Department of Environment and Agriculture

Hormonal Regulation of Mango Fruit Ripening

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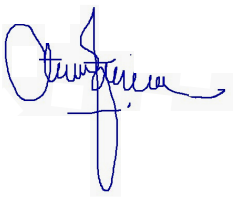
Declaration

To the best of my knowledge and belief this thesis contains no material previously published by any other person except where due the acknowledgement has been made. This thesis contains no material which has been accepted for the award of any other degree or diploma in any university.

Signature: 

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Dedication

To:

My father,
Hj. Sakimin Sakidin;

My mother,
Hajjah Poriah Hj. Mokti;

My father-in-law,
Mr. Iberahim Yusuf;

My mother-in-law,
Mrs. Tuan Minah Tuan Kadir;

My husband,
Dr. Ismail Iberahim;

My son,
Muhammad Zahin Iqbal Ismail;

&

All my family members

For

"A constant source of inspiration and doa'a during
the entire period of my PhD study
and my life..."

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Abstract

Mango fruit ripen quickly. It is highly perishable. Short shelf life of mango fruit limits its transportation to distant domestic and international markets. The objective of my research was to elucidate the role of changes in endogenous levels of brassinosteroids (BRs), ethylene, abscisic acid (ABA) and/or indole-3-acetic acid (IAA) in modulating the ripening processes of 'Kensington Pride' mango fruit. The endogenous levels of these regulators were regulated using inhibitors of their biosynthesis and/or action to unfold their mechanism in delaying/hastening mango fruit ripening, extending storage life and improving fruit quality as well as to underpin the mode of action of ABA and NO in modulating ethylene biosynthesis and activities of fruit softening enzymes in the pulp during ripening and/or alleviating chilling injury (CI) during cool storage.

Higher endogenous level of ABA at the climacteric-rise stage triggered the climacteric peak of ethylene production coupled with a significant quadratic relationship between both of them; suggest that ABA play a key role in modulating mango fruit ripening. The exogenous application of ABA (1.0 - 2.0 mM) promoted skin colour development and fruit softening during ripening, and the trend was reversed with its inhibitor of biosynthesis - nordihydroguaiaretic acid (0.1 - 0.2 mM NDGA). The endogenous level of IAA was higher at the initial stage of ripening and decline over ripening period. The exogenous application of 45 - 60 ng g^{-1} FW Epi-BL increased the climacteric peak of ethylene and respiration, promoted skin colour, but the changes in the endogenous level of BRs (castasterone and brassinolide) are unlikely to modulate mango fruit ripening as it is present in a trace amounts in mango pulp tissues throughout the ripening period.

Exogenous postharvest application of ABA (1.0 mM) increased the climacteric peak of ethylene production through promoting the activities of 1-aminocyclopropane-1-carboxylic acid (ACC) synthase (ACS), ACC oxidase (ACO) enzymes, and ACC content, decreased the fruit firmness with increased *exo*-polygalacturonase (*exo*-PG), *endo*-PG and e*ndo*-1,4-β-D-glucanase (EGase) activities, decreased pectinesterase (PE) activity in the pulp, higher total sugars and

sucrose, advanced degradation of total organic acids, citric and fumaric acid. The application of 0.2 mM NDGA showed reverse trends for these ripening indicator parameters.

NO fumigation (20 μL L^{-1} or 40 μL L^{-1}) was more effective in delaying fruit ripening when applied at the pre-climacteric (PC) stage, than at the climacteric-rise (CR) stage. NO (20 μL L^{-1}) fumigation delayed and suppressed the endogenous ethylene production, activities of ACS and ACO enzymes, and ACC content, rate of respiration, higher pulp rheological properties (firmness, springiness, cohesiveness, chewiness, adhesiveness, and stiffness) with lower activities of *exo*-, *endo*-PG, EGase, but maintained higher PE activity in pulp tissues during ripening at 21°C and cool storage (13°C). NO treatments (20 and 40 μL L^{-1}) significantly alleviated CI index during ripening at ambient temperature following 2- or 4-week of cold-stored (5°C) period. All NO fumigation treatments significantly suppressed ethylene production and respiration rates irrespective of cold storage period. NO–fumigated with above 5 μL L^{-1} significantly delayed fruit softening up to 2 days and retarded colour development, reduced total sugar and fructose concentrations, increased tartaric and shikimic acid at fully ripe stage during ripening period irrespective of cold-stored fruit.

In conclusion, the higher levels of endogenous IAA in fruit pulp during the PC stage and the accumulation of ABA prior to the climacteric stage might switch on ethylene production that triggers fruit ripening. There is a significant quadratic relationship between endogenous level of ABA in the pulp and ethylene production during fruit ripening period. Exogenous Epi-BL promoted fruit ripening, whilst, the changes in endogenous levels of BRs are unlikely to modulate mango fruit ripening. Moreover, the exogenous application of ABA (1.0 mM) promoted the activities of ethylene biosynthesis enzymes (ACS and ACO) and ACC content and ethylene biosynthesis as well as *endo*-PG activity in the pulp, whilst, the NDGA-treated fruit showed the reverse trends. The application of NO fumigation (20 μL L^{-1}) at PC stage can be effectively used to delay the fruit ripening up to 2 days at ambient temperature (21°C) and cool-storage (13°C) through suppression the activity of ethylene biosynthesis and softening enzymes and alleviate CI following 2- and 4-week cold storage (5°C) without any adverse effects on fruit quality.

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List of symbols and abbreviations

×	Multiply/interaction between
+	Plus
-	Minus
>	Greater than
<	Less than
≥	Greater than or equal to
≤	Less than or equal to
±	Plus minus
/	Divide
=	Equal to
~	Approximately
´	Minute(s)
°	Degree
°C	Degree celcious
%	Per cent
α	Alpha
β	Beta
λ	Lambda
β-Gal	*β*-galactosidase
Δ	Changes of
µg	Microgram(s)
µL	Microlitre(s)
µM	Micromolar(s)
µm	Micrometre(s)
µmol	Micromole(s)
1-MCP	1-Methylcyclopropene
2,4-D	2,4-Dichlorophenoxyacetic acid
3-Meox	3-methylene oxindole
A$	Australian dollar
ABA	Abscisic acid

ABS	Australian Bureau of Statistics
ACC	1-aminocyclopropane-1-carboxylic acid
ACO	ACC oxidase
ACS	ACC synthase
a.i.	Active ingredient
AI	Acid invertase
AMIA	Australian Mango Industry Association
ANOVA	Analysis of variance
AU	Absorbance units
AVG	Aminoethoxyvinylglycine
BL	Brassinolide
BC	Break colour
BRs	Brassinosteroids
BSTFA	*N*,*O*-Bis (trimethylsilyl)trifluoroacetamide
C	Carbon
Ca	Calcium
$CaCl_2$	Calcium chloride
C*	Chroma
CA^1	Controlled atmosphere
CA^2	California
CAS	Castasterone
C_2H_4	Ethylene
CH_3COONa	Sodium acetate
CI	Chilling injury
CIE	Commission International de L' Eclairage
CIS	Citrate synthase
cm	Centimetre(s)
Co.	Company
CO_2	Carbon dioxide / respiration
conc.	Concentration
$CoCl_2$	Cobalt chloride
Corp.	Corporation
CPPU	N-(2-chloro-4-pyridyl)-N′-phenylurea

CR	Climacteric-rise
CS	Climacteric stages
cv.	Cultivar
d	Day(s)
DAFB	Days after full bloom
DAFI	Days after flower induction
DAFS	Days after fruit set
dH_2O	Distilled water
DM	Dry matter
DPH	Day prior to harvest
DPPH	2, 2-diphenyl-1-picryl-hydrazyl
Dr.	Doctor
DTT	Dithiotheritol
e	Exponential
E	East
EC	Enzyme commission
EDTA	Ethylenediamine tetra-acetic acid disodium
EFE	Ethylene-forming enzyme
e.g.	For example
EGase	*Endo*-1,4-β-D-glucanase
Endo-PG	*Endo*-polygalacturonic acid
Epi-BL	Epibrassinolide
Eq.	Equivalent
et al.	et alia
EU	European Union
Exo-PG	*Exo*-polygalacturonic acid
f	Force
FAOSTAT	Food and Agriculture Organisation Statistic
$FeSO_4$	Ferrous sulphate
FID	Flame ionization detector
FR	Full-red
FW	Fresh weight
g	Gram(s)

g	Gravity
G	Green
GC	Gas chromatograph
GA	Gibberellic acid
h	Hour(s)
H	Hydrogen
h°	Hue angle
ha	Hectare(s)
HCl	Hydrochloric acid
$HgCl_2$	Mercury chloride
H_2O	Water
H_2O_2	Hydrogen peroxide
Homo-BL	Homobrassinolide
HPLC	High performance liquid chromatography
HWT	Hot water treatments
IAA	Indole-3-acetic acid
i.e.	That is
Inc.	Incorporated
Intl.	International
kg	Kilogram(s)
KOH	Potassium hydroxide
kPa	Kilo pascals
L	Litre(s)
L*	Lightness
lat.	Latitude
LDPE	Low-density polyethylene
LSD	Least Significant Difference
Ltd.	Limited
long.	Longitude
m	Metre(s)
M	Molar
MA	Massachusetts
MACC	1-malonyl aminocyclopropane-1-carboxylic acid

MAP	Modified Atmospheres Packaging
MeOH	Methanol
MG	Mature green
mg	Milligram(s)
$MgCO_3$	Magnesium carbonate
min	Minute(s)
MJ	Methyl jasmonate
mL	Millilitre(s)
mm	Millimetre(s)
mM	Millimolar(s)
mmol	Millimole(s)
MS	Mass Spectrometry
Mt	Metric tonnes
MV	Millivolt(s)
N	Newton(s)
N	Normality
n	Number of sample
N_2	Nitrogen
NA	Not available
NAA	Naphthalene acetic acid
NaCl	Sodium chloride
NaF	Sodium fluoride
$NaHSO_3$	Sodium hydrogen sulphite
NaOH	Sodium hydroxide
NaOCl	Sodium hypochlorite
NCED	9-cis-epoxycarotenoid dioxygenase
ND	Not detected
NDGA	Nordihydroguaiaretic acid
ng	Nanogram(s)
NIR	Near Infra Red
nL	Nanolitre(s)
nm	Nanometre(s)
Nmm	Newton millimetre(s)

nmol	Nanomole(s)
NO	Nitric oxide
NC	North Carolina
NS	Not significant
NSW	New South Wales
NT	Northern Territory
O_2	Oxygen
OR	Orange
P	Probability
PA	Pennsylvania
pA	Peak area
PCIB	α(p-Chlorophenoxy)isobutyric acid
PC	Pre-climacteric
PE	Pectinesterase
PEPC	Phosphoenol pyruvate carboxylase
PG	Polygalacturonic acid
pH	Symbol denoting hydrogen ion in a solution
PI	Pink
pmol	Picomole(s)
PL	Pectate lyase
PLP	Pyridoxal-5-phosphate
POD	Peroxidase
Prof.	Professor
psi	Pounds per square inch
Pty.	Proprietary
PVP	Polyvinylpyrolidone
PVPP	Polyvinylpyrolidone phosphate
QLD	Queensland
®	Registered
r	Correlation coefficient
RH	Relative humidity
RI	Refractive index
ROS	Radical oxygen species

RP	Ripening period
RR	Red ripe
S	South
s	Second(s)
SAM	S-adenosyl methionine
SAMDC	S-adenosyl methionine decarboxylic acid
SAS	Statistical Analysis System
SCO_2	Rate of CO_2 for sample
S.E.	Standard error
SI	Sucrose invertase
SLV	Scanning Laser Virometry
SNP	Sodium nitroprusside
SP	Storage period
sp.	Species
SUS	Sucrose synthase
SSC	Soluble solids concentration
St.	Saint
$StdCO_2$	Rate of CO_2 for standard
T	Treatment
TA	Titratable acidity
tan	Tangent
TAPP	Tanzania Agriculture Productivity Program
TEAC	Trolox Equivalent Antioxidant Activity
™	Trademark
TPA	Texture Profile Analyser
Trolox	6-hydroxy-2,5,7,8-tetramethychroman-2-carboxylic acid
UK	United Kingdom
USA	United States of America
US$	United States dollar
UV	Ultra-violet
V	Viscosity
VA	Virginia
VIC	Victoria

VIS	visible
Vol.	Volume(s)
vs.	Versus
v/v	Volume by volume
WA	Western Australia
WAFB	Weeks after full bloom
WAFF	Weeks after first flower
WAPF	Weeks after post flowering
WPH	Weeks prior harvest
w/v	Weight by volume

CHAPTER 1

General introduction

Mango (*Mangifera indica* L.) is one of the choicest fruits due to its delicacy, pleasant aroma and nutritional value. It's popularly known as 'the king of fruits' and rank the second most important tropical fruit crop in the horticulture industry of the world. Mangoes are produced commercially in at least 94 countries in the world with estimated area 5 million ha and annual production of 35.1 Mt (FAOSTAT, 2009). In Australia, mango production has substantially increased in the last decade with estimated production 60, 000 Mt (FAOSTAT, 2009). The international trade liberalization resulted in higher volume of mango fruit exported to various continents including European and North American countries. The world mango export has risen from 0.33 Mt (US$ 335 million) in 1995 to 1.1 Mt (US$ 924 million) in 2008 (FAOSTAT, 2008).

