel in 0.5X Tris-acetate EDTA (TAE) (Sambrook and

Russell, 2001) with a 1kb ladder (Promega) as the size marker and the amplicons were visualized by GelRed (Biotium Inc. Hayward, CA). Amplicons of desired size were gel eluted using GenElute™ Gel Extraction Kit (Sigma Chemical Co., USA) and cloned in pGEM-T Easy vector as per the manufactures' protocol (Promega, Madison, WI, USA) and the ligation reactions were transformed in *E. coli* cells (Top10, Invitrogen, USA). The recombinant colonies positive for presence of the complete ORF of *O-MTS* were confirmed by colony PCR followed by sequencing. To obtain the gene structure of *O-MTS*, the above mentioned terminal primers were amplified over Alphonso mango genomic DNA (30 ng) using similar conditions mentioned above. The obtained fragments were cloned in pGEM-T easy vector and sequenced. The sequences were aligned and the intron-exon junctions were identified by comparing with the cDNA sequence of the *O-MTS*. The complete ORF of *Mangifera indica O*-methyltransferase (*MiOMTS*) was amplified from pGEM-T easy clones using primers having flanking *BamHI* site (Table 4.1) for ligation into pGEX-4T-3 fusion vector (GE Healthcare Life Sciences, Little Chalfont, UK).

Table 4.1: Gene specific primers used for isolation of full length gene, expression cloning and for transcript quantification using qRT -PCR

Primer ID	Orientation	Group[a]	Sequence (5'-3')
MTS-RC-F	Forward	A	GATCTGCCACATGTTGTAGCTACTG
MTS-RC-R	Reverse	A	AATGGCATCAAACATGTTACCTCCAACG
MTS-F	Forward	B	ATGGGATCATTAGAAGTTAAGACATTG
MTS-R	Reverse	B	TTACAGTGGATAGGCCTCAATAATG
MTS-Exp-F	Forward	C	AAAGGATCCATGGGATCATTAGAAGTTAA GACATTG
MTS-Exp-R	Reverse	C	AAAGGATCCTTACAGTGGATAGGCCTCAA TAATG
MTS-RT-F	Forward	D	ATGAAGTGGATACTGCATGATTG
MTS-RT-R	Reverse	D	AGAACGATTTCAACTAGAACAACC

[a] Group A primers are gene specific used for 5' and 3' RACE reactions; Group B primers are gene specific terminal primers used to isolate complete ORF; Group C are primers used for the expression cloning; Group D are gene specific primers used for transcript quantification with qRT-PCR.

The ligation reaction was transformed into *E. coli* cells (Top10, Invitrogen, USA) and the positive transformants were selected by colony PCR and the orientation of the insert was confirmed by sequencing. The pGEX-4T-*MiOMTS* construct thus obtained was transformed in *E. coli* BL21 (DE3) cells (Invitrogen, USA) for expression of recombinant *O-MTS*. The starter culture was grown for 12-13hr at 37°C and was used as inoculum in the expression media at the final concentration of 1%. The expression of recombinant protein was induced by 0.1mM IPTG when the OD_{600} reached to 0.55. Thereafter the culture was grown for 5 hr at 16°C. The cells were harvested by centrifugation and the pellet was suspended in the lysis buffer containing 140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 1% glycerol and 2mM β-mercaptoethanol (pH 7.3). The clear lysate obtained after sonication and centrifugation was incubated with the pre-equilibrated GST-affinity purification resin (GE Healthcare Life Sciences, Little Chalfont, UK) for 2hr at 4°C. Unbound proteins were removed by washing the resin 5-7 times with the lysis buffer. Further, the cleavage of the recombinant protein from the GST-tag was carried out using thrombin according to the manufacturer's instructions (GE Healthcare Life Sciences, Little Chalfont, UK), for overnight at 4°C with gentle agitation. The recombinant protein was collected by fractionation and used for enzyme assays as well as molecular weight determination using SDS-PAGE.

4.2.5 Enzyme assays and identification of the assay products

The *in vitro* enzymatic assays were carried out in the lysis buffer containing appropriate amount of protein, 0.2mM substrate and 0.01mM *S*-adenosylmetheonine (SAM) in a final volume of 500µL reaction. The reaction was incubated at 30°C for 30min. For the determination of optimum pH for the enzyme activity, the assays were performed in 50mM citrate buffer (pH 4.0 and 5.0), 50mM phosphate buffer (pH 6.0, 6.5, 7.0 and 7.5) and 50mM Tris buffer (pH 8.0, 8.5 and 9.0) keeping other components in the assay same as above. Similarly, for optimum temperature the assays were performed at 15, 20, 25, 30, 35 and 40°C. Assay products were extracted twice with ethyl acetate; the organic layers were pooled, dried in a concentrator and constituted with methanol. An Accela™ ultra high performance liquid chromatography (UHPLC) system

(ThermoFisher, Waltham, USA), coupled online via heated electrospray ionization source (HESI) with a Q-Exactive-Orbitrap mass spectrometer (ThermoFisher), was employed with 2µL sample injection volume and the chromatograms were obtained by total ion monitoring in a positive ion mode. The products were separated and analysed on Hypercil GOLD C-18 reverse phase column (150mm X 3mm i. d., particle size 5 µm, Thermo Scientific, Waltham, MA, USA) with a linear gradient of 95% water acidified with 0.05% formic acid and 5% methanol to 80% methanol and 20% water with the flow rate of 0.5 µL min^{-1}. The sample manager was maintained at 4°C. Identification of the substrates as well as products were done by comparing the retention time and molecular mass of authentic external standards [furaneol (CAS No. 3658-77-3), mesifuran (CAS No. 4077-47-8), protocatechuic aldehyde (CAS No. 139-85-5) and vanillin (CAS No. 121-33-5)] procured from Sigma Aldrich, USA.

4.2.6 Quantitative real-time PCR

Quantitative real-time PCR was performed using FastStart Universal SYBR Green master mix (Roche Inc. Indianapolis, Indiana, USA) and elongation factor 1α as an endogenous control employing the primers mentioned earlier (Pandit et al., 2010). Transcripts of *MiOMTS* were amplified using gene specific primers (Table 4.1) and quantification was done by ViiA™ 7 Real-Time PCR System (Applied Biosystems, CA, USA) having thermal cycle programme of initial denaturation at 95°C for 10 min with subsequent 40 cycles of 95°C for 3 sec and 60°C for 30 sec followed by a dissociation curve analysis of transcripts. Considering the transcript abundance at ethylene treated preclimacteric stage (5 DAH) as 1, the fold difference in the transcript levels of *MiOMTS* gene in rest of the tissues was calculated. The quantification was done for three independent biological replicates separately, each of which was represented by at least three technical replicates. A semi-quantitative PCR was performed using above mentioned gene specific primers over control ripe (15 DAH) pulp and skin, pre-climacteric ethylene treated (5 DAH) pulp and skin, leaf and flower cDNAs. Twenty microliter PCR reaction contained 1 µL of the cDNA along with 1X final concentration of *Pfu* buffer (Promega, Madison, WI, USA), 2.0mM $MgCl_2$, 0.5mM dNTPs, 0.5µM of each gene specific primer and 1 unit *Pfu* DNA

polymerase (Promega, Madison, WI, USA). The thermal cycling programme consisted of initial denaturation at 95°C for 3 min with subsequent 30 cycles of 95°C for 30 sec, 60°C for 30 sec followed by extension at 72°C for 30 sec. The uniformity of expression across the tissues was monitored using elongation factor 1α (*EF 1α*) gene as an endogenous control. Equal amount of loading dye (6X) with 1X final concentration of GelRed (Biotium Inc. Hayward, CA) was added to the PCR products for visualisation and the PCR products were separated on 2% agarose gel.

4.2.7 Statistical analysis

Comparison of *MiOMTS* transcripts at various ripening stages of pulp and skin of control and ethylene treated fruits as well as correlations between the mesifuran content and transcript abundance during the ripening stages of pulp and skin of control fruits were carried out by ANOVA using fisher's LSD test at $p \leq 0.05$ using StatView software, version 5.0 (SAS Institute Inc., Cary, NC, USA).

4.2.8. Structural analysis of *O*-methyltransferase protein

Homology modeling of *MiO*MTS protein was carried out on the 'SWISS-MODEL' (Arnold et al., 2006) using *Pisum sativum* isoflavone 4-*O*-methyltransferase (PDB ID: 1zg3) as a template. Other quality parameters of the generated model were also assessed by 'SWISS-MODEL'. Ramachandran plot assessment of the structure was carried out on the RAMPAGE server (Lovell et al., 2002). The final structure was visualized in the program 'DeepView' (SPDBV 4.1.0).

4.3. Results

4.3.1 Isolation and identification of *O-MTS* coding gene from *Mangifera indica* (cv. Alphonso) fruits

Isolation of partial gene sequence of *O-MTS* from mango was done using degenerate primers reported previously by (Frick and Kutchan, 1999) using the cDNA of ripe stage (9 DAH) ethylene treated fruit pulp, as our previous study reported the highest accumulation of mesifuran at this stage (Results section in Chapter 3). The obtained PCR product was cloned and sequenced. The sequence

showed high similarity to *O*-methyltransferase sequences from other plants such as *Populus tricocarpa*, *Vitis vinifera* and *Ricinus communis*. Full length *O-MTS* gene was obtained by 5' and 3' RACE using the cDNA of ripe stage (9 DAH) pulp of ethylene treated fruit.

The complete ORF of *MiOMTS* was 1056 bp long with 30 bp and 95 bp 5' and 3' UTR, respectively. The *in silico* translated *MiO*MTS protein was 351 amino acids long with the predicted molecular mass of about 39kDa and pI of 5.71. The putative amino acid sequence of *MiO*MTS showed 41% identity to the strawberry *O*-methyltransferase (*FaO*MTS) which is the only characterized *O*-methyltransferase involved in the biosynthesis of mesifuran.