Mango is a climacteric fruit. It's highly perishable and ripens quickly within 7 to 9 days if harvested at mature green stage and kept at ambient temperature. A burst of respiration rate and ethylene production accelerated the ripening process (Burg and Burg, 1962; Mattoo and Modi, 1969b). The rise in the rates of respiration and ethylene production during ripening symbolises normal ripening behaviour of mango fruit. The ethylene production may rises when, before or after respiration rises during mango fruit ripening (Biale and Young, 1981; Burg and Burg, 1962). 'Kensington Pride' mangoes harvested at mature green stage, showed a respiratory peak on the 3rd day of ripening during ripening at ambient temperature (21°C) with a concomitant increase in the ethylene production on the same day (Lalel et al., 2003d). The climacteric ethylene production and respiratory patterns of mango are influenced by several pre- or post-harvest factors such as cultivar, harvest maturity, ethylene, storage temperature and atmosphere, disease incidence or heat treatments (Cua and Lizada, 1990; Esguerra and Lizada, 1990; Lalel et al., 2005; Mitcham and Mcdonald, 1993; Nair and Singh, 2003; Nair et al., 2004b)

During maturation process, Akamine and Goo (1973) claimed that ethylene production decreases and becomes undetectable for a short span of time and then reappears upon ripening. Ethylene biosynthesis is an essential feature of mango fruit ripening. Ethylene production is peaked at the onset of climacteric phase of fruit ripening and the small amount of ethylene present in the fruit at harvest is sufficient to initiate ripening (Burg and Burg, 1962; Mattoo and Modi, 1969b; Singh and Singh, 2011). Ethylene is either directly or indirectly involved in ripening associated changes. Earlier, it has been suggested that endogenous ethylene increases the activity of 1-amino cyclopropane-1-carboxylic acid (ACC) synthase (ACS) and the further conversion of ACC to ethylene through the ACC oxidase (ACO) (Atta-Aly et al., 2000). Exogenous application of ethephon has been reported to increase ethylene production in various fruit crops including mango and preceded the ripening process (Lalel et al., 2003e; Saltveit, 1999). Whilst, the application of inhibitor of ethylene biosynthesis [Aminoethoxyvinylglycine (AVG)] or action [1-methylcyclopropene (1-MCP) or nitric oxide (NO)] treatments suppressed its production and delayed fruit ripening in mango, banana, peach and strawberry (Cheng et al., 2009; Lalel et al., 2003e; Liu et al., 2007; Zaharah and Singh, 2011a; b; Zhu et al., 2006; Zhu and Zhou, 2007).

Beside ethylene, absisic acid (ABA) also plays an important role in regulating fruit ripening processes in various climacterics and non-climacteric fruit (Chen et al., 1999; Jiang and Joyce, 2003; Kitamura et al., 1983; Ruan et al., 2005; Tsay et al., 1984; Wendehenne et al., 2001). During initial stages of mango fruit development, the endogenous level of ABA increases (Kondo et al., 2004; Murti and Upreti, 1995), later on decreases or becomes constant during fruit maturation (Kondo et al., 2004). Additionally, higher endogenous level of ABA in peach, grape and tomato preceded ethylene production, suggests that ABA accumulation play key role in regulating fruit ripening and senescence (Zhang et al., 2009a; Zhang et al., 2009b). The exogenous application of ABA (10^{-6} M and 5 mg L^{-1}) hastened fruit ripening and softening in 'Alphonso', 'Langra' and 'Zihua' mango fruit (Palejwala et al., 1988; Parikh et al., 1990; Zhou et al., 1996). Similarly, the postharvest application of ABA (100 μM) to mature green tomato, increased ethylene production by up-regulating encoding ACS and ACO gene (*Le-ACO1* and *LeACS2*), thereby accelerated fruit ripening (Zhang et al., 2009b). Whilst, the application of 100 μM inhibitor of ABA

biosynthesis – nordihydroguaiaretic acid (NDGA) delayed tomato fruit ripening through suppression of *Le-ACO1* and *LeACS2* gene expression (Zhang et al., 2009b). The information on the changes of endogenous level of ABA as well as exogenous application and its inhibitor in regulating mango fruit ripening is still lacking and warrants to be investigated.

Higher endogenous level of auxins in mango fruit is contributing to the cell enlargement phase (Murti and Upreti, 1995). The changes in its endogenous level might influenced the ethylene biosynthesis (i.e. ACS) during fruit ripening (Vendrell and Palomer, 1997). A rapid degradation of endogenous level of indole-3-acetic acid (IAA) increased the climacteric peak of ethylene production in 'Yulu' peach on the 3rd day of ripening (Wu et al., 2003). In contrast, higher endogenous levels of IAA was detected during the fruit-beaker and ripe-fruit stages in the *rin* (ripening inhibitor) tomato mutant (Hong and Lee, 1993). No research work has been reported on the dynamics of levels of endogenous auxins during the ripening of mango fruit and warrants to be investigated.

Brassinosteroids (BRs) are a group of some 40 different steroids that are synthesized by plants and regulate plant growth, development and fruit ripening in climacteric and non-climacteric fruit (Mandava et al., 1981; Montoya et al., 2005; Symons et al., 2006). The concentrations of endogenous BRs such as catasterone (CAS) was found higher in the early stage of fruit development and then declined during maturation stage in tomato fruit (Montoya et al., 2005) and grape (Symons et al., 2006). A dramatic increase in the level of CAS and 6-deoxocastasterone was also coincided with the onset of grape berry ripening (Symons et al., 2006). The pre-harvest application of BRs (0.4 mg L^{-1} and 1.0 mg L^{-1}) promoted fruit softening in litchi (Peng et al., 2004) and fruit ripening in passion fruit (Gomes et al., 2006). Whilst, the post-harvest application of 3.0 µM of 28-homobrassinolide or 24-Epibrassinolide to tomato pericarp discs increased ethylene production and promoted ripening (Vardhini and Rao, 2002). Apparently, no information is available on the changes of endogenous level of BRs during mango fruit ripening period and the effects of their exogenous application in regulating mango fruit ripening and warrants to be investigated.

Nitric oxide (NO) is a free radical gas and acts as a multifunctional signalling molecule in plants, involving in many physiological processes including ripening of climacteric and non-climacteric fruit (Lamattina et al., 2003; Leshem et al., 1998; Manjunatha et al., 2010; Wendehenne et al., 2004). The endogenous levels of ethylene and NO during fruit development and maturation have inverse and stoichiometric relationships, thereby offering an opportunity for modulation of their levels with exogenous application to exert the opposite effects. The exogenous application of NO gas or donor compounds such as sodium nitroprusside (SNP) have been shown to delay the process of ripening and senescence in various fruit crops such as plum, banana, kiwifruit, peach, pear, strawberry, and tomato (Aboul-Soud, 2010; Cheng et al., 2009; Eum et al., 2009; Flores et al., 2008; Liu et al., 2007; Singh et al., 2009; Sozzi et al., 2003; Wills and Bowyer, 2003; Zhang et al., 2005; Zhu et al., 2006; Zhu et al., 2010b; Zhu and Zhou, 2007) through inhibition of ethylene biosynthesis thus suppressed ethylene production during ripening or cold storage. However, the effects of NO fumigation in delaying the ripening of mango fruit and its potential in alleviating chilling injury (CI) development needs to be elucidated.

Fruit softening is a major limitation to extend postharvest storage and shelf life of mango (Chourasia et al., 2006; Chourasia et al., 2008), and for its export through sea freight. During fruit softening, pectin and hemicelluloses in cell wall undergo solubilisation and depolymerisation as a result of fruit softening enzyme such as polygalacturonase (PG), pectinesterase (PE), *endo*-1,4-β-D-glucanase (EGase) and pectate lyase (PL) (Abu-Sarra and Abu-Goukh, 1992; Ali et al., 1990; Ali et al., 1995; Ali et al., 2004; Ashraf et al., 1981; Chourasia et al., 2006; Chourasia et al., 2008; Lazan et al., 1986; Lazan et al., 1993; Mitcham and McDonald, 1992; Prasanna et al., 2005; Prasanna et al., 2003; Roe and Bruemmer, 1981). Increased activities of fruit softening enzymes have been reported to be influenced by the endogenous level or the exogenous application of ethylene (Burg and Burg, 1962). The exogenous application of ABA has also been reported to promote softening in mango fruit during ripening (Zhou et al., 1996). The application of NO reduced PG activity and delayed fruit ripening in peach and banana (Cheng et al., 2009; Yang et al., 2010; Zhu et al., 2010a). The mode of action of ABA and its biosynthesis inhibitor or NO in regulating the fruit softening and softening enzymes

activity during ripening or cold storage in 'Kensington Pride' mango fruit warrants to be investigated.

It was therefore hypothesized that the changes in endogenous levels of BRs, ABA, IAA, and/or ethylene may plays a role in regulating the ripening processes of 'Kensington Pride' mango fruit. The endogenous levels of these regulators were regulated using inhibitors of their biosynthesis and/or action to unfold their mechanism in delaying/hastening mango fruit ripening, extending storage life and improving fruit quality.

Therefore in this study, my research was aimed:

1. To examine the changes in the endogenous level of BRs, ethylene, ABA and IAA during the ripening peiod of 'Kensington Pride' mango fruit and their exogenous application in regulating fruit ripening.

2. To investigate the mode of action of exogenously applied ABA and its inhibitor of biosyntheis in modulating ethylene biosynthesis, activities of fruit softening enzymes in the pulp and quality in mango fruit.

3. To evaluate the effects of NO fumigation at the pre-climacteric and climacteric-rise stages on the production of ethylene and respiration rate, fruit softening, colour development and quality during mango fruit ripening at ambient temperature.

4. To underpin the mode of action of NO in modulating ethylene biosynthesis and activities of fruit softening enzymes in the pulp during ripening and cool storage.

5. To unfold the the role of NO in alleviating CI symptoms, ethylene production, respiration rate, fruit softening and skin colour development following cold storage.

CHAPTER 2

General literature review

2.1 Introduction

Mango (*Mangifera indica* L.) is an important tropical fruit. It belongs to family Anacardiaceae (Bompard, 2009). Mango fruit is delicious with pleasant aroma and high nutritional value. It is rich in dietary fibre and has a protective effects against degenerative diseases, especially with regards to the heart; may help prevent certain types of cancer, as well as lowering blood cholesterol levels (Brown, 2011). Mango fruit contain high pectin, vitamin C and A (β-carotene), which play an important role in lowering blood cholesterol and boost the immune system.

Mango fruit mature between 11 to 14 weeks after fruit set depending upon a cultivar. Green hard mature mango fruit ripen very quickly. Consumers and retailers expect ripe mango fruit to have a yellow background skin colour, preferably with some pink/red blush. The fruit quality including its appearance, taste and flavour is important for both growers and consumers. Mango fruit ripening under go various biochemical changes including increased respiratory climacteric, endogenous ethylene production, fruit softening, changes in carbohydrates, organic acids, phenolic compounds as well as production of aroma volatile compounds (Gomez-Lim, 1997; Lalel et al., 2003e; Lizada, 1993). The changes in endogenous level of plant growth regulators are considered as an important factor that may play a role in regulating mango fruit ripening. This review will focus on the research work reported on the changes in endogenous level of plant growth regulators and their exogenous application on mango fruit ripening particularly on postharvest life and quality.

2.2 Economic importance of mango

Mango is grown over an area of 5.1 million ha in at least 94 countries, with an annual production of 35.12 Mt in the world (FAOSTAT, 2009). Presently, among different mango producing countries, India dominate the mango production over 38.60% of total production in the world, followed by China, Thailand, Indonesia, Mexico,

Pakistan and Brazil, which contribute about 11.79%, 7.03%, 6.12%, 4.30%, 4.92% and 3.41% share to world mango production, respectively (Table 2.1).

Table 2.1. The production of mangoes (including mangosteen and guava) and area in the leading mango producing countries in the world during 2009.

Country	Area harvested (ha)	Production (Mt)
India	2, 356, 700	13, 557, 100
China	466, 637	4, 140, 290
Thailand	308, 026	2, 469, 810
Indonesia	186, 000	2, 150, 000
Pakistan	173, 731	1, 728, 000
Mexico	170, 027	1, 509, 270
Brazil	75, 178	1, 197, 690
Nigeria	128, 874	831, 489
Bangladesh	166, 000	828, 161
Philippines	188, 100	771, 441
Viet Nam	49, 036	540, 000
Kenya	28, 700	474, 608
Egypt	130, 000	450, 000
Yemen	25, 818	404, 573
Cuba	50, 311	354, 200
United Republic of Tanzania	31, 750	320, 000
Haiti	41, 121	244, 607
Madagascar	21, 161	221, 286
Democratic Republic of the Congo	11, 683	210, 000
Peru	13, 000	165, 634
Australia	9, 936	60, 000
Other	461, 013	2, 495, 968
World (Total)	5, 092, 802	35, 124, 127

Source: FAOSTAT (2009).

An increase in the export of mango has rose from 0.33 million tonnes (US$ 335 million) in 1995 to 1.1 million tonnes (US$ 924 million) in 2008 (FAOSTAT, 2008). India ranks first in the world in exporting mangoes in quantity (274, 854 Mt) worth of US$ 224.98 million (FAOSTAT, 2008) (Figure 2.1). The expansion in mango industry, the trade liberalization in the world, development of taste to mango fruit by European and North American consumers are major factors contributing to the expansion of international trade of mango fruit. The United States (USA) and European Union (EU) including Netherland, United Kingdom (UK), Germany, France, Belgium and Portugal together accounted for 75% of major world mango import country (Figure 2.2), with the total quantity of 266, 807 Mt (US$ 212.13

million) and 134, 258 Mt (US$ 221.27 million), respectively (TAPP, 2010). Among different mango producing countries, Mexico is the 2nd major exporting country to major import country USA comprising of the value of about US$ 129.13 million, followed by Brazil, Peru and Ecuador with mango export values of US$ 22.11, US$ 22.13 and US$ 22.30 million, respectively (TAPP, 2010). In comparison to rest of the world, Australian mango industry is very small. However, during 2008 out of 57, 947 Mt of mango production in Australia, about 109 Mt of mango with a value of US$ 0.63 million were exported to EU country (TAPP, 2010).

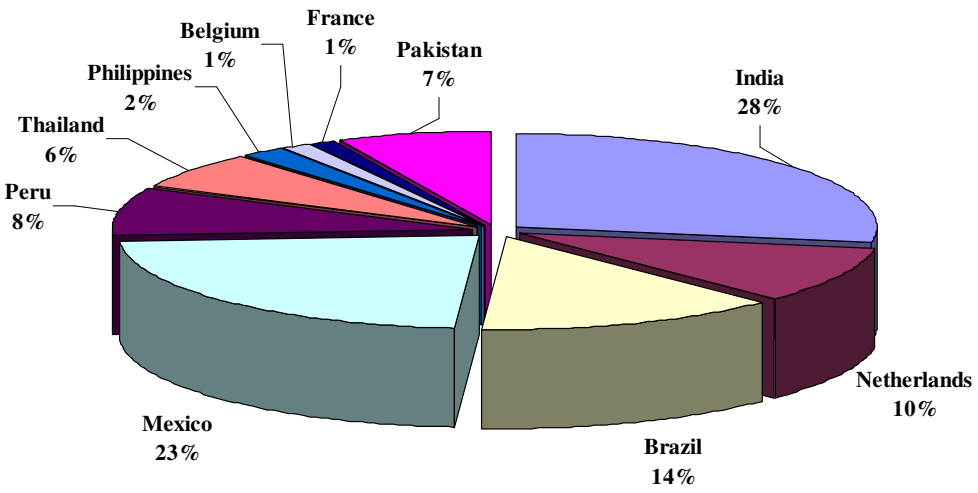

Figure 2.1. World top ten major mangoes exporting countries during 2008 (FAOSTAT, 2008).

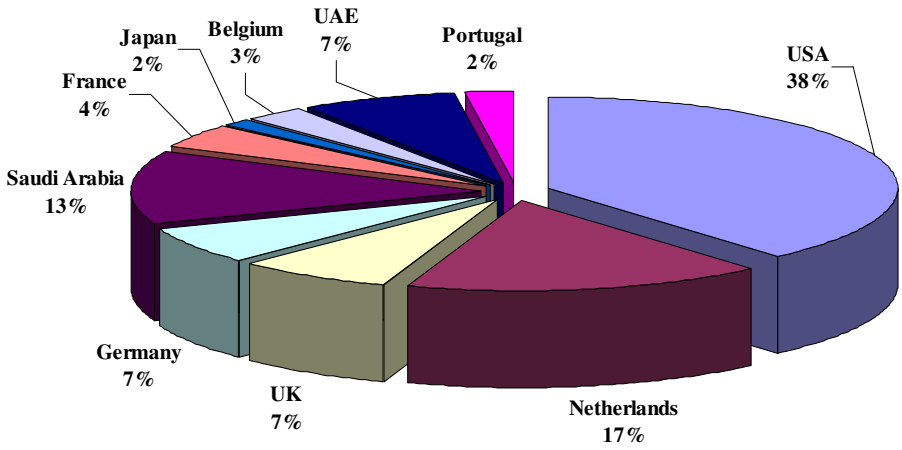

Figure 2.2. World top ten major mangoes importing countries during 2008 (FAOSTAT, 2008).

Mango (cv. 'Kensington Pride') accounts for 90% of trees grown commercially in Australia (AMIA, 2006). In 2009/10, Queensland is the major mango producing state, accounting for 66% by volume of domestic production. The Northern Territory is also a dominant mango producer accounting for 29% of production. Western Australia (WA) and New South Wales (NSW) are the third and fourth largest mango producing state in Australia, contributing 4% and 1% of total mango production in the country, respectively (Table 2.2). The total Australian mango production in 2009, was 44, 343, 000 Mt, and the gross value was approximately A$ 121.95 million (Table 2.2).

Table 2.2. State wise distribution of total mango production and gross value during 2009 in Australia.

State	Production (´000 Mt)	Value (A$)
Queensland (QLD)	29, 436	82, 554, 802
Northern Territory (NT)	12, 758	26, 020, 875
Western Australia (WA)	1, 713	13, 249, 924
New South Wales (NSW)	436	119, 977
Total (Australia)	44, 343	121, 945, 578

Source: ABS (2009).

During 2007-2008, most of the Australian mango production has been exported to the potential Asian markets (Table 2.3). Australian mango has been exported to Japan, Singapore and New Zealand at the proportion of 34.27%, 19.20% and 11.92%, respectively. Total exports in that year were valued at A$ 11.01 million (ABS, 2009). In 2009-10, mango exports increase 25%, with Australia exporting 4, 000 Mt of mangoes valued at A$ 15 million to South Korea, with approximately 80% from QLD based companies (Mulherin, 2010).

The WA mango industry has been expanding significantly over the past 10 years, but remains small from a national perspective. WA mango production in year 2008/09 was valued at A$ 13.25 million (ABS, 2010). The mango production took place in two regions, the Central and Kimberley region, mainly in Carnarvan and Kununurra, which produced 1, 330 Mt and 1, 309 Mt in 2002/03, respectively (FAOSTAT, 2004). The dispersion of producers across the state causes a lengthy

picking time, and a continuous supply to the Perth Market from October through to April (McCoy, 2007).

Table 2.3. Major export markets for mangoes from Australia in 2007/08.

Destination	Value (A$ ´000)
Hong Kong	3, 716
Japan	2, 082
Singapore	1, 293
New Zealand	885
United Arab Emirates	679
Malaysia	547
Qatar	314
The Netherlands	312
Lebanon	305
Canada	95
Kuwait	94
Other	521
Total	11, 015

Source: ABS (2009).

Figure 2.3. Major mango growing region in Australia.
Source: http://www.australiafresh.com.au/

2.3 Harvest maturity

Harvesting at optimum maturity is a critical step, which determines the potential storage life, flavour and consumer acceptance of mango fruit (Medlicott et al., 1988; Seymour et al., 1990; Singh and Singh, 2011). Fruit harvested at immature are more susceptible to mechanical damage (Chonhenchob and Singh, 2003), such as chilling

injury (CI), with unacceptable quality including less skin colour development when ripe (Ledger, 1995; Medlicott et al., 1988). However, some physiological disorder, for instance internal breakdown as a result of delay in picking 'Chiin Hwang' mango fruit has also been reported earlier by Lee et al. (1998). Mango fruit had better aroma quality (Bender et al., 2000), if harvested at advanced maturation, but reduces the storage potential (Seymour et al., 1990). In order to meet the quarantine requirements, mango fruit must be harvested at optimum harvest maturity to suit to the postharvest vapour heat or hot water treatments (HWT) (Jacobi et al., 2001; Jacobi and Wong, 1992). HWT to immature mango fruit show severe heat injury in the form of skin scald, but advanced maturity fruit was found to be more tolerant (Jacobi et al., 2001). The development of flavour and aroma will be stunted, uneven ripening and lower sugar:acid ratio, if mango fruit are not harvested at right maturation stage (Lakshminarayana, 1975). However, commercial harvesting decision by farmers are usually influenced towards achieving better shelf life and receiving a good market price by harvesting fruit at appropriate maturity stage. There is a guideline of maturity indices which can be applied to determine fruit maturity. The maturity indices for different mango cultivars including morphological characteristic (skin colour and shape), chemical attributes such as soluble solids concentration (SSC), titratable acidity (TA), SSC:TA ratio, starch content, starch:acidity ratio and phenolics content, specific gravity, aroma (gas chromatography and mass spectroscopy or electronic nose), computational methods, non-destructive methods for skin colour or fruit firmness such as Acaustic Resonance Spectroscopy and Acaustic Tester are summarised in Figure 2.4.

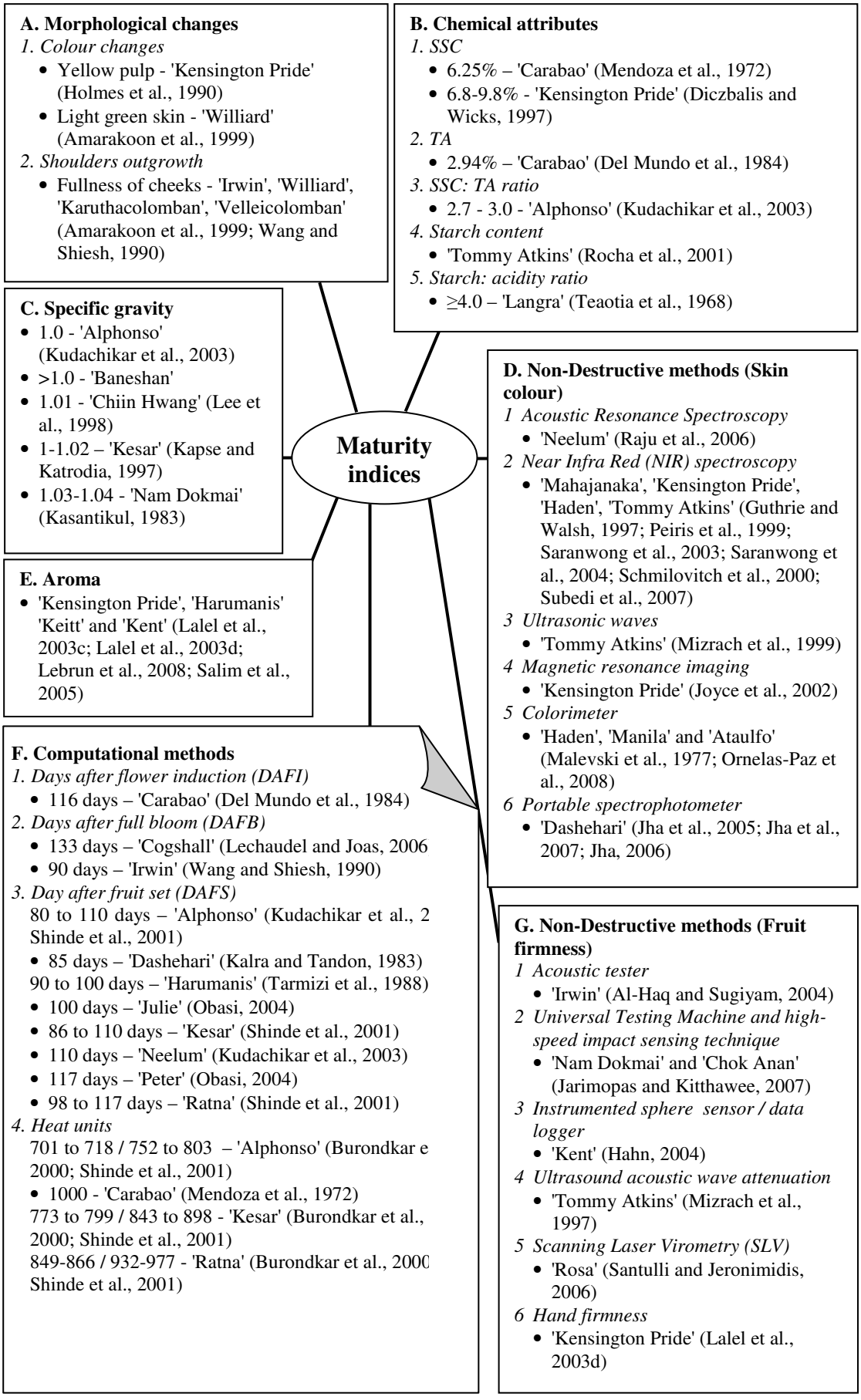

Figure 2.4. Maturity indices attributes of different cultivars of mango fruit.

2.4 Roles of phytohormones in fruit ripening

2.4.1 Ethylene

Fruit ripening is controlled by ethylene, which is auto catalytically synthesized in small concentration prior to the initiation of ripening, which in turn triggers the entire array of changes during ripening. Ethylene as low as 0.01 μL L^{-1} triggered the ripening process in mango fruit (Johnson et al., 1997).

The pathway of ethylene biosynthesis was first established in apple fruit (Adams and Yang, 1979). Since then it has been shown to operate in other climacteric fruit, such as mangoes, avocadoes, bananas and tomatoes. The two key enzymes in the pathway are those catalysing the conversion of S-adenosyl methionine (SAM) to 1-aminocyclopropane-1-carboxylic acid (ACC) and ACC to ethylene, called ACC synthase (ACS) and ACC oxidase (ACO) (ethylene-forming enzyme, EFE), respectively. At the onset of fruit ripening, expression of multiple ACS genes are activated, resulting in increased production of ACC. ACC is then oxidized to ethylene by ACS. In most cases, it is the ACS activity that determines the rate of ethylene biosynthesis (Tharanathan et al., 2006). An overview of fruit ripening, with special reference to textural softening, has been presented in Figure 2.5.

Two major systems exist for ethylene synthesis in plants. System I occurs in non-climacteric and pre-climacteric (PC) fruit. Enzymes for system I are constitutive, meaning they are continually produced within fruit regardless of the ripening stage. The system II ethylene synthesis pathway is present only in climacteric fruit, and is inducible. System II ethylene synthesis can be switched on by a range of internal and external signals for senescence, and is sensitive to ethylene from system I (Owusu-Apenten, 2005).

Climacteric fruit show a sharp increase in climacteric ethylene production at the onset of ripening which is considered to contribute in controlling the initiation of changes in colour, aroma, texture, flavour and other biochemical and physiological attributes. This accelerated ethylene production rate may be required to raise the internal content of ethylene to a stimulatory level, or it may be due to the autocatalytic response initiated when tissue becomes sensitive to the low level of

ethylene present throughout the early PC period (Burg and Burg, 1965; Dominguez and Vendrell, 1993). In climacteric fruit, the transition to autocatalytic ethylene production appears to result from a series of events where developmentally regulated *ACO* and *ACS* gene expression initiates a rise in ethylene production, setting in motion the activation of autocatalytic ethylene production. Besides the differences in their rates, the pattern of respiration, climacteric and non-climacteric fruit also differ in their internal ethylene concentration during the growth and ripening phases.