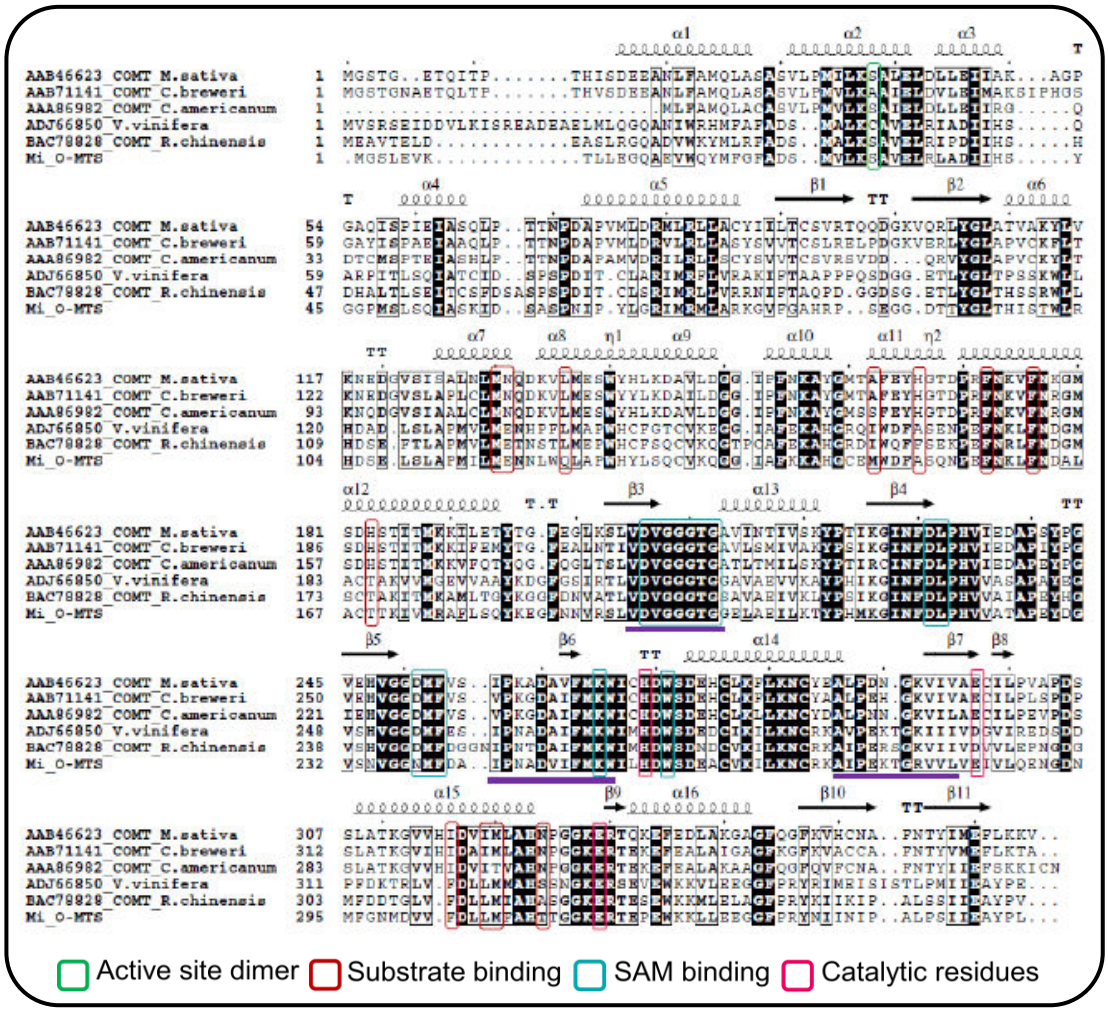


Figure 4.1: Alignment of *in silico* translated sequence of *MiO*MTS with *O*-MTS reported from other plants. The predicted secondary structures are indicated on the top of the alignment. The conserved SAM binding motifs are underlined in purple.

*MiO*MTS also showed 74% identity to *Ricinus communis* and *Populus trichocarpa O*-methyltransferases which are however, not yet characterized for their enzymatic activity. The alignment of *in silico* translated amino acid sequence of *MiO*MTS with other representative plant *O*-methyltransferases showed the presence of highly conserved motifs for substrate and SAM binding along with important catalytic residues. The prediction of secondary structure was performed using the primary (GenBank protein database) and secondary structure of COMT from *M. sativa* (alfalfa; AAB46623) depicting α-helices and β-strands in the structure using ESPript 3.0 software (Figure 4.1). The amplicons obtained after the PCR of gene specific terminal primers of *O*-MTS on genomic DNA template revealed a size of about 1.3 kb and the sequence analysis showed the presence of two introns with 165 bp and 95 bp length. Both the introns followed the "GT-AG" rule (Breathnach and Chambon, 1981) (Figure 4.2). Both, the complete cDNA and genomic sequence of *MiOMTS* were deposited to NCBI data base with the accession numbers KP993176 and KP993177, respectively.

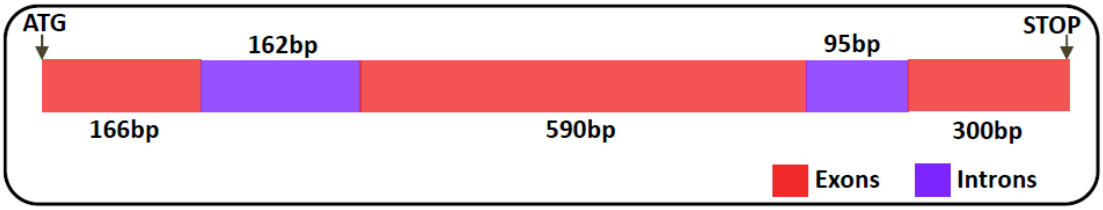


Figure 4.2: Gene organization of *MiOMTS*. Numbers on the top and the bottom indicate sizes (bp) of the introns and the exons, respectively.

4.3.2. Homology modelling

A homology based model was generated for *MiO*MTS using isoflavone 4-*O*-methyltransferase from *P. sativum* having 36.8% identity with 98% coverage with *MiO*MTS. When the structure of *MiO*MTS was assessed by Ramchandran plot, 92.2% residues were in favoured region while 6.8% residues were in allowed region. The QMEAN score of the generated structure as evaluated by SWISS-MODEL was -5.02 (Figure 4.3A). The homodimer structure of *MiO*MTS displayed the catalytic fold of conserved SAM binding domains and presence of dimerization domain. The dimerization of protein has been reported as critical feature for the activity of *O*-MTS (Zubieta et al., 2001). The substrate binding residues placed on helices and coils formed the binding pocket along with

presence of vital residues His 256, Glu 285 and Glu 316 responsible for the catalytic activity of *O*-methyltransferase as also reported in case of *Medicago sativa* (Zubieta et al., 2001) (Figure 4.3B).

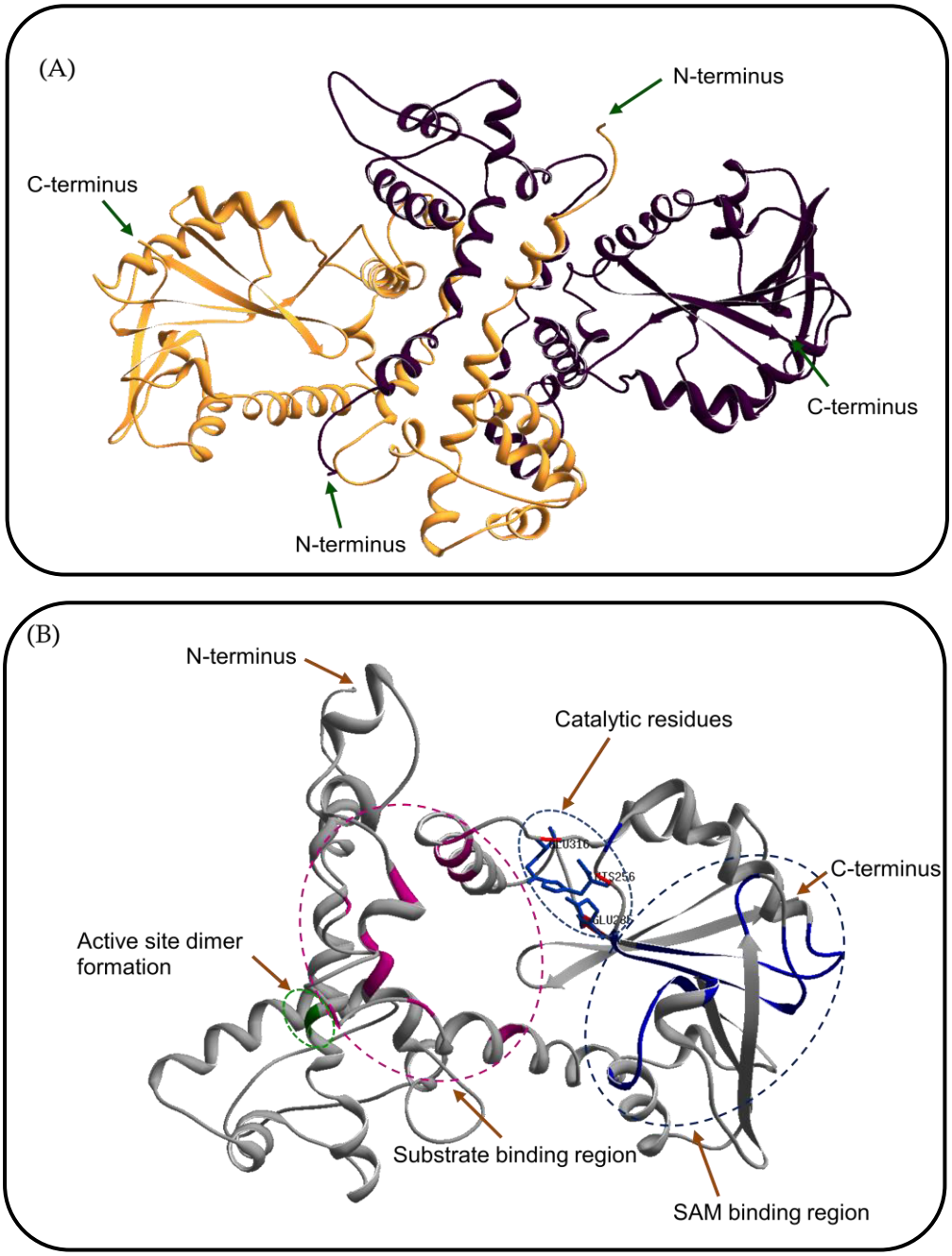


Figure 4.3: Homology model for *MiO*MTS generated using *Pisum sativum* isoflavone 4-*O*-methyltransferase (PDB ID: 1zg3) as a template. (A) Homodimer structure of *MiO*MTS with two monomers in yellow and violet colors. (B) A monomer showing the substrate binding pocket in magenta color, a SAM binding domain in blue color and an active site dimer forming residue in green color. The presence of catalytic residues is shown by red color with their side chains in navy blue color.