Environmental factors to which fruit are exposed during transportation, storage and postharvest ripening have the potential to influence the level of ethylene biosynthesis (Lelievre et al., 1997). The best quality is maintained when the concentration of ethylene, carbon dioxide (CO_2), and oxygen (O_2) in the atmosphere and the duration of exposure, temperature and humidity are carefully controlled and maintained at optimal levels. The exogenous application of ethylene (10 to 1, 000 μL L^{-1}) are used commercially to promote ripening of mangoes, bananas, melons, and tomatoes (Saltveit, 1999).

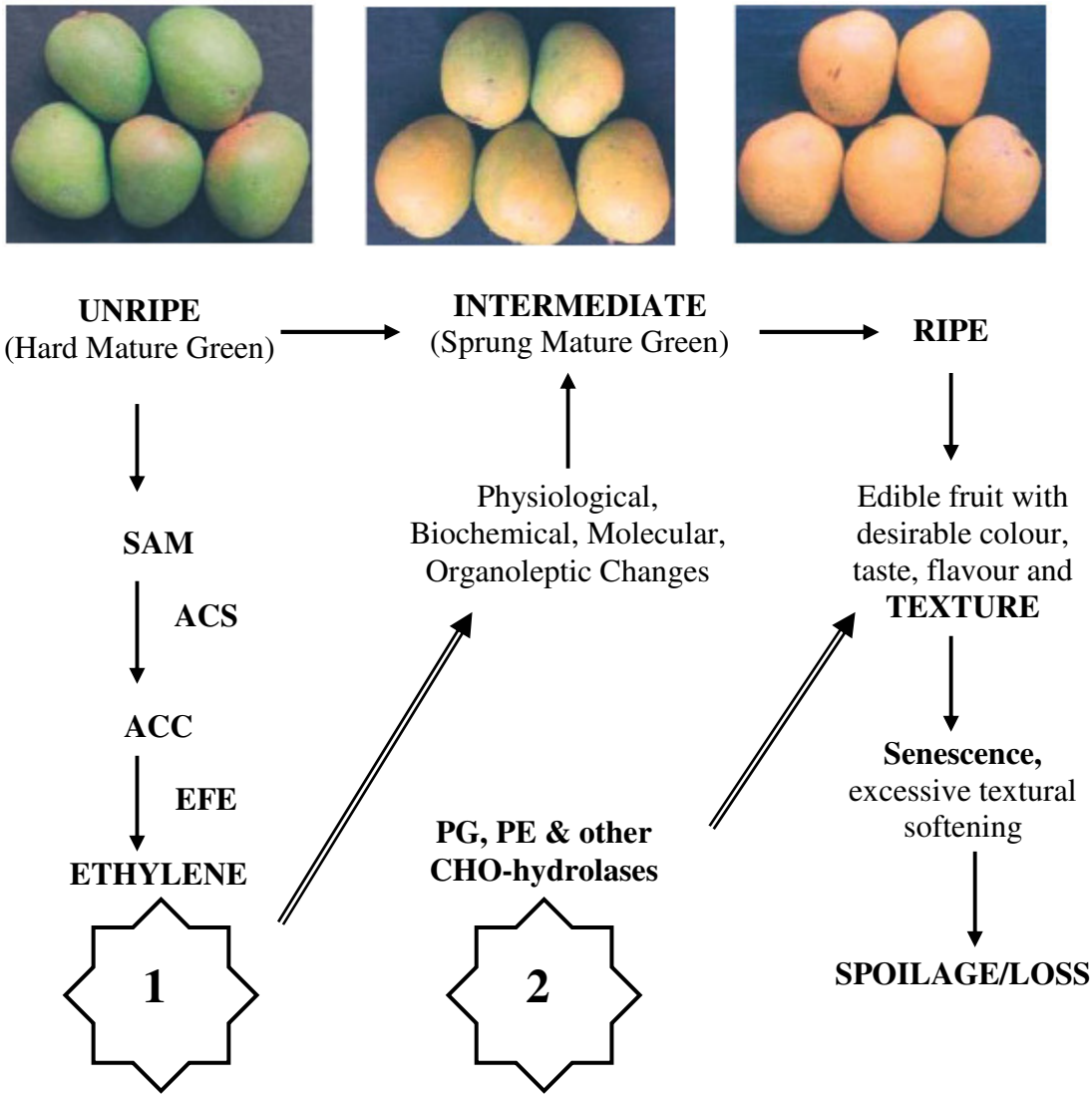

Figure 2.5. Overviews of fruit ripening with particular emphasis on textural softening, control point are at ethylene (1) and post-ethylene (2) levels (Prasanna et al., 2010).

2.4.1.1 Endogenous ethylene

Ethylene plays a very important role in fruit ripening (Lelievre et al., 1997). It has been suggested that endogenous ethylene increases the activity of ACS and the further conversion of ACC to ethylene through ACO (Atta-Aly et al., 2000). However, it has been reported that self-inhibition may occur in the fruit, with a concomitant drop in ACS and ACO activities (Atta-Aly et al., 2000). It has been reported that the 'Ataulfo' mango increased the ACC content to 23.94 nmol g^{-1} during the PC stage (1 - 4 days postharvest) at room temperature (25 ± 1°C) causing

increases in SSC, decreases in acidity, lost of firmness and changes in colour (Montalvo et al., 2007).

2.4.1.2 Exogenous application of ethylene

Over 3 decades ago, in order to produce good quality fruit, exogenous applications of ethephon, a kinds of synthetic ethylene, have been commercially used to hasten fruit ripening processes. Higher concentrations of ethephon have been used, in the range of 250 - 500 mg L^{-1}, exhibiting slow rate and uniform ripening when applied on fully mature 'Dashehari', 'Amrapali' and 'Neelum' mangoes (Joon et al., 2001; Kulkarni et al., 2004; Kumar and Dhawan, 1995; Singh et al., 1995), whilst Pandey et al. (1980) and Chundawat et al. (1973) found that range of concentration hastened fruit ripening in 'Dashehari' mango. Shanmugavelu et al. (1976) has reported that the lower concentrations of ethephon (104 mg L^{-1}), reduced the fruit ripening in 'Muloga' and 'Baneshan' mangoes. The application of 5, 000 mg L^{-1} ethephon hastened fruit ripening by reducing the number of days taken for ripening from 9 days in the control compared to the 3 days in the treated fruit (Singh et al., 1979). Post-harvest treatment by spraying or dipping in a concentration of over 1, 000 mg L^{-1} has been documented to hasten fruit ripening or, in some cases, it caused over ripening in mango fruit (Ruiz and Guadarrama, 1992; Sergent et al., 1993; Singh et al., 1979; Valverde et al., 1986) and the process became faster when the fruit were placed under ambient or room temperature. It has also been reported that exogenous application of ethephon (1, 500 mg L^{-1}), in combination with modified atmosphere packaging (MAP) at 13.5°C, reduced the fruit ripening time of 'Kensington Pride' mangoes by decreasing the respiration rate and increasing the ethylene as well as the oxygen production rate (Singh and Janes, 2001). At ambient temperature, exogenous application of ethephon (500 mg L^{-1}) of hard mature green 'Kensington Pride' mangoes in combinations of ‘Tween 20’ hastened fruit ripening by advancing climacteric peak of ethylene production and triggering accumulation of other free compounds, such as putresine, spermidine and spermine, which are believed to contribute to the fruit ripening process (Malik and Singh, 2003).

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2.4.1.3 Regulation of ethylene biosynthesis

An understanding of ethylene biosynthesis regulation is very important to find out the best method to either retard or promote fruit ripening. There are many intrinsic and extrinsic factors that promote or inhibit ethylene synthesis in higher plants. Fruit ripening involves the transitory increase in ethylene production (Rhodes, 1980) and inhibition of ethylene production or its action is the potential method of retarding fruit ripening (Saltveit, 1999). Many well-known compounds have been successfully used to suppress or inhibit ethylene production in climacteric fruits, for instance, 1-methylcyclopropene (1-MCP) and aminoethoxyvinylglycine (AVG) (Adkins et al., 2005; Bregoli et al., 2002; Jiang and Joyce, 2000; Jobling et al., 2003b; Lohani et al., 2004; Mathooko et al., 2004; Mir et al., 2004; Pelayo et al., 2003; Rath et al., 2006; Shellie, 1999; Torrigiani et al., 2004).

2.4.1.3.1 1-Methylcyclopropene (1-MCP)

Inhibition of ethylene biosynthesis and/or action is one of the potential methods of retarding fruit ripening (Saltveit, 1999). 1-MCP is well-known to suppress or inhibit ethylene production in various non-climacteric and climacteric fruit (Watkins, 2008; Watkins and Miller, 2004) including mangoes (Hojo et al., 2006; Jiang and Joyce, 2000; Lalel et al., 2003e; Pandey and Singh, 2007; Santos et al., 2004; Singh and Dwivedi, 2008). The threshold concentration of 1-MCP required to suppress ethylene production and action in mango has been reported to be 1.0 μL L^{-1}, depending upon cultivar and time of its application (Singh and Neelam, 2008; Vilas-Boas and Kader, 2007). The onset of ethylene production was delayed and a reduced respiration rate in the 'Nam Dok Mai' mango post-harvest treated with 0.5 μL L^{-1} and 1.0 μL L^{-1} 1-MCP also prolonged the shelf life of mangoes to 15 days of storage at 20°C (Penchaiya et al., 2006). Similarly, the exogenous application of 1-MCP (5.0 μL L^{-1}) for 6 h in 'Guifei' mango extended the shelf life for 8 to 12 days (Wang et al., 2006). Post-harvest exposure of hard mature green 'Rosa', 'Jasmim' and 'Espada' mangoes to 1-MCP (100 μg L^{-1}) for 24 h delayed ethylene production (Silva et al., 2004). The inhibition of ethylene production with exogenous application of 1-MCP has also been reported in 'McIntosh' and 'Scarletspur' apples (DeEll et al., 2008; Elfving et al., 2007), 'Monroe' avocados (Pereira et al., 2008) and 'Barlett' pears (DeEll et al., 2008). The ripening processes in avocados treated with 225 g L^{-1}, 450 g L^{-1} and 900

g L^{-1} aqueous formulation of 1-MCP for 2 min during hard mature stages were delayed from 2 to 16 days (Pereira et al., 2008). However, mango fruit do not respond very well to 1-MCP gas application. Now, new formulation of 1-MCP is available where it can be used as a liquid for a spraying or dipping. The effect of this new formulation of 1-MCP on 'Kensington Pride' mango fruit ripening is yet to be investigated.

2.4.1.3.2 Aminoethoxyvinylglycine (AVG)

Aminoethoxyvinylglycine (AVG) is an ethylene biosynthesis inhibitor which blocks the conversion of methionine to ACC (Yu and Yang, 1979), by competitively inhibiting the activity of ACS, a key enzyme in the ethylene biosynthesis pathway (McGlasson, 1985). AVG treatment appeared to delay and suppress the ethylene production depending upon cultivar, harvest maturity, age, and the physiological stage of fruit (Jobling et al., 2003a). The effectiveness of the AVG application also depends on the time of application and the level of ethylene receptor site or level of ACS activity. Fruit with high ethylene production required higher AVG concentration than fruit with low ethylene production to delay fruit ripening and softening (Amarante et al., 2002). It has also been reported that fruit with higher ACS activity requires a greater AVG concentration to delay ripening than fruit with lower ACS activity (Autio and Bramlage, 1982). AVG might act differently during pre- and post-harvest treatment. Pre-harvest application of AVG has been reported to reduce and inhibit ethylene activity, delay fruit maturity, and allows fruit to ripen more slowly in peaches, melons, apples, pears, nectarines and plums (Bregoli et al., 2002; Greene, 1996; Hayama et al., 2008; Jobling et al., 2003b; Lu et al., 2007; McGlasson et al., 2005; Romani et al., 1983b; Shellie, 1999). The post-harvest applications of AVG have been reported to reduce ethylene production, respiration rate and total fatty acids in the 'Kensington Pride' mango (Lalel et al., 2003e). However, no research work has been done on the role of pre-harvest applications of AVG on the post-harvest quality of 'Kensington Pride' mangoes and this is yet to be investigated.

2.4.1.3.3 Nitric oxide (NO)

Nitric oxide (NO) is a gaseous free radical and emerged as an important signaling molecule associated with many biochemical and physiological processes in plants

and animals (Hayat et al., 2010; Lamattina et al., 2003; Manjunatha et al., 2010). In plants, NO can be synthesized through basically two different pathways (Kaiser et al., 2006; Salgado et al., 2006) following the Eq. 1 and Eq. 2.

First, the L-arginine-dependent pathway uses NAD(P)H and O_2 as co-substrates and is catalyzed by nitric oxide synthase (NOS).

$$\text{L-Arg} + \text{NAD(P)H} + H^+ + O_2 \xrightarrow{\text{NOS}} \text{L-Citr} + \text{NAD(P)}^+ + \text{NO} \quad \text{.....(Eq. 1)}$$

Second, the nitrite-dependent pathway uses NADH or "electrons" as reductands and is catalysed by a number of different enzymes such as nitrate reductases (NR).

$$NO_2^- + e^- + 2H^+ \xrightarrow{\text{NR}} 2NO + H_2O \quad \text{.....(Eq. 2)}$$

Nitric oxide (NO) bring a new concept on how its involvement have antagonizing effects on ethylene production (Manjunatha et al., 2010) (Figure 2.6). A number of research reports demonstrate the involvement of NO in altering ripening-related ethylene that would assist in developing the new technique for shelf life extension processes either during normal ripening or cool/cold storage. It gave promising effect to delay the ripening processes in various fruit crops such as mango, plum, kiwifruit, apple, strawberries, jujube, tomato and peach during ripening or storage depending upon the maturity at harvest or stage of ripening (Eum et al., 2009; Zaharah and Singh, 2011b), concentration applied (Pristijono et al., 2008; Singh et al., 2009; Wills et al., 2000; Zaharah and Singh, 2011b; c; Zhu et al., 2006; Zhu et al., 2009; Zhu et al., 2010b; Zhu and Zhou, 2007), duration and storage condition (Singh et al., 2009; Wills et al., 2000; Zaharah and Singh, 2011c; Zhu et al., 2006), time and method of NO application (Pristijono et al., 2008).

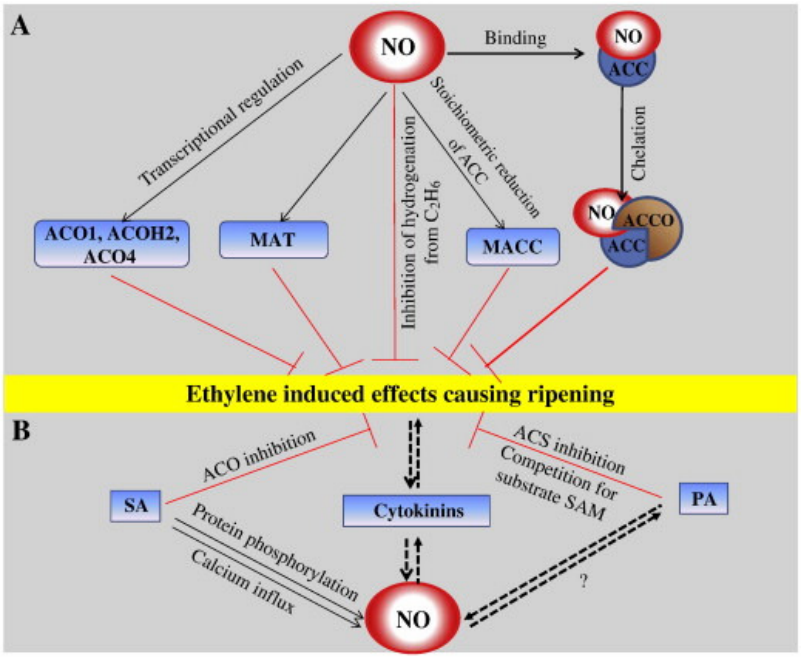

Figure 2.6. The mechanisms through which NO possibly antagonize ethylene causing ripening inhibition. (Dark lines with '→' mark indicate the established phenomenon with indirect link, red lines ended with '⊣' mark indicate ethylene inhibition by NO and '---›' indicate the unidentified mechanisms). Adaptation from Manjunatha et al. (2010).

Further explanation on the role of endogenous level of NO and its exogenous application in regulating ethylene production, ethylene biosynthesis enzymes and its encoding gene expression of various fruit types is discussed in Section 2.4.6.1 and Section 2.4.6.2. The effects of NO treatment in regulating fruit softening and physico-chemical changes during ripening of fruit crops is discussed further in Section 2.6.7.1 to 2.6.7.3.

2.4.2 Abscisic acid (ABA)

2.4.2.1 Endogenous ABA

Besides ethylene, higher levels of abscisic acid (ABA) has been reported to play a role as a ripening promoter in tomatoes (*Lycopersicon esculentum*) (Ruan et al., 2005; Sheng et al., 2000), peaches (*Prunus persica*) (Wendehenne et al., 2001), kiwifruits (*Actinidia* sp.) (Chen et al., 2005; Chen et al., 1999; Chen et al., 1997), apples (*Malus domestica*) (Lara and Vendrell, 2000; Plich and Nowacki, 1987), plums (*Prunus salicina*) (Kitamura et al., 1983), pears (*Pyrus communis*) (Kochankov et al., 1975; Tsay et al., 1984), strawberries (*Fragaria* sp.) (Jiang and Joyce, 2003) and grapes (*Vitis vinifera*) (During et al., 1978). However, lower

endogenous level of ABA has been found during fruit maturation in mango (cv. 'Dashehari', 'Chausa', 'Langra' and 'Alphonso') (Murti and Upreti, 1995; Ram et al., 1992). In peaches, the increased levels of endogenous ABA were found 3 days after harvest accompanied with a peak in ethylene production (Wu et al., 2003), whilst in 'Zaoxian' and 'Hayward' kiwifruits, the peak of ethylene production were only detected 6 and 10 days later (Chen et al., 2005; Chen et al., 1997). This suggested that ABA may play a role during fruit ripening by regulating the production of ethylene. Cold temperatures suppressed the increase in endogenous ethylene production in 'Tainong' mangoes and the maximum level was only found after 7 days the fruit was transferred to ambient temperature (Wang et al., 2008a). The reported literature suggests that endogenous ABA plays an important role in regulating fruit ripening after harvest. The information on the endogenous level of ABA in mangoes during fruit ripening is still lacking and warrants to be investigated.

2.4.2.2 Exogenous application of ABA

Post-harvest treatments of ABA has been found to be highly effective in hastening fruit ripening in various types of fruit, such as mangoes (Palejwala et al., 1988; Parikh et al., 1990), bananas (Jiang et al., 2000), kiwifruit (Chen et al., 2005), apples (Plich and Nowacki, 1987) and apricots (Zhang and Lu, 1983) by inducing ethylene production and enhancing respiration rates. In contrast, inhibition or no responses in ethylene production were found during the ripening of 'Naebyungjangsu' tomatoes and 'Bigarreau Napoleon' cherries (Hartmann, 1992; Hong et al., 1999). The exogenous application of 10^{-6} M ABA to unripe mangoes has been found to enhance fruit ripening in various mango cultivars such as 'Alphonso' and 'Langra' (Majmudar et al., 1981; Palejwala et al., 1988; Parikh et al., 1990) by decreasing acid levels, increasing sugar contents and sugar:acid ratios (Palejwala et al., 1988), as well as the transformation of chloroplasts to chromoplasts and carotenoid pigment (Parikh et al., 1990). Similarly, mature green 'Zihua' mangoes dipped in 5 mg L^{-1} ABA showed an increase in polygalacturonase (PG) and the biosynthesis of carotenoid, but decreased pectinesterase (PE) activity (Zhou et al., 1996). In apricots, different timings of application of ABA resulted in different responses in ripening. The exogenous application of ABA (100 mg L^{-1}) at the PC stage showed the best effect in accelerating fruit ripening by enhancing the respiration rate, rather than being treated during the post climacteric stage (Zhang and Lu, 1983). Apparently, no research

work has been reported on the role of exogenously applied ABA in regulating 'Kensington Pride' mango fruit ripening.

2.4.2.3 ABA inhibitor

There are several compounds that have been used as inhibitors of ABA such as cycloheximide, actinomycin-D, Ca chelator, and fluridone, which delayed fruit ripening in 'Alphonso' and 'Langra' mangoes (Palejwala et al., 1988), inhibited ethylene production in 'Lichun' and 'Zongza 101' tomatoes (Zhu et al., 2003) and reduced the rate of fruit softening during storage in 'Zaoxian' and 'Hayward' kiwifruits (Chen et al., 1997). The vacuum infiltrated application of cycloheximide (1.0 mg mL^{-1}) to mature unripe mango delay fruit ripening and softening by blocking the increase in the level of hydrolytic and gluconeogenic enzymes (Palejwala et al., 1988). The effects of exogenously applied inhibitors of ABA biosynthesis on ripening of 'Kensington Pride' mango are yet to be investigated.

2.4.3 Auxins

2.4.3.1 Endogenous auxins

Endogenous auxins are among the naturally occurring plant hormones which may act as inhibitors of fruit ripening (Mapson, 1970). The levels of auxin-like substances were relatively higher during the period of rapid fruit growth and possibly contribute to the cell enlargement in 'Dashehari' and 'Alphonso' mango (Murti and Upreti, 1995; Murti and Upreti, 1999; Ram et al., 1983). Similarly, higher levels of auxin-like substances during rapid fruit growth phase have also been reported in peaches, kiwifruits, tomatoes and grapes (Buta and Spaulding, 1994; Chen et al., 1999; Chen et al., 1997; Nilgun and Nihat, 2005; Sheng et al., 2000; Wu et al., 2003), but declined during fruit ripening. The inhibition of ethylene action by auxins is possibly through the suppression of ACO and the induction of ACS gene expression (Kim et al., 2001; Ludford, 1987). No research work has been reported on changes in endogenous levels of indole-3-acetic acid (IAA) during fruit ripening period in mango and warrants to be investigated.

2.4.3.2 Exogenous application of auxins

The exogenous application of auxins in the form of 2,4-Diclorophenoxyacetic acid (2,4-D), 1-naphthaleneacetic acid (NAA), IAA, inhibited fruit ripening and softening

in mangoes (Kobiler et al., 2001; Wang et al., 2008a), pears (Frenkel and Dyck, 1973), peaches (Ohmiya, 2000), and bananas (Lohani et al., 2004). The exogenous dipped application of IAA (10^{-6} M) to mature green 'Alphonso' mango delayed ripening (Majmudar et al., 1981). Later on, the application of 175 mg L^{-1} and 150 mg L^{-1} 2,4-D to hard mature 'Tommy Atkins' and 'Keitt' (Kobiler et al., 2001) and 'Tainong' mango (Wang et al., 2008a) respectively, reduced fruit firmness and increased SSC. Furthermore, the vacuum infiltration of 1.0 mM IAA and/or 2,4-D to mature green 'Bartlett' pear inhibited softening and de-greening by preventing climacteric-rise (CR) in respiration but stimulated ethylene biosynthesis (Frenkel and Dyck, 1973).

In 'Harichal' bananas, the application of IAA (0.1 mM) delayed fruit ripening and softening by reducing the ethylene production and suppressed the activity of PE, PG, pectate lyase (PL) and cellulose (Lohani et al., 2004). Whilst, the application of higher concentration of 2,4-D (100 mM) on the same variety hastened ripening by advancing respiration rates, PG and PL activity (Payasi et al., 2004). The initiation of fruit ripening after the application of higher concentration of 2,4-D (150 mg L^{-1}) may due to the enhancement of the endogenous ABA as reported earlier in 'Tainong' mango fruit (Wang et al., 2008a). Apparently, no research has been reported on effects of the exogenously applied IAA on ripening of 'Kensington Pride' mango fruit.

2.4.3.3 Antiauxins

The post-harvest application of different concentrations (0.1 - 1.0 μM) of 3-methylene oxindole (3-Meox) to hard mature 'Bartlett' pears enhanced fruit ripening and softening by advancing the climacteric peak of ethylene production (Frenkel, 1975). Another compound that has been classified as acting as an antiauxin is α-(p-chlorophenoxy) isobutyric acid (PCIB), which has been proposed by Burstrom (1950). The ripening of mature green pears from the same variety was hastened after treatment with 0.02, 0.2, and 2.0 mM PCIB by accelerating the onset of chlorophyll degradation, softening, and CO_2 evolution (Frenkel and Haard, 1973). It is surmised that exogenous application of antiauxin may influence ripening of mango fruit, but not research work has been reported on this aspect.

2.4.4 Cytokinins

2.4.4.1 Endogenous cytokinins

Endogenous cytokinins present both in the seed and in the pericarp of mango and have been found to increase during rapid seed and fruit growth (Ram, 1983). Ram et al. (1983) have reported that eleven fractions of endogenous cytokinins-like substance were successfully isolated from the pericap and seed of 'Dashehari' mango. The increase in cytokinin-like substances proceeded the period of rapid cell division and cell enlargement and coincided with the rapid cell enlargement until 3 weeks after pollination; however, the concentration was low during maturation (Ram et al., 1983). In 'Alphonso' mangoes, cytokinin activity increased in the pulp from 15 days after fruit set (DAFS), and peaked at 45 DAFS (Murti and Upreti, 1997). A similar trend was found in 'Delaware' grapes when the endogenous cytokinins activity increased at the early stage of fruit development, but then declined to lower levels before the initiation of ripening (Inaba et al., 1976). No research work has been reported on the changes in the endogenous levels of various cytokinins in pericarp, mesocarp and seed of mango fruit during ripening period.

2.4.4.2 Exogenous application of cytokinins

The pre-harvest application of 10 mg L^{-1} N-(2-chloro-4-pyridyl)-N′-phenylurea (CPPU) in combination with 100 mg L^{-1} gibberellic acid (GA_3) from the early stage of fruit growth of 'Irwin' mango, promoted fruit enlargement, but decreased sugar content during full-ripe stage (Sasaki and Utsunomiya, 2002). The application of 10 mg L^{-1} CPPU sprayed during 14 days after blooming improved skin colour, taste, aroma, and retained better fruit firmness in 'Arumanis' mango during ripening (Notodimedjo, 2000). Pre-harvest application of 20 mg L^{-1} CPPU, 2 weeks after full bloom (WAFB) delayed fruit ripening by 6 weeks in 'Elliot' blueberries compared to the control (Koron and Stopar, 2006). In addition, the lower concentrations of CPPU (10 mg L^{-1}) applied between 10 to 15 days after 50% full bloom have also been reported to delay ripening in 'Elliot' blueberries (Serri and Hepp, 2006). On the other hand, a pre-harvest application of CPPU (20 mg L^{-1}), 2 WAFB or 15 DAFB, hastened the ripening of 'Hayward' kiwifruit by advancing the ethylene production, decreasing fruit firmness, and showing better SSC (Antognozzi et al., 1997; Costa et al., 1995). No research work has been reported on the effects of postharvest application of cytokinins on the ripening of 'Kensington Pride' mango.

2.4.5 Brassinosteroids (BRs)

Brassinosteroids (BRs) are a group of some 40 different steroids that are biosynthesized by plants and are potent hormones affecting many aspects of plant growth. Brassinolide (BL) (Figure 2.7) is the first BRs isolated from rape pollen in nature (Mitchell et al., 1970). BL and synthetic analogs were subsequently used in various bioassays, which indicated that, in addition to leaf bending, cell elongation, and cell division, BL also effects source/sink relationships (Krizek and Mandava, 1983), proton pumping and membrane polarization (Cerana et al., 1983; Romani et al., 1983a), photosynthesis (Braun and Wild, 1984; Krizek and Mandava, 1983), stress responses such as thermo-tolerance (Dhaubhadel et al., 1999; Wilen et al., 1995) and senescence (Mandava et al., 1981). In addition to these effects, BRs promote vascular differentiation (Iwasaki and Shibaoka, 1991; Yamamoto et al., 1997) and reorientation of microtubules (Mayumi and Shibaoka, 1995). Key observations in BRs's interaction with other hormones in stem elongation include a synergistic response with auxins and an additive effect with gibberellins (Mandava et al., 1981). However, BRs inhibit root elongation (Guan and Roddick, 1988) that may be the consequence of BRs-induced ethylene biosynthesis (Arteca and Bachman, 1987), and promoted fruit ripening (Symons et al., 2006).