4.3.3 Heterologous expression, purification and characterization of recombinant *MiOMTS*

The complete ORF of *MiOMTS* cDNA was cloned in pGEX-4T vector and the protein was expressed as GST-tag fusion protein in *E. coli* BL21 (DE3) cells. The protein was purified using GST affinity chromatography and released from the tag using thrombin. The fusion protein showed the size of ~66kDa confirming the expression of recombinant *MiO*MTS (Figure 4.4). The purified protein was assayed for its activity with furaneol, caffeic acid, catechol and protocatechuic aldehyde as substrates and *S*-adenosyl-L-metheonine (SAM) as methyl group donor. The methylation products of these substrates upon *MiO*MTS enzymatic reaction were identified and analysed on high resolution mass spectrometer. The recombinant *MiO*MTS successfully used only furaneol and protocatechuic aldehyde as the substrates converting them to mesifuran and vanillin, respectively, in independent reactions.

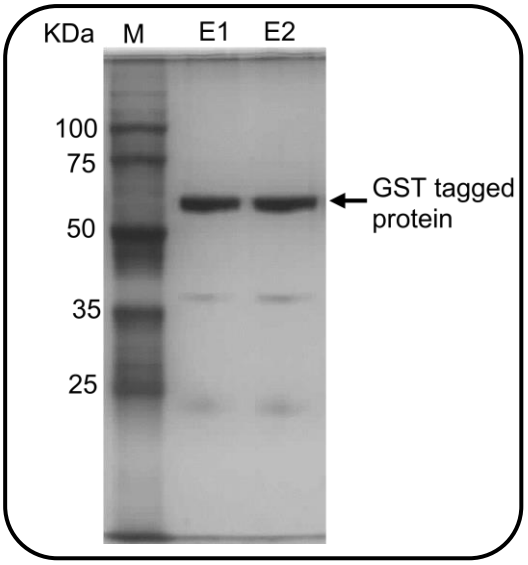


Figure 4.4: SDS-PAGE profile of GST-bound *MiO*MTS protein, E1 and E2: elution fractions and M: marker protein

The activity of *MiO*MTS was confirmed by carrying out the similar assay reaction with the protein of *E. coli* BL21 (DE3) cells transformed with empty pGEX-4T vector which did not show the formation of methylation products in the respective assay reactions (Figure 4.5). In contrast to furaneol and protocatechuic aldehyde, *MiO*MTS could not use caffeic acid and catechol as the methyl group acceptor. In order to evaluate the optimum biochemical conditions for the activity

of recombinant *MiO*MTS, the assays were carried out at varied pH and temperatures using furaneol as a substrate. The exercise displayed optimum activity of *MiO*MTS at pH 7.0 with the retention of 70 and 90% activity at pH 6.0 and 8.0, respectively. However, no activity was detected at pH 4, 5 and 9 with more than 50% reduction in the activity at pH 8.5. In case of temperature, optimum activity was detected at 25°C, with retention of 60% activity at 30°C. However, more than 50% reduction in the activity at 20°C and complete inactivation of the enzyme at 40°C was clearly evident (Figure 4.6).

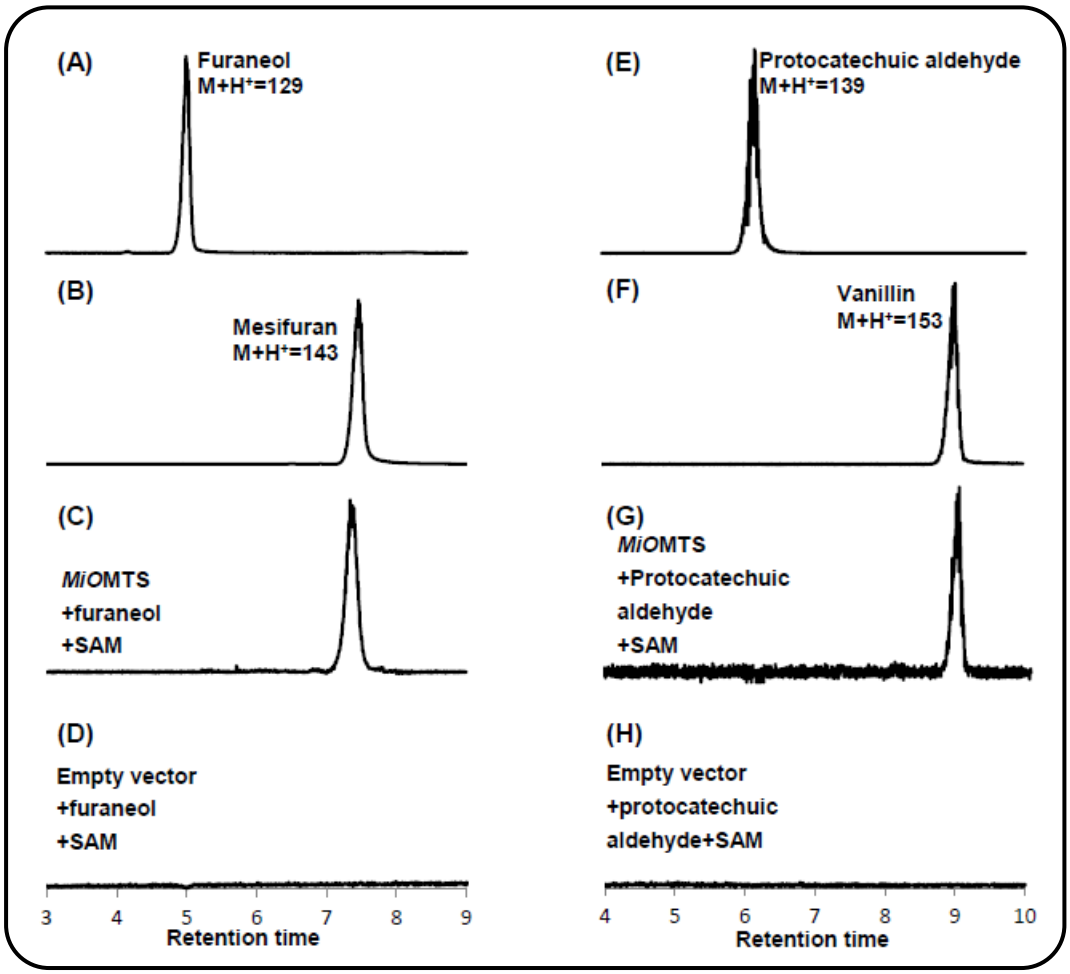


Figure 4.5: Chromatograms of *MiO*MTS enzymatic reactions analysed on Q-Exactive-Orbitrap mass spectrometer. Extracted Ion Chromatograms (EIC) representing the authentic standards of substrates and their expected products (A, B and E, F). The assay product of *MiO*MTS with furaneol (C) and protocatechuic aldehyde (G). Also, the EIC of assay reactions of protein from empty vector with furaneol and protocatechuic aldehyde monitored for ($M+H^+$) ion of their respective product (D and H).

4.3.4 Correlation of *MiOMTS* transcript abundance and mesifuran synthesis during the ripening of Alphonso mango and role of pre-climacteric exogenous ethylene treatment

To evaluate the tissue specificity of *MiOMTS* transcripts, a semi-quantitative PCR performed among pulp and skin of control and ethylene treated fruits along with leaf and flower tissues showed expression of *MiOMTS* only in the pulp and skin of Alphonso fruits suggesting fruit specific expression of the *MiOMTS* transcripts (Figure 4.7). To get better understanding of synthesis of mesifuran during late ripening stages of Alphonso mango and its regulation at transcriptional level, the transcripts of *MiOMTS* were profiled during the conventional ripening of Alphonso mango. Our previous study showed exceptionally high synthesis of mesifuran in the ethylene treated fruits during ripening compared to conventionally ripened fruits (Results section in Chapter 3). To further assess this at transcript level, the transcripts of *MiOMTS* were analysed in the fruits which were subjected to the pre-climacteric ethylene treatment. Both pulp and skin of the control and ethylene treated fruits were analyzed separately for the transcript abundance.