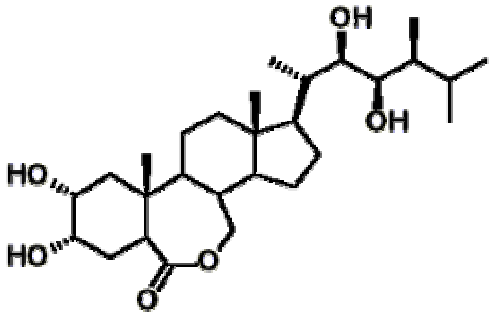

Figure 2.7. Brassinolide (Sakurai, 1999).

2.4.5.1 Endogenous BRs

Brassinosteroids (BRs) are a new class of plant hormones. As a prelude, BRs not only function as growth promoters, but also influence other physiological effects on the growth and development of plants (Braun and Wild, 1984; Mandava et al., 1981; Sakurai and Fujioka, 1993), and are promising synthetic plant regulators for practical application in agriculture. BRs has also been reported to affect ethylene biosynthesis

in mung bean (Yi et al., 1999) and there was a positive relationship between the increased BRs at the onset of ripening and the increased endogenous ethylene levels prior to veraison in grape berry (Chervin et al., 2004). Later on, the accumulation of endogenous BRs play a key role during fruit development and the onset of ripening in grapes (Symons et al., 2006). A clear pattern of changes in the endogenous levels of bioactive BRs - castasterone (CAS) was only evident throughout grape berry development. The endogenous level of CAS increased from ~ 0.9 ng g^{-1} fresh weight (FW) on 0 week after post flowering (WAPF) to ~ 2.3 ng g^{-1} FW on 2 weeks after post flowering (WAPF), following a mark decreased (10-fold lower) by 6 WAPF. CAS levels remained low, between 6 and 8 WAPF. However, a dramatic increase in the levels of CAS and its direct precursor, 6-deoxocastasterone in grape berry, was detected between 8 and 10 WAPF. This change coincided with the onset of grape berry ripening, as indicated by the increase in berry weight and SSC (Symons et al., 2006). However, no research work has been reported on the changes in endogenous levels of BRs in mango fruit during ripening and warrants to be investigated.

2.4.5.2 Exogenous application of BRs

Earlier study of exogenous application of epibrassinolide (Epi-BL) in rice before and at heading accelerated ripening and significantly increased starch content in hulled grain (Fujii and Saka, 2001). Later on, the exogenous application of 3.0 µM of 28-homobrassinolide (28-homo-BL) and 24-epibrassinolide (24-epi-BL) to tomato pericarp discs increased ethylene production, elevated levels of lycopene, lowered chlorophyll and ascorbic acid, increased reducing sugars and total sugars during fruit ripening (Vardhini and Rao, 2002). In litchi, exogenous application of 1.0 mg L^{-1} BL at full blossom and early fruit stage promote fruit softening processes by increasing the changes in pectin, protopectin, PE and PG activities (Peng et al., 2004). The exogenous application of Epi-BL has also been tested in grape berry (Symons et al., 2006). The application of 5.0 µL Epi-BL on 'Cabernet Sauvignon' grapes hastened berry ripening and increased SSC (Symons et al., 2006). In climacteric fruit, the foliar spray of BRs analogue (BB-16) (0.3 mg L^{-1}) in three consecutive weeks after the appearance of the first flowers (WAFF), has been reported to promote fruit ripening through the increased SSC of passion fruit (Gomes et al., 2006). No research work has been reported on the effects of postharvest application of BRs on the ripening of 'Kensington Pride' mango.

2.4.5.3 BRs inhibitor

The application of an inhibitor of BRs - brassinazole (10 µg g^{-1}), 5 WAPF, retarded the anthocyanin production and delayed the first appearance of coloring in the grape berry skin as an indicator of the onset of ripening and also reduced sugar accumulation (Symons et al., 2006). Apparently, no information is available on the role of BRs inhibitor in regulating mango fruit ripening, and this warrants further investigations.

2.4.6 Nitric oxide (NO)

2.4.6.1 Endogenous level of NO

Endogenously-produced NO gas appears to be a natural plant growth regulator in a wide variety of climacteric and non-climacteric fruits, flowers and vegetables (Leshem et al., 2001). The endogenous levels of NO were reported to be higher in immature or unripe than in mature or ripe climacteric and non-climacteric fruits such as in strawberry, avocado, banana, cherry tomato, kiwifruit, persimmon and orange (Leshem, 2000a; Leshem and Pinchasov, 2000; Leshem et al., 1998). However, the difference between unripe and ripe stages was most marked in the avocado and in the banana, where the unripe tissue contained approximately 10-fold and 4-fold more NO than in the ripe stage, respectively (Leshem et al., 1998). The endogenous levels of ethylene and NO during fruit development, maturation and senescence in horticultural crops (Corpas et al., 2004; Leshem and Pinchasov, 2000; Leshem and Wills, 1998), thereby offering an opportunity for modulation of their levels with exogenous application to exert the opposite effect.

2.4.6.2 Exogenous application of NO

3.4.6.2.1 NO on ethylene production and ripening

NO has been reported to cause disturbances in ethylene formation by inhibiting hydrogenation of ethylene from ethane (Burnham and Pease, 1940). It was supported by Leshem and Wills (1998), and claimed that NO play an important role in regulation of fruit ripening and senescence. It was well documented that ethylene promotes the fruit ripening process; a reduction in its biosynthesis can be helpful to extend ripening period and shelf life of fruit. The exogenous applications of NO have been reported to suppress and/or delay the climacteric peak of ethylene production, thus delay the fruit ripening either during ripening or cold storage in various types of

fruits (Aboul-Soud, 2010; Cheng et al., 2009; Eum et al., 2009; Flores et al., 2008; Liu et al., 2007; Singh et al., 2009; Sozzi et al., 2003). The application of NO treatment in the range of concentration (5, 10, and 20 μL L^{-1}) has been reported to reduced and/or delayed the climacteric peak of ethylene production during ripening at ambient temperature (20°C - 27°C) in apple (Wang et al., 2008b), plum (Singh et al., 2009), peach (Flores et al., 2008; Liu et al., 2007), and pear (Sozzi et al., 2003). NO fumigation (5 μL L^{-1} and 10 μL L^{-1}) also resulted in suppressing ethylene production during 35 days of cold storage (5°C) and ripening period for 7 days at 25°C (Zhu et al., 2006). The reduction in ethylene production was noticed in banana slices infiltrated with sodium nitroprusside (SNP) during ripening for 5 days at ambient temperature (Cheng et al., 2009). Recently, the suppression of ethylene production in kiwifruit dipped with 1.0 μmol L^{-1} NO during ripening has also been reported by Zhu et al. (2010b). However, the application of NO did not show any effect on the rate of ethylene production in apple discs during 5 h after treatment at 20°C (Rudell and Mattheis, 2006). In general, the post-harvest application of NO treatment suppress of ethylene production, however, its effects on the rate of ethylene production during ripening and cold storage needs to be elucidated in mango fruit.

2.4.6.2.2 NO and ethylene biosynthesis

The mechanism of action of NO in delaying ripening and senescence of post-harvest horticultural produces was not completely understood and earlier it has been suggested due to the inhibition of ethylene biosynthesis (Leshem, 2000b; Leshem and Haramaty, 1996; Leshem et al., 1998). The exogenous application of NO has been reported to inhibit ethylene biosynthesis in both climacteric and non-climacteric fruit such as banana, peach and strawberry (Cheng et al., 2009; Liu et al., 2007; Zhu et al., 2006; Zhu and Zhou, 2007), indicating a possible stoichiometrically competitive relationships. The decrease in ethylene production is possibly through the mechanism of the binding of NO to ACO forming binary ACO-NO complex, which is then chelated by ACC to produce stable ACC-ACO-NO complex (Manjunatha et al., 2010). As reported in peach, the prevention of autocatalytic ethylene biosynthesis in NO-treated fruit appeared due to inhibition the activities of ACO (Zhu et al., 2006) and/or ACS (Liu et al., 2007) with higher 1-malonyl

aminocyclopropane-1-carboxylic acid (MACC) and ACC content during ripening or cold storage. In strawberry – a non-climacteric fruit, exhibited an inhibition of ethylene biosynthesis as well as suppression and delayed the peak of ACS activity and ACC content, without any significant changes in ACO activity of NO-treated fruit during ripening at ambient temperature (Zhu and Zhou, 2007). The application of 200 μL L^{-1} NO to mature green (MG) and break colour (BC) of tomato fruit exhibited lower ACC content during the first 9 and 4 days of ripening at 20°C, respectively, as compared to untreated fruit, respectively (Eum et al., 2009).

The application of NO treatment also regulates the expression of gene encoding for ACO and ACS enzymes. The application of NO treatment (5 mM SNP) to the slices of banana cultivar 'Brazil' ripen at 24°C for 5 days reduced ACO activity and also down-regulated the expression of gene encoding for ACS and ACO (*MA-ACS1* and *MA-ACS1*, respectively) (Cheng et al., 2009), expansion gene (*MaExp1*) as well as ethylene receptor gene (*MaERS2*) (Yang et al., 2010). In tomato, the expression of ACO gene (*LeACO1*, *LeACOH2* and *LeACO4*) decreased and/or delayed in NO-treated fruit applied at MG and BC stage during ripening period than control fruit (Eum et al., 2009). However, the effects of post-harvest application of NO on regulation of ethylene biosynthesis, activities of ACS and ACO enzymes and ACC content during ripening or cold storage have not been studied in mango, except Zaharah and Singh (2011a) which is based on the experimental results presented in Chapter 7.

2.5 Physico-chemical changes during ripening

2.5.1 Respiration

Respiration is a central process in all living cells that mediates the release of energy through the breakdown of carbon compounds and the formation of carbon skeletons necessary for maintenance and synthetic reactions after harvest (Kays and Paull, 2004b). In climacteric fruit, the CR in respiration, which reflects enhanced metabolic activities, occurs at the transition from the growth phase of the fruit to its senescence phase. It coincides with increased rate of ethylene production and changes associated with ripening, such as colour, softening, increased tissue permeability, and development of characteristic aroma (Kader and Saltveit, 2003). The relationship between respiration and ethylene production in climacteric fruit was shown in Figure

2.8. Bashir and Abu Goukh (2003) have reported that increased respiration in climacteric fruit showed an increased SSC, total sugars and a decrease in flesh firmness. Another study, on post-harvest behaviour of respiration and ethylene production in papaya fruit (*Carica papaya* L.) harvested at different maturity stages, showed the period to climacteric peak after harvest differ among maturity stages of the fruit (YaHui and LihShang, 2005). Rate of respiration during ripening in mangoes is influenced by cultivar (Araiza et al., 2005), maturity stages at harvest (Mohammed and Brecht, 2002), pre- and post-harvest environment factors (Chonhenchob and Singh, 2004), storage temperature (Nakamura et al., 2003), atmospheric composition (Nakamura et al., 2004), exposure to ethylene (Lalel et al., 2003d; Montalvo et al., 2007; Nair and Singh, 2003), mechanical injury and decay (Mohammed and Brecht, 2002).

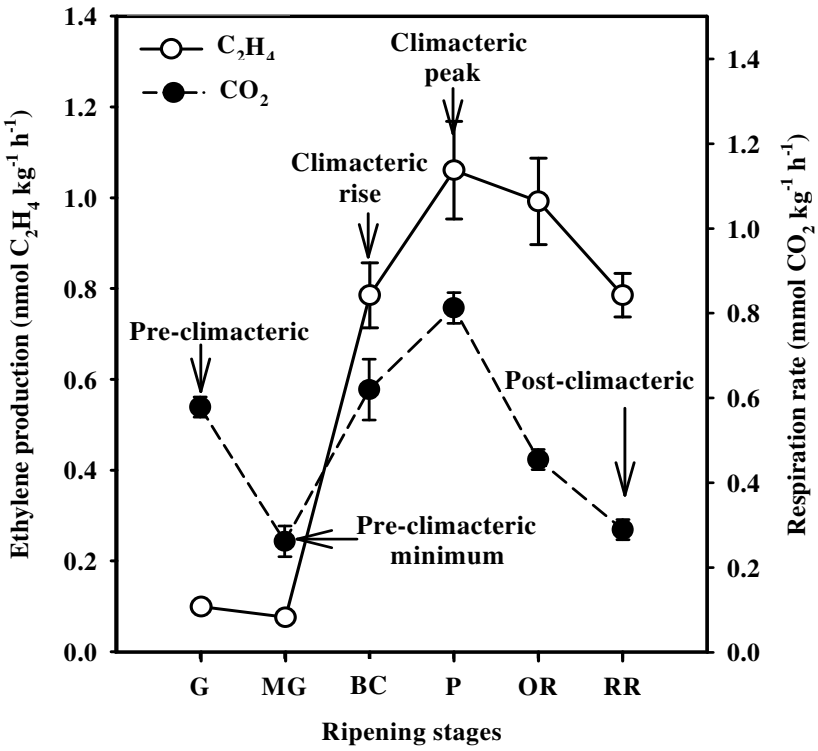

Figure 2.8. Relationship between respiration and ethylene production in climacteric fruit at different ripening stages G = Green; MG = Mature Green; BC = Break Colour; OR = Orange; RR = Red Ripe. Adapted from Watada et al. (1984), and Rothan and Nicolas (1989).