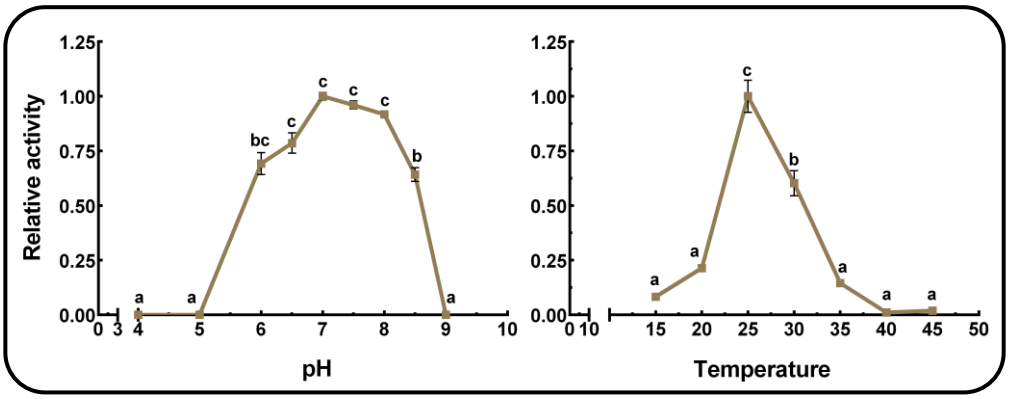


Figure 4.6: Optimum conditions of pH and temperature for the activity of *MiO*MTS. In case of both the parameters, the peak area of the product at the highest activity was considered 1 and relative activity was calculated for rest of reactions. Letters at each point indicate the significance of ANOVA ($p \leq 0.05$) analysed by Fisher's LSD test independently for both the parameters; the values with different letters are significantly different from each other.

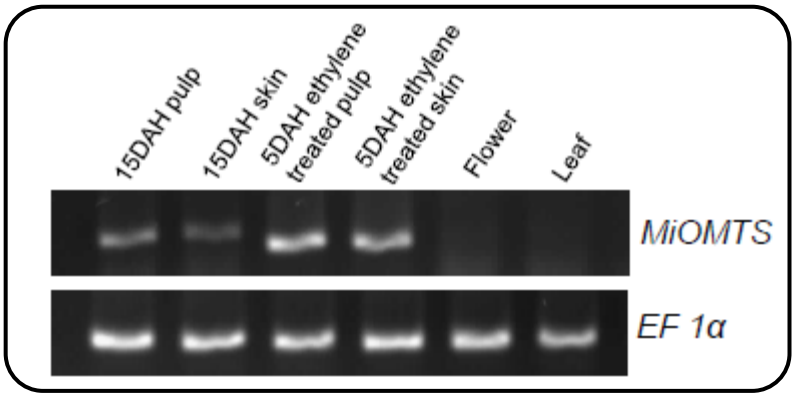


Figure 4.7: Tissue specific expression of *MiOMTS* transcripts as evaluated by semi-quantitative PCR. Elongation factor 1α (*EF 1α*) was used as endogenous control.

In case of control fruits, no transcripts were detected in both pulp and skin till mid-ripe (10 DAH) stage. Thereafter, a steady increase in the transcript level was observed in both the cases till overripe (20 DAH) stage. This can be easily correlated to the synthesis of mesifuran during the conventional ripening of Alphonso mango wherein mesifuran was detected at mid-ripe, ripe and overripe stages only (10, 15 and 20 DAH) (Figure 4.8). However, in the pulp of ethylene treated fruits, the transcript of *MiOMTS* were detected even at mature raw (1 DAH) and table green stages (3 DAH), although at relatively low level. Moreover, a sudden surge in the transcripts was observed at pre-climacteric (5 DAH) stage of pulp as well as skin of ethylene treated fruits which was 19 and 82 fold higher than the abundance at overripe (20 DAH) pulp and skin of conventionally ripened fruits, respectively. Thus, pre-climacteric (5DAH) stage of ethylene treated fruits remained the highest transcript accumulation stage among the entire data set. Past this stage, a decline in the transcripts was observed both in pulp and skin ripening stages. Such a high expression level of *MiOMTS* in the ethylene treated fruits clearly explained exceptionally high mesifuran content in the fruits treated by ethylene (Figure 4.8). The correlation analysis performed suggested strong positive correlation between the mesifuran content and *MiOMTS* transcripts in the pulp (R^2=0.95) and skin (R^2=0.94) of the control fruits. Although, no such significant correlation was observed in the ripening stages of the ethylene treated fruits, it can be noted that ethylene treatment results in strong up-regulation of both, *MiOMTS* transcript levels and mesifuran content (Figure. 4.8).

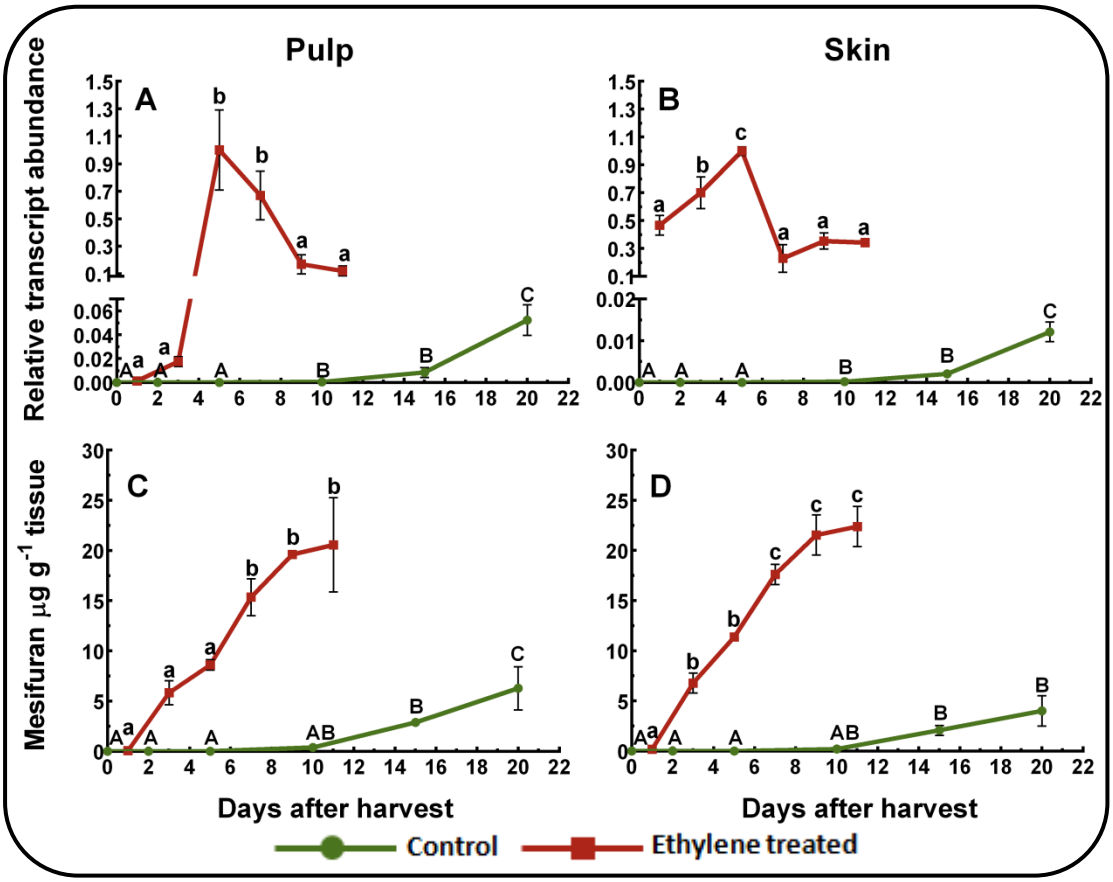


Figure 4.8: The relative transcript abundance of *MiOMTS* transcripts in pulp (A) and skin (B) of control and ethylene treated fruits along with the quantity of mesifuran in pulp (C) and skin (D) of ethylene treated fruits. Vertical bars at each point represent standard error of measurement among the biological replicates and letters at each point indicate the significance of ANOVA ($p \leq 0.05$) analysed by Fisher's LSD test.

4.4. **Discussion**

Comprehensive analysis of volatile composition of Alphonso mango pertaining to various aspects such as during the development and ripening (Pandit et al., 2009b), cultivation locality based variations (Kulkarni et al., 2012a) and effect of ethylene treatment on volatile composition (Chapter 3) have displayed terpene hydrocarbons as quantitatively major shareholders in the volatile profile of fruits of Alphonso mango. However, the similar pattern of terpenes is also detected in the flowers and leaves of Alphonso mango (Pandit et al., 2009b). It is the ripening specific appearance of lactones and furanones which makes the flavor of ripe fruit of Alphonso mango so unique. Furanones which comprise two compounds *viz.* furaneol and mesifuran in Alphonso mango are of special importance mainly due to their sweet, fruity, caramel like flavor characters and low odour detection thresholds (ODTs). Previously, we have isolated and characterized an enone

oxidoreductase from Alphonso mango which catalysed the biosynthesis of furaneol (Kulkarni et al., 2013a). A step forward in the same pathway, the present study clearly demonstrated the synthesis of mesifuran from furaneol by the activity of *MiO*MTS using SAM as a methyl group donor. Thus, mango is the only second plant after strawberry (Wein et al., 2002) from which the furaneol methyltransferase has been isolated and characterized.

4.4.1. Isolation and functional characterization of *MiOMTS*

The amino acid alignment of *MiO*MTS with other plant *O*-MTSs showed the presence of conserved motifs essential for the SAM and substrate binding (Zubieta et al., 2002) (Figure 4.1). The gene structure of the *MiOMTS* displayed presence of two introns which is in congruence with the caffeic acid *O*-methyltransferase from *P. tomentosa* which also showed presence of 104 bp and 81 bp introns (Du et al., 2013). However, *O*-methyltransferase gene from *Vitis vinifera* reported presence of single intron of 77 bp (Dunlevy et al., 2010). Nonetheless, introns from both the genes followed "GT-AG" rule. As per the classification based on the length of plant *O*-MTSs, proteins in Pl-OMT I group are of 231-248 amino acids long while that of Pl-OMT II group possess 344-383 amino acids (Joshi and Chiang, 1998). Thus, *MiO*MTS can be classified in to Pl-OMT II group. Although, enzymes from this group are known to utilize variety of substrates, *MiO*MTS utilized only furaneol and protocatechuic aldehyde as substrates, producing mesifuran and vanillin, respectively. We have shown presence of mesifuran in Alphonso mango, however; the presence of vanillin has not yet been reported (Pandit et al., 2009a and 2009b). The most obvious reason for this could be that protocatechuic aldehyde has also not yet been reported in Alphonso fruits. This suggests that out of the four substrates tested, furaneol might be the only *in vivo* substrate of *Mi*OMTS. Thus, all the further experiments were carried out by monitoring furaneol-methyltransferase activity of *Mi*OMTS. The optimum activity parameter for *MiO*MTS depicted pH optimum 7.0 while the enzyme retained its 90% activity at pH 8.0 with no activity detected below pH 6.0 and above pH 8.5. As ripening of Alphonso mango is accompanied by decreased titratable acidity (Tharanathan et al., 2006), the optimum activity of *MiO*MTS at neutral pH *in vitro* justified its activity and thereby synthesis of

mesifuran during the late ripening stages of Alphonso fruits (Figure 4.6). Similar observation was also reported for strawberry *FaOMT* which was also active during pH 6.0 to 10 having activity optima at pH 8.5 (Wein et al., 2002)

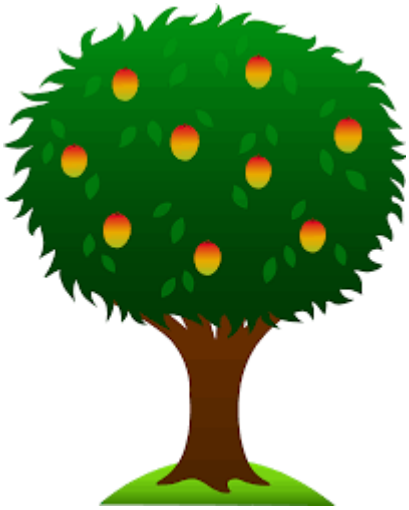
4.4.2. Expression profiling of *MiOMTS* transcripts

In conventionally ripened Alphonso mango, mesifuran was detected at 10 DAH (mid-ripe) stage both in pulp and skin separately with subsequent increase in its content till 20 DAH (over-ripe) stage. However, in ethylene treated fruits, it was detected although with very minor quantities at 1 DAH (mature-raw) stage with significantly enhanced synthesis thereafter till 11 DAH (over-ripe) (Results section in Chapter 3). The expression pattern of *MiOMTS* in the pulp and skin of conventionally ripened Alphonso mango showed prominent correlation with the synthesis of mesifuran (Figure 4.8). Moreover, in a semi-quantitative PCR analysis, *MiOMTS* transcripts depicted fruit specific expression while no transcripts were detected in leaf and flowers of Alphonso (Figure 4.7). These findings were in congruence with the strawberry *FaOMT* which also displayed fruit specific expression and high correlation between its expression and mesifuran synthesis during strawberry ripening (Wein et al., 2002). Singh et al. (2011) have also shown the ripening related expression profile of caffeic acid 3- *O* methyltransferase (*comt*) from Dashehari mango in a semi-quantitative PCR analysis. However, the study did not attempt molecular and functional characterization of the gene. In case of ethylene treated fruits the transcript accumulation was seen even at 1 DAH (mature-raw) with steady but significant increase in its expression till 5 DAH (pre-climacteric). After 5 DAH (pre-climacteric), steady decline in the transcript abundance was evident despite a continuous increase in the mesifuran synthesis. This might be due to the substantial synthesis of *MiOMTS* transcripts and thereby the corresponding protein at preceding stage which continuously catalyzed the production of mesifuran from furaneol *in vivo* during the later ripening stages. Such sensitivity to ethylene was also reported in case of strawberry *FaOMT* which displayed diminished expression levels in achenes and receptacles of transgenic *etr1-1* lines having mutated ethylene receptor (Merchante et al., 2013). Thus, the fruit specific and 10 DAH onwards expression of *MiOMTS* in conventionally ripened

Alphonso mango and its early and enhanced expression in ethylene treated fruits strongly suggested that *MiO*MTS catalyses mesifuran biosynthesis in Alphonso mango and the synthesis of mesifuran is regulated at transcriptional level by ethylene.

Chapter 5

Summary and Future Directions



SUMMARY AND FUTURE DIRECTIONS

Alphonso is one of the highly important mango cultivar due to its generous success at the national and international trading market. It generates sizeable amount of revenue and aids in the agro-rural development in India. However, the industry also witnesses considerable postharvest losses during handling, packaging and transport. To overcome these, various postharvest treatments have been employed by farmers as well as traders. Amongst these, pre-climacteric ethylene treatment has gained popularity recently due to the obvious reasons such as; it does not have any harmful effects and reduces ripening period without affecting the cosmetic features of the fruits.

To analyze the effect of such pre-climacteric ethylene treatment on the softening related cell wall hydrolysis process, ethylene treated and control fruits were studied for the changes in the activity profile and transcript abundance of three glycosyl hydrolases *viz*. β-D-galactosidase, α-D-mannosidase and β-D-glucosidase. Mature green fruits collected from mango orchards in Dapoli were given ethylene treatment. Another set of untreated fruits was considered as control. The assays performed for the activity profile of all the three enzymes during ripening of ethylene treated and control fruits displayed early induction of the activities of all the three enzymes upon ethylene treatment. The activity chart of β-D-galactosidase showed the highest share to the glycosidase activity in both, control and ethylene treated fruits, while the lowest share has been displayed by β-D-glucosidase activity. The findings suggested significant role of former and minimal involvement of later in the cell wall hydrolysis process of Alphonso mango. This was supported by the histochemical assay of β-D-galactosidase activity in ethylene treated and control ripe fruits using X-gal as a chromogenic substrate. To get insight in the regulation of their activities at molecular level, the transcript abundance of all three glycosidases was studied which showed early and enhanced transcript accumulation of β-D-galactosidase and three transcript variants of α-D-mannosidase in ethylene treated fruits. Moreover, β-D-galactosidase transcripts showed significantly increased accumulation in ethylene treated fruits compared to others. However, β-D-glucosidase transcripts were not detected throughout the ripening stages of pulp of control and ethylene treated

fruits pointing towards the probable involvement of other transcript variant/variants for the effective glucosidase activity during ripening. Analysis of reducing and non-reducing sugar content upon ethylene treatment revealed substantial alteration in the pool of reducing sugars than non-reducing pointing towards variation in the sugar metabolism of Alphonso mango upon exogenous ethylene treatment. The study exemplified the alteration of cell wall hydrolysis process and sugar metabolism upon pre-climacteric ethylene treatment reducing the shelf-life of Alphonso mango.

Effect of pre-climacteric ethylene treatment on the volatile accumulation of Alphonso mango during ripening was studied wherein the volatile content of skin and pulp were separately analyzed by gas-chromatography. The analysis showed accelerated ripening in terms of early appearance of ripening specific compounds *viz*. lactones and furanone. However, quantitatively lactones remain unaffected in ethylene treated fruits. Alphonso pulp was found to be lactone rich while skin has high terpene content. This was clearly evident by the separate clustering of pulp and skin ripening stages across PC1 in the Principal Component Analysis score plot. The volatile profile of both pulp and skin showed significantly enhanced synthesis of mesifuran in ethylene treated fruits contributing high sweet fruity caramel like character to the treated fruits as compared to control thus altering the flavor blend of Alphonso mango fruits. The study demonstrated the role of ethylene as a vital modulator of the biosynthesis of ripening specific volatile compounds in Alphonso mango. It has also suggested the need for the standardization of ethylene doses with suitable ripening parameters for the better trade of Alphonso mango.

The biosynthesis of important flavor volatile, mesifuran was studied by isolation and characterization of *O*-methyltransferase gene (*MiOMTS*) and the encoded product. *O*-methyltransferase enzyme uses furaneol as a substrate and S-adenosyl-L-methionine (SAM) as methyl group donor producing mesifuran which is an important flavoring compound of ripe Alphonso fruits. After obtaining the complete open reading frame of *MiOMTS*, it was cloned in pGEX-4T expression vector and heterologously expressed in *E. coli* cells to obtain recombinant protein. The recombinant protein was assayed for its activity using

furaneol, protocatechuic aldehyde, caffeic acid and catechol as substrates. Of these, *MiOMTS* didn't use caffeic acid and catechol but successfully converted furaneol to mesifuran and protocatechuic aldehyde to vanillin in an *in vitro* assay reaction. The optimum activity parameter for *MiO*MTS was found to be pH 7.0 and temperature was at 25°C. To evaluate the transcriptional regulation of mesifuran synthesis the relative transcript abundance of *MiOMTS* throughout the ripening stages of skin and pulp of control and ethylene treated fruits was studied. Early and significantly enhanced accumulation of its transcripts was evident in ethylene treated fruits while the high correlation of mesifuran synthesis and its transcript abundance was displayed by control fruits. The observation strongly suggested the regulation of mesifuran synthesis at transcriptional level by ethylene trigger. The study was only second after strawberry demonstrating the isolation and characterization of furaneol *O*-methyltransferase from Alphonso mango and carries immense importance for the flavor production industries.