2.5.2 Fruit Softening

Pulp firmness is important for the evaluation of fruit maturity, and determines the consumer acceptance. The principle changes during fruit ripening associated with the increased cell wall activity leading to fruit softening. The changes in fruit texture are cultivar dependent and occur due to cell walls changes and pectic substances in the middle lamella (Selvaraj and Kumar, 1989). Higher solubility of cell wall pectins is important in promoting mango fruit softening (Lazan et al., 1986; Nasrijal, 1993; Roe and Bruemmer, 1981; Tandon and Kalra, 1984). There is a comparable rate of pectin solubility, which begins earlier in the inner of mesocarp tissues than in the outer part (Lazan et al., 1993). Mitcham and McDonald (1992) reported that the outer mesocarp of 'Keitt' remains firm for a longer period than 'Tommy Atkins' mangoes, and both cultivars had softer inner part than the outer mesocarp at ripening stage.

The cell wall hydrolases implicated in pectin depolymerization in mango are polygalacturonase, pectinesterase (EC 3.1.1.11), *endo*-1,4-*β*-D-glucanase (EC 3.1.1.4), *β*-galactosidase (EC 3.2.1.23), pectate lyase (EC 4.2.2.2), cellulase (EC 3.2.1.4), galactanase (EC 3.2.1.145), arabinanase and xylanase (EC 3.2.1.8) (Abu-Sarra and Abu-Goukh, 1992; Ali et al., 1990; Ali et al., 1995; Ali et al., 2004; Ashraf et al., 1981; Chourasia et al., 2006; Chourasia et al., 2008; Lazan et al., 1986; Lazan et al., 1993; Mitcham and McDonald, 1992; Prasanna et al., 2005; Prasanna et al., 2003; Roe and Bruemmer, 1981). The role of polygalacturonase, pectinesterase, *endo*-1,4-*β*-D-glucanase and *β*-galactosidase in mango fruit softening during ripening is further discussed in Section 2.5.2.1 – 2.5.2.4.

2.5.2.1 Polygalacturonase

The presence of polygalacturonase (PG), the enzyme responsible for degrading the (1→4)-linked galacturonic acid residues, has been reported in ripening mangoes (Lazan et al., 1993). PG exists as *exo*-PG (EC 3.2.1.67) or *endo*-PG (EC 3.2.1.15). In general, PG tends to be absent or barely detectable in green mango fruit; its activity appears only with the onset of ripening and increases dramatically during ripening (Abu-Sarra and Abu-Goukh, 1992; Ali et al., 2004; Chaimanee, 1992; Lazan et al., 1986; Mitcham and McDonald, 1992; Prasanna et al., 2003; Roe and Bruemmer, 1981; Suntornwat et al., 2000). There was a differential rate of PG activity in mango

fruit with higher activity in the inner mesocarp than the outer tissue, thus accelerating fruit softening (Mitcham and McDonald, 1992). The increase in the activity of *exo*-PG was more pronounced than the activity of *endo*-PG in 'Nam Dok Mai' mango during ripening (Suntornwat et al., 2000). Similarly, Chaimanee et al. (2000) reported the increase in *exo*-PG was highly correlated with ripening, whereas *endo*-PG activity was constant. A significant correlation between loss of firmness and PG activity was found in 'Keitt' mango (Roe and Bruemmer, 1981). On the other hand, Abu-Sarra and Abu-Goukh (1992) and Lazan et al. (1986) suggested that there was a non-significant correlation and contradictory reports between PG activity in relation to mango fruit softening. The *exo*-PG enzyme hydrolyses the terminal α(1–4)-linked galacturonic acid residues while *endo*-PG hydrolyses the same linkage but randomly (Abu-Sarra and Abu-Goukh, 1992; Lazan et al., 1986; Lazan et al., 1993; Suntornwat et al., 2000). However, the information on the changes in PG activity influence by endogenous or exogenous application of plant hormones during mango fruit ripening is lacking and warrant to be investigated.

2.5.2.2 Pectinesterase

Pectinesterase (PE) has been detected to catalyse the de-esterification of methyl groups from acidic pectins during mango fruit ripening (Abu-Sarra and Abu-Goukh, 1992; Ali et al., 1990; Roe and Bruemmer, 1981; Tahir and Malik, 1977). The activity of PE has been reported to decline or in some cases it showed constant trend in mango fruit during ripening (Ali et al., 2004; Ashraf et al., 1981; El-Zoghbi, 1994; Prasanna et al., 2003; Roe and Bruemmer, 1981; Lelyveld and Smith, 1979). Ashraf et al. (1981) noticed that peel of some Pakistan mango varieties exhibit higher PE activity than pulp. Similarly, the activity of PE has been reported to increase in 'Irvingiagabonensis' African mango during ripening (Aina and Oladunjoye, 1993). The role of PE in softening of mango fruit in relation to plant growth regulation is still not well understood.

2.5.2.3 Endo-1,4-β-D-glucanase

Endo-1,4-*β*-D-glucanase (EGase) are belong to a multigene family and classified as one of the major cell-wall hydrolases which contributes to mango fruit softening during the later stages of ripening (Chourasia et al., 2008). Earlier, the trend of EGase activity has been reported to increase in various mango varieties during

ripening or cool storage (Abu-Sarra and Abu-Goukh, 1992; Ketsa and Daengkanit, 1999; Roe and Bruemmer, 1981). The information reported on the roles of phytohormones in regulating EGase activity in mango fruit during ripening or cold storage is sporadic and inconclusive.

2.5.2.4 β-galactosidase

There was a significant correlation between the changes in the activities of *β*-galactosidase (*β*-Gal) and fruit softening. Moreover, the increased pectin solubility and degradation suggests that *β*-Gal play an important role in cell-wall pectin modification in mango fruit softening during ripening (Ali et al., 1995). *β*-Gal activity exhibits a parallel increase to fruit softening during ripening. Additionally, *β*-Gal which having galactanase activity through hydrolysis of galactans and arabinogalactans may increase the loss of neutral sugars during ripening in 'Sensation' mangoes (Seymour et al., 1990).

2.5.3 Soluble sugars

Increased fruit sweetness due to higher soluble sugars concentration during ripening is considered as important compositional change in relation to mango taste. Determination of SSC is a simple method to determine the sweetness of mango fruit during ripening. Basically, mango fruit has a tremendous increase in the SSC from harvest to ripe stage. SSC increased from 6.2% to 14.0% in 'Kensington Pride' (O'Hare, 1995), from 4.9% to 11.6% in 'Keitt' (Medlicott and Thompson, 1985) and from 7.0% to 15.0% in 'Alphonso' mangoes (Thomas, 1975).

Sugar accumulation has a close relationship with starch content. During mango developmental stage, starch content accumulates in chloroplasts and completely hydrolysed to sugars during ripening (Ito et al., 1997; Kumar et al., 1994; Medlicott et al., 1986; Selvaraj et al., 1989). In most of mango cultivars including 'Alphonso', starch content is higher (14%) at the immature stage than at the ripe stage (0.3%). Similarly, in 'Irwin' mangoes, starch is almost undetectable at ripe stage, with high or small increases in sucrose or fructose, respectively (Ito et al., 1997). Starch content shows only small decreases during ripening of 'Haden' mango, and it was insufficient for sucrose conversion (Castrillo et al., 1992). Ripe mango fruit contains 10–20% total sugars depending upon cultivar and stage of ripening (Litz, 2009).

During early ripening stage, fruit contain higher reducing sugars, whilst at ripe stage non-reducing sugars dominate in mango fruit. Medlicott and Thompson (1985) reported that ripe 'Keitt' mango fruit contain sucrose (57%), fructose (28%) and (glucose 15%). A simultaneous increase of glucose, fructose and sucrose during mango ripening has been reported by Krishnamurthy et al. (1971), Lakshminarayana (1973; 1975) and Shashirekha and Patwardhan (1976). 'Haden', 'Irwin', 'Kent' and 'Keitt' mangoes exhibits a gradual reduction in glucose and fructose and a continuous increase in sucrose concentrations during fruit ripening (Vazquez-Salinas and Lakshminarayana, 1985).

Increased starch hydrolysis through amylase (EC 3.2.1.1) activity resulted in higher sucrose accumulation during mango fruit ripening (Fuchs et al., 1980; Mattoo and Modi, 1969a; Tandon and Kalra, 1984). Active sucrose metabolism (Kumar et al., 1994), indicating that mango mesocarp had higher activities of sucrose synthase (SUS, EC 2.4.1.13) and invertase (SI, EC 3.2.1.26) during ripening period. Castrillo et al. (1992) reported ten times higher activity of SS enzyme during the rapid phase of sucrose accumulation, whilst fructose as a reducing sugar showed slightly increase during ripening. The activity of SS enzyme was found to be higher as compared to the activities of neutral and acid invertases (AI, EC 3.2.1.26). The effects of exogenous application of phytohormones on sugar metabolism in mango fruit during ripening warrants to be investigated.

2.5.4 Organic acids

The changes in the concentrations of organic acids have important role for flavour constituents and also respiratory activity. Substantially losses occur in organic acids from maturation to ripening stages. Citric and malic acid are predominant acids in mature mango fruit; other organic acids such as tartaric, succinic, shikimic, and fumaric acid are present only in low concentrations (Medlicott and Thompson, 1985; Sarker and Muhsi, 1981; Shashirekha and Patwardhan, 1976; Singh and Singh, 2011). During fruit development, citric acid content steadily increased in 'Irwin' mangoes synthesized by AI activity, attaining a higher level at the early stage of the endocarp-hardening, and decreases during fruit maturation and ripening (Ito et al., 1997). Citric and malic acids are major organic acids in 'Keitt' mango, but tartaric, oxalic, ascorbic and α-ketoglutaric acids are present in a small amount. The

substantial decrease in acidity is due to higher citric acid losses, with a small proportion loss in malic acid (Medlicott and Thompson, 1985). The major organic acid in 'Badami' mangoes is citric acid, but malic and succinic acids present at low concentration (Shashirekha and Patwardhan, 1976). Oxalic, citric, malic, pyruvic and succinic acids have been detected in 'Fazli', whilst tartaric acid in 'Zardalu' mangoes (Kumar et al., 1993). Lizada (1993) reported that the concentration of citric and succinic acids reduced during ripening with no clear trend in malic acid depending on cultivar.

The organic acids (oxalic, succinic, pyruvic, oxaloacetic and α-ketoglutaric acids) in mango fruit were synthesized through tricarboxylic acid cycle reactions. The maximum concentration of α-oxoglutaric and pyruvic acids in 'Pairi' mangoes, appear prior the climacteric peak. The concentrations of aspartic and glutamic acid shows the highest level after 3 days of harvest (Krishnamurthy et al., 1971). Malic enzyme (EC 1.1.1.40) transform to pyruvic acid via oxidative decarboxylation catalyses from L-malic in 'Alphonso', 'Banganpalli', 'Dasheri', 'Fazli', 'Langra', and 'Suvarnarekha' mangoes during three-quarter-ripe and ripe stages (Selvaraj and Kumar, 1994). The levels of malic dehydrogenase (EC 1.1.1.37) and succinic dehydrogenase (EC 1.3.5.1) increase during the onset of ripening; whereas, the level of citrate synthase (CIS, EC 2.3.3.1) increases several-fold during maturation in 'Alfonso' mangoes (Baqui et al., 1974). Dubery et al. (1984) claimed activity of malic enzyme was maximum just after the climacteric peak and decline during postclimateric stage. The activity of phosphoenol pyruvate carboxylase (PEPC; EC 4.1.1.49) and pyruvate decarboxylase (EC 4.1.1.1) exhibits differential pattern during ripening which are cultivar dependant. The activity of PEPC is relatively high in 'Alphonso' and 'Langara', but the activity is low in 'Dashehari' and 'Totapuri' mangoes during ripening period (Selvaraj and Kumar, 1994). However, the role of plant growth regulator in regulating metabolism of organic acids during ripening warrants to be investigated.

2.5.5 Fruit colour

Mango skin colour is important indication of harvest maturity, for visual and overall quality, processing and consumption (Cocozza et al., 2004a; Gonzalez-Aguilar et al., 2001; Jha et al., 2007; Mahayothee et al., 2004). The good indication of fruit ripen

rightly is when the skin be able to change from dark to olive green, sometimes reddish, orange-yellow or yellowish colour. All mango cultivars show green colour losses, but some cultivars retain green colour when it ripe. Development of reddish blush in some of the cultivars has been attributed to the accumulation of anthocyanins in fruit skin.

The skin pigmentation occurs when chloroplasts are transformed to chromoplasts containing yellow or red colour (John et al., 1970; Lakshminarayana, 1980; Lizada, 1993; Parikh et al., 1990). In unripe mango, chloroplasts of cells in the peel was developed from well-arranged grana and osmiophilic globules (Parikh et al., 1990), and the integrity lose as the ripening proceeds. Carotenoids and xanthophylls are the predominant pigments in mango cultivar which has yellow skin colour. Whilst, skin with reddish colour mango cultivars contain anthocyanin paenoidin-3-galactoside (Proctor and Creasy, 1969). The concentration of chlorophyll decreases substantially in 'Keitt' mangoes, while the concentration of carotenoids increases and anthocyanin decreases gradually in 'Tommy Atkins' during ripening period (Medlicott et al., 1986). Mitcham and McDonald (1992) reported that 'Tommy Atkins' develop more red and yellow pigmentation in the peel and mesocarp than 'Keitt' mangoes. Peel colour is not an appropriate indicator of maturity index, because some of the cultivars, the fruit softening occurs prior to the changes in skin colour.