Future Directions

Post harvest ethylene treatment of Alphonso mango has popularly being used by the farmers and traders for early ripening. The present study has evaluated the effect of this treatment on the crucial attributes of Alphonso mango at biochemical and molecular level. The work has generated important findings and laid the base for further research which includes;

- Significantly high activity and transcript level of β-D-galactosidase in ethylene treated fruits also suggested regulation of its activity at transcriptional level by ethylene trigger. Molecular and biochemical characterization of mango *MiOMTS* also revealed regulation of mesifuran synthesis at transcriptional level by ethylene. This can be further analysed by mining the promoter region of these genes for ethylene response elements (EREs) such as '*GCC* box' which are well known mandatory elements for regulation of transcription by ethylene signal.

- α-D-Mannosidase which has largely been implied in *N*-glycan processing, showed ripening related activity and transcript profile. This hinted *N*-glycan processing as an important event during the ripening of Alphonso

mango. Thus, analysis of the *N*-glycan content and its processing during the ripening of Alphonso mango will aid to understand its probable role during the ripening process.

- Alphonso pulp was found to be lactone rich tissue and ethylene treatment resulted in their early synthesis during the ripening. Nonetheless, lactones remained quantitatively unaltered upon ethylene treatment as compared to the control fruits. Thus, Alphonso fruit can be considered as an ideal system to study the yet unidentified intermediates of the lactone biosynthesis pathway and their probable regulation by ethylene signal.
- Studying *in planta* functions of the *MiOMTS* gene by transformation in model plant systems such as tomato or *Arabidopsis*. As mesifuran carries immense importance from flavor point of view, *MiO*MTS has potential biotechnological application in the flavor production industries which can be delved into.

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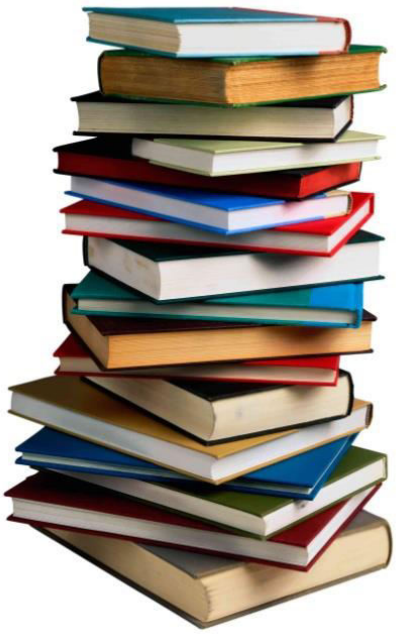

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APPENDIX 1

Nucleotide sequences of *Mangifera indica* genes

> KM100127 *Mangifera indica* alpha-D-mannosidase isoform1, mRNA, partial cds

TATGCATGATGAGGCAACCCCACATTACATTGACTTAATTGATCAGACTACTCTGGGGCATCAATTTAT
CAAAGATGAGTTTGGGAAGCTTCCAAGAGTTGGCTGGCAGATTGACCCTTTTGGACATTCTGCTGTTCA
AGCTTACTTGCTTGGTGCAGAGTTAGGATTTGACTCTCTCTTTTTTGCCCGAATTGATTACCAAGACAG
AGCTAAGCGGTTAAAGGAGAAGACTCTTGAGGTTGTATGGAGGGGCTCTAAATCACTTGGTTCATCTTC
ACAAATATTTACTGGTATATTTCCTAGGCATTATGACCCTCCTGATGGTTTCACATTCGAAATTAATGA
TGTATCCCCTCCTATTCAGGATGATATTCTTCTATTTGACTATAATGTTCAAGAACGAGTCAATGACTT
TGTAGCTGCTGCTTTAGCCCAGGCCAACGTGACCAGGACAAATCATGTTATGTGGCTGATGGGGACAGA
TTTTCGCTATCAATATGCAAATTCATGGTTCAGACAGATGGACAAATTCATTCATTATGTTAATAAGGA
TGGGCGTGTAAATGCTCTATATTCAACTCCATCCATCTACACTGATGCAAAATATGCTGCAAATGAAGA
ATGGCCGATCAAAACTGAAGATTTCTTTCCGTATGCAGATCGTGCGAATGCATATTGGACAGGTTACTT
CACTAGCAGGCCATCCTTCAAAGGCTATGTGAGAATGCTGAGTGGTTACTATGTTGCGGCAAGGCAATT
AGAATTCTTTAAAGGCAGGAGTGGTTCAGGGTCAAACACCGATGCATTGGCGGATGCTTTGGCAATTGC
TCAACATCATGATGCAGTTAGTGGTACAGAAAGGCAGCATGTTGCTGCTGATTATGCTTTACGACTCTC
AATAGGCTATATTGAGGCTGAGCAATTGGTTGCCTCTTCACTTGCTTTCTTGGCAGAGTCAAGATCAAG
TACTGGACATGGGGATCCAGTCACAAACTTTCAGCAGTGCCCTCTTCTTAATATAAGTTACTGTCCTCC
AACTGAAGCGGCCTTCACTGATGGCAGAAGCTTGGTTGTTGTCATCTACAATCCTCTAGGATGGAAGAG
AGAGGAAGTAGTACGAATTCCTGTGGTCTCCACTCAAAAAGTTACTGTTAAGGATGCTGGTGGGAGAAC
AATTGACTCTCAGCTCCTACCTCTTTCAAATGTGACGTTGGGCATAAGAAATCTCTATGTTAAAGCATA
TCTGGGTAAAAGCCCCAGTGAAACGGTTAAATATTGGCTTGCATTTTCAGCATCTGTACCACCTCTTGG
TTTCAGCACTTATGTTGTCTCAATTGCAAAACAGACAGATCGTGGTTCAACCATTTCAACAGTTTACTC
ACCAAAAGGAAGTATGAGTAATAACATAGAAATTGGGCAAGGAAATTTAAAGCTACTTTATTCTGGAGG
GAAAGTCACTCATTATGTTAACAACAGAAATCTGGTGACAGAAACTGTTGAACAGTCATACAGTTATTA
TTCTGGATATAGTGGAACTGATAAAGATCCTCAGGCATCCGGTGCATATGTTTTCCGTCCAAATGGTTC
GTCTCCAATAAAATGGGAAAACCAGGTTCAGTTAACTGTTGTGCGGGGTCCTTTGTTGGATGAACTGCA
TCAAAAGCTTAGTCCTTGGATATCACAGATTACCAGGGTCTACAGAGAAAAGGAGCATGCTGAAGTTGA
GTTCACAGTTGGGCCTATACCAGTAGATGATGGGATTGGGAAGGAAGTCATAACTCAAATAACCGCAAG
CATGAAGACCAACAAAACATTCTACACAGATTCTAATGGGCGGGACTTCATTAAAAGGATTCGAGATTT
CAGGACAGACTGGGACCTGGAAGTGACCGAACCAATTGCTGGAAATTTTTACCCAGTCAATCTTGGAAT
TTACATGCAAGATGACACCACAGAATTATCTGTGTTAGTGGATCGCTCGGTGGGAGGATCCAGTTTAGT
GGATGGTCAAATTGAACTGATGCTCCACAGGAGGTTGATTTATGATGACAAAAGAGGTGTTGGTGAGGT
ATTAA

> KM100128 *Mangifera indica* alpha-D-mannosidase isoform 2*,* mRNA, partial cds

TATGAATGACGAGGCTAATTCACATTATTTTGCTATCATCGAGCAGATTGCAGAAGGCAACATGTGGTT
GAATGACACCGTTGGGTTTATTCCTAAGAATTCTTGGGCAATTGATCCATTTGGTTATTCGGCTACAAT
GGCATATCTCCTCCGTCGTATGGGTTTCGAAAACATGCTTATTCAACGAACCCACTATGAGCTGAAGAA
AGAACTTGCTTTGCACAAAAATTTGGAATATGTATGGCGTCAAACTTGGGATGCTGATGAAACTACTGA
CATTTTTGTGCACATGATGCCCTTTTATTCATATGATGTTCCGCATACTTGTGGACCAGAGCCTGCTAT
CTGTTGTCAGTTTGATTTTGCTCGAATGCTTGGATTTTCTTATGAACGGTGTCCATGGGGACAACATCC
AGTTCAGATCAAGGAGGAAAATGTGCAGGAGAGGGCACTAAAACTTTTGGATCAGTATAGGAAGAAATC
AACGTTGTACCGGACAAATACACTTCTCATCCCTCTTGGAGATGATTTCCGTTACGTCACCAATGATGA
AGCTGAAGCACAGTTTAAGAATTACCAACTGTTGTTTGATTATATCAACTCAAACCCCAGTTTAAACGC
AGAAGCAAAATTTGGTACTTTGGAGGACTACTTTCAAACACTTCGTGAGGAGGCTGACAGAATAAATTA

TTCTAGTCCTGGTGAGGGTGGCTCTAGTCAGATTGTAGGTTTTCCTTCTCTGTCGGGTGACTTCTTTAC
TTATGCTGATAGACAACAGGACTACTGGAGTGGCTATTATGTTTCAAGACCTTTCTTCAAGGCTGTAGA
TCGGGTGCTAGAGCAAACTCTTCGTGCAACTGAAATAATGTTCGCTTTGTTGCTTGGTTATTGTCAAAA
GCCACACTGTGAGAGGTACCCAACAGGTTTCGCTTACAAGCTGACAGCAGCAAGGAGGAATTTAGCTCT
TTTTCAGCATCATGATGGGGTAACCGGTACTGCAAAAGATCATGTTGTTCAGGACTATGGAACTCGGAT
GCATACTTCTTTGCAGGATTTGCAGATTTTCATGTCTAAAGCTATTGAAGTACTGATTGGAATGCGTGA
AAAATTTGATCAGAATGTATCCCAGTTCGAGCCAATACAAGTGAGAAATAGATATGATGCTCAGCCAAT
GTACAGAGCAATAAGTGCTCATGAAGATACCTCACAATCCGTGGTCTTTTTTAATCCTTCGGAGCAAAA
AAGGGAAGAGGTTGTAATGGTTATTGTTAACAGGCCAGATGTTACTGTTTTGGACTCAAATTGGACCTG
TGTACAAAGCCAGGTGTCTCCTGAATTGCAGCATGATAGGAACAAAATCTTTACTGGGAGACACCGTCT
TCACTGGAAAGCTTCTATTCCTGCCATGGGGTTGCAAATATATTACATTGCTAAAGATTTTGTTGGGTG
TGAGAAAGCAAAACCATCAAAACTGAAGTACTCATCAAAGTCCAATTCATTCTCTTGCCCCACTCGTTA
TGCTTGCTCAAAATTAGAAGGTGATGTGGCTGAAATCCAGAATAGACATCACATTCTCACTTTTGATGT
CCAGCATGGGCTATTGCAGAAAGTAAGCCATATAAATGTCTCCCAAAATCTTGTGCACGAAGAAATTGG
TATGTATTCTACTCAGGAAAGTGGGGCCTACCTGTTCAAACCCATTGGTGATGTTCAGCCTATTATCAA
AACTGGTGGCTTGATCATTATTTCTGAAGGCCCTTTGATGCAGGAAGTATACTCTTATCCAAGGACTGA
ATGGGAGAAAAGCCCCATATCCCATAGTACTCGCATTTATAATGGAGATAATACGATACAGGAATTTGT
CATTGAGAAAGAATATCATGTTGCGCTTCTTGGTTATGATTTCAATGACAAGGAGCTGATAGTAAGGTA
CAAAACTGATATTGATAATAGCAGGACATTCTATTCTGACTTAAATGGCTTTCAGATGAGCAGGAGAGA
AACCTATGACAAAATCCCATTGCAGGGAAATTACTACCCTATGCCTTCACTTGCATTCATGCAAGGTTC
CAGTGGTCAGCGATTTTCTGTTCATACTCGGCAGTCACTGGGTGTGGCAAGCCCCAAGGATGGATGGTT
GGAGATTATGCTTGACCGACGTTTGGTAAGAGATGATGGTCGTGGTCTTGGGCAAGGAGTGT

> KM100129 *Mangifera indica alpha-D-mannosidase isoform 3*, mRNA, partial cds

CGGCAGATGGACAAGTTCATTCATTATGTGAACATGGATGGACGAGTCAATGCTCTGTACTCTACCCCA
TCCATATATACTGATGCAAAATATGCTGCAAATGAATTCTGGCCATTGAAGACTGATGACTTCTTTCCT
TATGCGGACCGTGCAAATGCTTACTGGACAGGATATTTCTCAAGTAGACCAGCCTTAAAACGCTATGTT
AGAATGATGAGTGGTTACTATTTGGCGGCAAGGCAACTAGAGTTCTTTAAAGGTAGGAGTAATGCAGGG
CCTAACACAGATTCATTAGCGGATGCTCTGGCTATTGCTCAGCATCATGATGCAGTTACTGGTACTGAG
AAGCAACATGTGGCTGATGATTATGCAAAACGGCTATCAATAGGCCACATGGAGGCTGAAGAGGTAGTT
GCAACATCACTTGCTTGCTTGGCTGACTCGATGTCCTATGATGGATGTAAACGGGCGACTCTGAAATTT
CAACAGTGTCCACTACTGAATATTAGTTACTGTCCCGCATCAGAAATAGATCTGTCTCATGGGAAAAAC
TTGATTATTGTGTTCTACAATTCCCTCGGATGGAAGAGAGATGATGTTATTCGAATTCCGGTTGACAAT
GAAGATATTAGTGTTTTTGATTCCAAGGGAAAAGTAATCGAATCGCAGCTTCTTCCTCTTACTGATTCT
TACATTGATCTAAGGAACTACCATGTTAGGGCATATTTGGGTAGAACTCCAAGCCTGACACCTAAATAC
TTGCTTGCGTTTGCGGTCTCTGTACCTCCTCTTGGTTTTGGCACTTATACCATCAGAAGTGTCGAAACA
ACAGGGGCTAGTTCAACAAAATCATCTGTACACACATTTGAAAGTAGTGAAAAATCTTCTGTTGAAGTT
GGTCCCGGGAATTTAAAGCTTACTTTCTCCTCAGATCAAAGCAAACTCATTAATTATACTAATAGCAAA
AGCTCAGTTCAGGAATTGGTTGAGCAGTCATACAGTTTTTACCCCGGATATAATGGAACTAATGACAAA
GCTCCTCAGAATGCT

> KM100130 *Mangifera indica beta-D-galactosidase,* mRNA, partial cds

GGTCCATCAGCATCCTCCCTGACTGCAAAACTGCAGTTTTTAACACTGCTAGGCTTGGTGCCCAAAGCT
CCCTGAAACAGATGACACCTGTCAGCACATTTTCTTGGCAGTCATACATTGAAGAAAGTGCCTCTTCTA
GTGATGATAAGACTTTTACCACTGATGGGCTGTGGGAACAATTAAATGTGACTAGAGATGCTTCGGACT
ACTTGTGGTATATGACAAATATAAATATAGACTCTAACGAAGGATTTCTGAAGAATGGACAAGATCCTC
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TTTCTTTATTAAGTATATCTGTGGGTCTTCAGAATGTCGGCACACATTTTGAGCAATGGAATACTGGGG
TGCTAGGTCCAGTAACATTGAGGGGTCTCAATGAGGGGACGAGAGACTTGTCTAAACAGCAATGGTCTT
ACAAGATTGGTCTAAAAGGTGAGGATTTAAGCCTTCATACTGTTAGTGGGAGCTCCTCTGTTGAATGGG
TAGAAGGATCATCATTGGCCCAAAAACAACCATTGACATGGTACAAGACTACTTTCAATGCACCGGCG

>KM100131 *Mangifera indica beta-D-glucosidase*, mRNA, partial cds

TTGCGCCTGGCAGGTGTTCCAAATGGGTAAATGCAGCATGTCAGGCGGGAAACTCCGCCACCGAACCTT
ACATTGTTGGCCACCATCTGCTACTTTCTCATGCCGCAGCCGTCAAACTATACAAGCAAAAATACCAAG
TTATTCAAAAGGGCAAGATTGGTATTACACTGGTGACACCCTGGATAGTACCTTACTCCAAAAAGAAAC
CTCATATTGAGGCAGCATATAGAGCCCTTGACTTCATGTTTGGATGGTATATGGACCCAATTATCTATG
GCGATTATCCATTCAGTATGAGAAATATTGTGAGACAAAGACTCCCAAAGTTCACCAAGAAGCAATCAG
ATATGGTGAAAGGATCTTTTGATTTTATCGGTATAAATTATTACACTGCAGATTATGCAGCTAACATTC
CTGTTGCCAATACCGTCAACATTAGCTACTCAACAGATTCTCTTGCTACTTTGACCACCAGTCGCAATG
GCATTCTAATCGGTTCACAGGCGGCTACAAGTTGGCTTCATGTCTATCCTAGGGGTTTACGAGATCTTC
TATTATACGTGAAAGAAAAATACAACAATCCTCTTGTTTACATAACTGAAAATGGGATTGATGAATTCA
ATAATGCAACCTTATCGCTAGAGCAGGCATTGAAGGATCCCATGAGGATAGATTATTACCACCGCCATC
TTTTGTTCCTTGAAAGAGCCATTAAGGAGGGTGTAAATGTGAAGGGATACTTTGTGTGGTC

> KP993176 *Mangifera indica O-methyltransferase*, mRNA, complete cds

ACCGGGGATCAGAGAGATCGGAGAGAGAAAATGGGATCATTAGAAGTGAAGACATTGCTAGAAGGCCAA
GCAGAAGTATGGCAATACATGTTTGGCTTTGCAGACTCCATGGTTTTGAAGTCTGCTGTGGAGCTTCGC
TTAGCTGACATTATACATTCCTATGGTGGCCCAATGTCGTTGTCCCAAATCGCCTCAAAAATTGACTCC
GCTTCACCAAACATTCCCTACCTTGGTCGCATCATGAGAATGCTTGCCCGTAAAGGAGTTTTTGGGGCA
CATCGTCCATCGGAAGGAGGCGACACTACCTACGGGTTGACTCACATATCAACATGGCTTCGACACGAC
TCGGAGCTCAGTCTTGCTCCAATGATACTAATGGAAAACAATCTATGGCAATTAGCACCATGGCATTAT
CTTAGCCAATGTGTCAAACAAGGTGGAATTGCTTTCAAGAAGGCGCACGGGTGTGAGATGTGGGACTTC
GCATCACAAAACCCCGAATTCAACAAGCTGTTCAATGATGCCCTTGCATGCACGACCAAGATTGTGATG
AGGGCATTTTTATCACAATACAAAGAGGGGTTTAACAATGTCAGATCACTGGTTGATGTGGGTGGTGGC
ACCGGAGGCGAGCTGGCCGAGATTTTGAAAACCTATCCACACATGAAAGGCATAAATTTTGATCTGCCA
CATGTTGTAGCTACTGCTCCTGAATATGATGGGGTCTCAAACGTTGGAGGTAACATGTTTGATGCCATT
CCTAATGCAGACGTCATTTTCATGAAGTGGATACTGCATGATTGGAGTGATGAAGCTTGTGTGAAGATT
CTGAAAAATTGTAGGAAAGCAATACCGGAGAAAACTGGGAGGGTTGTTCTAGTTGAAATCGTTCTGCAG
GAAAATGGTGACAACATGTTTGGGAACATGGACGTAGTGTTTGATCTACTGATGTTTGCACACACTACA
GGGGGGAAAGGAAAGGACTGAACCAGAATGGAAAAAATTATTGGAGGAAGGAGGCTTTCCTCGCTACAAT
ATCATCAACATCCCAGCTTTACCATCCATTATTGAGGCCTATCCACTGTAACGTCATTAATATATTGTT
GTTGCCTGTCATGTCATCTGCTTGCGAGCAGGGCCGTGGAAGTTCTCAAATGAATTAATAAATACATTT
CAAAAGAT

> KP993177 *Mangifera indica O-methyltransferase*, gene sequence

ATGGGATCATTAGAAGTGAAGACATTGCTAGAAGGCCAAGCAGAAGTATGGCAATACATGTTTGGCTTT
GCAGACTCCATGGTTTTGAAGTCTGCTGTGGAGCTTCGCTTAGCTGACATTATACATTCCTATGGTGGC
CCAATTTCTTTGCCCCAAATCGCCTCAAGTATTCACTCTCTCTATATATCCTCATCTCTTGTTCTTTTA
GTAGATTCATCACCTAAACTAAATTAAGGATCATTCATGTGTGCCTATTTATCAATTTCTTATTCAGAA
TAGTGAAACATTCCGTAGCAGTTTTGATCTTTTCCTTTGTTTTTTGTGACAGAAATTGACTCCGCTTCA
CCAAACATTCCCTACCTTGGTCGCATCATGAGAATGCTTGCCCGTAAAGGAGTTTTTGGGGCACATCGT
CCATCGGAAGGAGGCGACACTACCTACGGGTTGACTCACATATCAACATGGCTTCGACACGACTCGGAG
CTCAGTCTTGCTCCAATGATACTAATGGAAAACAATCTATGGCAATTAGCACCATGGCATTATCTTAGC
CAATGTGTCAAACAAGGTGGAATTGCTTTCAAGAAGGCGCACGGGTGTGAGATGTGGGACTTCGCATCA
CAAAACCCCGAATTCAACAAGCTGTTCAATGATGCCCTTGCATGCACGACCAAGATTGTGATGAGGGCA
TTTTTATCACAATACAAAGAGGGGTTTAACAATGTCAGATCACTGGTTGATGTGGGTGGTGGCACCGGA
GGCGAGCTGGCCGAGATTTTGAAAACCTATCCACACATGAAAGGCATAAATTTTGATCTGCCACATGTT
GTAGCTACTGCTCCTGAATATGATGGGGTCTCAAACGTTGGAGGTAACATGTTTGATGCCATTCCTAAT
GCAGACGTCATTTTCATGAAGGTAAAAGTTTAACTGTTCAGATTTCTTTTTATAATTGATTATCAATTG
CCTTACATTTGAAACAATAACAGTATGTGCATAATTTATTAATGCAGTGGATACTGCATGATTGGAGTG

ATGAAGCTTGTGTGAAGATTCTGAAAAATTGTAGGAAAGCAATACCGGAGAAAACTGGGAGGGTTGTTC
TAGTTGAAATCGTTCTGCAGGAAAATGGTGACAACATGTTTGGGAACATGGACGTAGTGTTTGATCTAC
TGATGTTTGCACACACTACAGGGGGAAAGGAAAGGACTGAACCAGAATGGAAAAAATTATTGGAGGAAG
GAGGCTTTCCTCGCTACAATATCATCAACATCCCAGCTTTACCATCCATTATTGAGGCCTATCCACTGT
AA

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Educational Background

- **Master of Science:** Biotechnology, 2004, First Class, Sant Gadge Baba Amaravati University,
- **Bachelor of Science:** Biochemistry, Microbiology, Chemistry, 2002, First Class, R.L.T. College of Science, Akola

Other Qualifications

- Completed the 'Patent Certificate Course for Scientists and Engineers' organized at IPFACE Venture Centre, Pune, India form 7-14 February,
- Test of English as a Foreign Language (TOEFL) 2007; Score: 90/120
- Senior Research Fellowship (2009-2012) from the Council of Scientific and Industrial Research, India

Professional work experience

- Worked at National Chemical Laboratory, Pune as a Project Assistant II from Jan 2005- May 2006 on project "Biomolecule prospecting of *Symplocos* and *Gaultheria* species".
- Also worked on "Revealing the secrets of mango: Studies of genetic mechanisms involved in Alphonso flavor biogenesis" as a project assistant II from July 2006- March 2009. The project involved GC-MS-FID based analysis of mango fruit volatiles. Isolation, cloning and characterization of genes involved in flavor biogenesis. Development of microsatellite markers using genomic DNA library approach for Alphonso mango. Also, developing the ISSR based SCAR markers for mango cultivars.

Projects Undertaken

- As a part of M. Sc. Degree: Initiation of root for establishment of root organ culture of four different medicinally important plants, 2003, Under the guidance of Dr. Wadegaonkar at Department of Biotechnology, Sant Gadge Baba Amaravati University, Amaravati, India.
- Summer training research project on "Effect of methyl jasmonic acid on alkaloid accumulation in *Nothapodytes foetida* (Weigh) Sleumer" at Plant Tissue Culture Division, National Chemical Laboratory, Pune, India from May-Aug 2003.

Publications

- **Chidley HG**, Kulkarni RS, Pujari KH, Giri AP, Gupta VS. (2013) Spatial and temporal changes in the volatile profile of Alphonso mango upon exogenous ethylene treatment, *Food Chemistry*, 136, 585–594.
- Kulkarni RS, Pandit SS, **Chidley HG**, Nagel R, Schmidt A, Gershenzon J, Giri AP, Gupta VS. (2013) Characterization of three novel isoprenyl diphosphate synthases from the terpenoid rich mango fruit*Plant Physiology and Biochemistry,*71, 21-131.
- Kulkarni RS, **Chidley HG**, Deshpande A, Schmidt A, Pujari KH, Giri AP and Gershenzon J and Gupta VS. (2013) An oxidoreductase from 'Alphonso' mango catalyzing biosynthesis of furaneol and reduction of reactive carbonyls, *SpringerPlus*, 2, 494-501
- Kulkarni RS, **Chidley HG**, Pujari KH, Giri AP and Gupta VS. (2012), Geographic variation in the flavor volatiles of Alphonso mango, *Food Chemistry*, 130 58–66
- Pandit SS, **Chidley HG**, Kulkarni RS, Pujari KH, Giri AP, Gupta VS. (2009) Cultivar relationships in mango based on fruit volatile profiles, *Food Chemistry*, 144, 363-372.
- Pandit SS, Kulkarni RS, **Chidley HG**, Giri AP, Köllner TG, Degenhardt J, Gershenzon J, Gupta VS. (2009) Changes in volatile composition during fruit development and ripening of 'Alphonso' mango, *Journal of Science of Food and Agriculture*, 89, 2071-2081

Under preparation

- **Chidley HG**, Deshpande AB, Oak PS, Pujari KH, Giri AP, Gupta VS. Pre-climacteric ethylene treatment modulates the ripening related glycosidase activities, their transcript abundance and sugar content in Alphonso mango fruit.
- **Chidley HG**, Deshpande AB, Oak PS, Pujari KH, Giri AP, Gupta VS. Molecular cloning and expression of *O*-methyltransferase from Alphonso mango catalysing the synthesis of mesifuran, a key aroma volatile in Alphonso mango fruit

Book chapters

- Kulkarni RS, **Chidley HG**, Pujari KH, Giri AP, Gupta VS. (2012) Flavor of mango: A pleasant but complex blend of compounds, In ***Mango Vol. 1: Production and Processing Technology*** (Eds. Sudha G Valavi, K Rajmohan, JN Govil, KV Peter and George Thottappilly) Studium Press LLC.
- Gupta VS, Giri AP, Pandit SS, Kulkarni RS, **Chidley HG**, Deshpande AB, Dar MS, Oak PS. (2013). Alphonso mango flavor: Blend and Biosynthesis, In: ***Biotechnology Beyond Borders*** (Eds M. V. Deshpande and J. Ruiz-Herrera). CSIR-NCL, Pune, India, pp 103-121

Conferences/Workshops

- **Chidley HG,** Kulkarni RS, Deshpande AB, Pujari KH, Giri AP, Gupta VS. Effect of Pre-climacteric Ethylene Treatment on the Volatile Profile of Alphonso Mango. (Poster) Indo-Mexico workshop on Biotechnology: Beyond Borders, 7-9 October, 2013, CSIR-NCL, Pune, India
- **Chidley HG,** Kulkarni RS, Deshpande AB, Pujari KH, Giri AP, Gupta VS. Effect of Pre-climacteric Ethylene Treatment on the Volatile Profile of Alphonso Mango. (Poster) National science day poster presentation session on 28th Feb 2012 CSIR-NCL, Pune, India.
- Kulkarni RS, **Chidley HG**, Pujari K, Schmidt A, Gershenzon J, Giri A and Gupta V. Biochemistry and Molecular Biology of Alphonso Mango Flavor. (Poster) World Congress on Biotechnology, 21-23 March 2011, Hyderabad, India
- Pandit SS, Kulkarni RS, **Chidley HG**, Giri AP, Pujari KH, Patil BP, Jambhale ND, and Gupta VS. Revealing the Secrets of Mango Flavor. National science day poster presentation session on 28th Feb 2008 CSIR-NCL, Pune, India.
- Pandit SS, Mitra SS, Pujari KH, Giri AP, Patil BP, **Chidley HG**, Jambhale ND and Gupta VS. Diversity in Indian Mango Assessed Using ISSR and Morphological Markers. International Conference on "Plant Genomics and Biotechnology: Challenges & Apportunities" organized by Indira Gandhi Agricultural University, Raipur, Chhattisgarh, India during October 26th-28th 2005.

Personal Details

- **Date of Birth :** 17th Apr 1982
- **Marital Status:** Married
- **Nationality:** Indian