Due to a high carotenoid contents (~ 90 mg kg^{-1}), the development of an intense yellow to orange colour occur in mango pulp which is rich in vitamin A. The level of carotenoid in the pulp is dependent on cultivar. For example, in 'Alphonso' mango, 50% from 16 fractions of carotenoid are β-carotene (John et al., 1970; Jungalwala and Cama, 1963). The carotenoid composition did not show any qualitative changes from mature-green to the ripe stage in 'Keitt' and 'Tommy Atkins' mangoes, but it has quantitative changes during ripening (Mercadante and Rodriguez-Amaya, 1998). In 'Badami' mangoes, about 15, 14 and 17 carotenoids has been detected at mature-green, partially ripe and fully ripe stages, respectively (John et al., 1970). Other than cultivar differences, the variation in pigment types and quantities is due to geography and climate, maturity stages and pre- as well as post-harvest treatments and analytical methods used for their determination.

2.5.6 Aroma

Aroma influences the consumer perception. Because different type of volatile compound contributes to mango aroma, it's considered as a very complex quality attribute. There are many factors which influence the aroma production in mango fruit such as cultivar, harvest maturity, CI, post-harvest treatments, growth regulators for example ethylene and jasmonates, ripening and storage conditions (Lalel, 2002; Lalel and Singh, 2006; Lalel et al., 2001; Lalel et al., 2003a; Lalel et al., 2003b; Lalel et al., 2003c; Lalel et al., 2003d; Lalel et al., 2003e; Lalel et al., 2003f; Lalel et al., 2004a; Lalel et al., 2004b; Nair et al., 2003; Singh et al., 2004). Other factors such as rootstock (Dang, 2007), polyamines, hot water dip (Dea et al., 2010), fungicide treatments (Dang et al., 2008b), and edible coatings (Dang et al., 2008a) have also been reported to influence aroma volatiles production in ripe mango fruit. In mango, terpenes are the most abundant group of compounds among other major volatile compounds, followed by esters, ketones and lactones. Fatty acids increases during ripening of mango fruit. Lalel et al. (2003a) identified 35 new compounds from the total of 61 aroma volatile in 'Kensington Pride' mango. In their study, (+)-Spathulenol and β-maaliene were reported for the first time in mango fruit. Hydrocarbons and esters, accounting for about 59% and 20% of the total identified compounds, respectively, has been detected the most abundant group of volatile compounds. The major compound produced during the first 7 days of ripening was α-terpinolene, followed by ethyl octanoate which became the major compound. The concentration of major monoterpenes (except for car-3-ene) increased for the first 3 or 4 days and decreased afterwards. During early ripening process, most of the major sesquiterpenes were intensively synthesised. Moreover, the production of three major esters has been detected to increase quite sharply during fruit ripening. The production of terpenes and esters appeared parallel with production of ethylene and fatty acids, respectively (Lalel et al., 2003a). Various pre- and post-harvest factors affecting production of aroma volatiles have been investigated in detail and has implications in international mango trade (Singh et al., 2004).

2.6 Roles of phytohormone on fruit softening and physico-chemical changes

2.6.1 Ethylene

Ethylene has been used to enhance the visual appearance of fresh commodities by accelerating ripening and rapid development of colour with the degradation of

chlorophyll. Inhibition, suppression or removal of ethylene delay the colour changes in storage and prolong the shelf life of stored commodities (Saltveit, 1999). For instance, the application of as low as 100 mg L^{-1} ethephon or higher than that up to 2, 500 mg L^{-1} ethephon has been reported to accelerate the reduction of fruit firmness and improve fruit quality by increasing the rate of respirataion SSC, SSC:TA ratio, reducing and non-reducing sugar, ascorbic acid, carotenoid content and better flavour and aroma score in various types of mango cultivars including the 'Kensington Pride' (Lalel et al., 2003e; Nair and Singh, 2003; Singh and Janes, 2001), 'Dashehari' (Chundawat et al., 1973; Mann, 1985), 'Muloga' and 'Baneshan' (Shanmugavelu et al., 1976), 'Keitt' (Coneglian and Rodrigues, 1994; Sergent et al., 1993), 'Kent' (Centurion et al., 1998), 'Neelum' (Kulkarni et al., 2004) and 'Amrapalli' (Singh et al., 1995). On the other hand, Kumar and Dhawan (1995) found that when 'Dashehari' mango were treated with 500 mg L^{-1} ethephon in conjunction with hot water (50 ± 5°C for 10 min) they exhibited a slow rate of ripening, low carotenoid concentration, maintained better texture and colour, as well as 8 days prolonged shelf life than untreated fruit. Similarly, 100 mg L^{-1} ethephon treated fruit following storage at room temperature (25°C - 28°C, 85 - 90% RH) had maintained better fruit firmness in 'Dashehari' mangoes, than the application of 200 or 400 mg L^{-1} ethephon (Pal, 1998). Pre-harvest application of 500 – 1, 000 mg L^{-1} ethephon a week prior to harvest did not show any difference in pH, TA, SSC and total sugars during ripening at 28°C (Coneglian et al., 1993).

2.6.1.1 1-Methylcyclopropene (1-MCP)

1-MCP has been reported to reduce fruit softening in climacteric and non-climacteric fruit, including mangoes (Watkins, 2008; Watkins and Miller, 2004). Influence of 1-MCP on fruit softening and the quality of mango fruit depends on various factors, including cultivar, concentration used, storage duration and condition, time, number of application and stage of maturity (Alves et al., 2004; Cocozza et al., 2004a; Hojo et al., 2006; Lalel et al., 2003e; Lima et al., 2006; Santos et al., 2004; Silva et al., 2004; Singh and Neelam, 2008; Wang et al., 2006).

The application of 1-MCP (1, 10, and 25 µL L^{-1}) for 6 h exposure reduced the respiration rate, fatty acids content, and the biosynthesis of total aroma volatiles, monoterpenes, sesquiterpenes, aldehydes, total esters, alcohols and tetradecane of

'Kensington Pride' mango fruit during ripening at ambient temperature (Lalel et al., 2003e). The exogenous application of 300 nL L^{-1} 1-MCP to 'Kent' mangoes delayed fruit ripening by maintaining fruit firmness and prolong the shelf life by 4 days compared to untreated fruit (Osuna et al., 2005). In 'Nam Dok Mai' mangoes, post-harvest application of 1-MCP (250, 500, and 1, 000 µg L^{-1}) delayed fruit softening and skin colour development (Penchaiya et al., 2006).

In 'Tommy Atkins' mango, the exogenous application of 900 nL L^{-1} 1-MCP stored at 11°C retarded fruit softening, regardless of number of applications, and the application of higher concentrations (1, 200 nL L^{-1} 1-MCP) at 25°C gave the same responses (Lima et al., 2006). Application of 1-MCP (1, 500 nL L^{-1}) at the end of refrigeration time has been reported to increase chroma value (C*) with a gradual reduction in the hue angle (h°) of the 'Tommy Atkins' mango's skin, and when the treatment was applied at the beginning of the refrigeration it delays the reduction of TA (Lima et al., 2007). In another study, mango cultivar 'Tommy Atkins' was treated with 100 nL L^{-1} 1-MCP without MAP which was effective in controlling losses in fruit firmness losses and improved pulp colour, aroma and taste (higher TA and ascorbic acid) than mangoes treated with 100 nL L^{-1} under MAP (Cocozza et al., 2004a; b).

The reduction in fruit softening losses in mango fruit together with the delay in the increase of SSC when fruit was treated with 1.0 mg L^{-1} 1-MCP has been ascribed due to the inhibition of PE and PG activity, with higher inhibition in PG activity was reported in the 'Chausa' compared to the 'Langra' and 'Dashehari' mango. The reduction of PE activity was found to be higher in the 'Dashehari' than in the 'Chausa' mango (Singh and Neelam, 2008). Similarly, the activities of total and soluble pectin, pectic solubilisation, PE, PG, and α-D-galactosidase in the 'Palmer' mango were suppressed with the application of 150 nL L^{-1} 1-MCP for 12 h at ambient temperature, thus reducing fruit softening until the 18^{th} days of storage (Hojo et al., 2006). Better external appearances has been reported after the 'Jasmim', 'Rosa', and 'Espada' mango fruit was incubated with 100 µg L^{-1} 1-MCP for 24 h during the PC stage, followed by ripening at 23°C (Silva et al., 2004). The effect of 1-MCP dip application on fruit softening enzymes such as *endo*-, *exo*-PG, PE, and EGase has yet not been investigated in the 'Kensington Pride' mango.

2.6.1.2 Aminoethoxyvinylglycine (AVG)

Pre-harvest application of AVG before anticipated harvest significantly delayed fruit softening during ripening and caused fruit quality changes in various types of fruit crops including peaches (*Prunus persica* L. Batch), melons (*Cucumis melo*), nectarines (*Prunus persica*), apples (*Malus domestica*), and plums (*Prunus salicana*) (Amarante et al., 2005; Amarante et al., 2002; Brackmann and Waclawovsky, 2001; Byers, 1997; Chun et al., 1997; Drake et al., 2005; Greene and Schupp, 2004; Jobling et al., 2003b; Kang et al., 2002; Kondo and Hayata, 1995; Lu et al., 2007; Shellie, 1999; Silverman et al., 2004; Torrigiani et al., 2004).

Pre-harvest application of 150 mg L^{-1} AVG at 3 and 6 weeks prior to harvest in the 'Rubiduox' peach reduced fruit softening with better retention of skin background, but TA and SSC increased slowly and developed splitting on the surface of the skin during ripening in CA storage at 2°C (Amarante et al., 2005). In 'Premier' peaches, the flesh firmness and SSC increased at first harvest date following 150 mg L^{-1} AVG, 10 days prior to harvest than untreated fruit (Lu et al., 2007).

Pre-harvest spray application of Retain™ (250 mg L^{-1}) in 'Gala' and 'McIntosh' apples during 4 and 7 weeks prior harvest (WPH) delayed fruit softening by maintained flesh firmness for about 20 and 31 days respectively, retarded colour development, decreased SSC, starch index and TA (Amarante et al., 2002; Greene, 2000). In another study, starch degradation and red colour development of 'McIntosh' apples was delayed after spraying with 225 mg L^{-1} AVG during 2 and 4 weeks before commercial harvest (Greene and Schupp, 2004; Schupp and Greene, 2004). However, the pre-harvest application of AVG (125 mg L^{-1}) at 21 and 28 days prior harvest of 'Gala' apples did not show any difference on flesh firmness as compared to untreated fruit (Phan-Thien et al., 2004). Interestingly, pre-harvest application of 50 g $acre^{-1}$ AVG (4 WPH) on 'Redchief Delicious' apples did not affect the levels of amylopectin, fructose, malate, ascorbate, citrate, anthocynin, and soluble starch synthase, but reduced the accumulation of sucrose, glucose and sorbitol content (Silverman et al., 2004). The flesh firmness, SSC and fresh mass of 'Primo' melons exhibited no difference compared with untreated fruit at harvest and after storage when sprayed with 260 mg L^{-1} AVG between 12 and 18 days before harvest (Shellie, 1999). The inhibition of fruit softening due to the suppression of ethylene production

without affecting fruit quality occurred when 'Makuwa' melons were sprayed with AVG (100 mg L^{-1}), 20 days prior to harvest (DPH), decreased L* and C* values, but retained higher h° value in 'Mission' melons (Chung et al., 2000).

The exogenous application of AVG (100, 500 and 1, 000 a.i. mg L^{-1}) suppressed the respiration rate, fatty acids content, and the biosynthesis of total aroma volatiles, monoterpenes, sesquiterpenes, aldehydes, total esters, alcohols as well as tetradecane of 'Kensington Pride' mango fruit during ripening at ambient temperature (Lalel et al., 2003e). 'Arctic Snow' nectarines, sprayed with 125 mg L^{-1} AVG a week before commercial harvest followed by spraying with 30 µL L^{-1} ethylene after harvest kept at cold storage (1°C), exhibited higher fruit firmness than untreated fruit during the ripening period at 20°C (McGlasson et al., 2005), although no significant difference in sugar levels was reported (Rath and Prentice, 2004). Apparently, no information is available on the effects of pre-harvest application of AVG on the activity of fruit softening enzymes and fruit quality in mangoes.

2.6.1.3 Nitric oxide (NO)

Short period of fumigation of intact and fresh-cut horticulture commodities to a very low concentration of NO play a role in regulating fruit softening and the changes in fruit softening enzymes activities in various fruit crops such as in apple (Wang et al., 2008b), banana (Cheng et al., 2009; Yang et al., 2010), kiwifruit (Zhu et al., 2010b), plum (Singh et al., 2009; Zhang et al., 2008) peach (Flores et al., 2008; Sun et al., 2011; Zhu et al., 2010a; Zhu et al., 2006), and pear (Sozzi et al., 2003). The effects of NO treatment on respiration, fruit softening and physico-chemical are discussed in Section 2.6.7.1, 2.6.7.2 and 2.6.7.3 respectively.

2.6.2 Abscisic acid

Fruit softening of mangoes occur due to the degradation of the pectin component of the cell wall, therefore causing galactosyl and deoxyhexosyl residue losses (Lamikanra, 2002). The increases in gluconeogenic enzymes are also accompanied with increases in mango ripening (Lamikanra, 2002). Earlier, abscisic acid (ABA) has been reported to reduced fruit firmness and improved fruit quality in mango (Parikh et al., 1990; Zhou et al., 1996), banana (Jiang et al., 2000; Lohani et al.,

2004), kiwifruit (Chen et al., 2005; Chen et al., 1999; Chen et al., 1997) and strawberry (Jiang and Joyce, 2003).

The application of 5 mg L^{-1} ABA under ambient temperature to 'Zihua' mango promoted fruit softening by decreasing PE and increasing PG activity followed by the incremental rise in carotenoid content (Zhou et al., 1996). Exogenous application of ABA (10^{-6} M) reduced fruit firmness, increased starch degradation through increasing glucose-6-phosphatase enzyme activity and carotenoid pigment of 'Alphonso' mangoes thus hastening fruit ripening compared to untreated fruit (Parikh et al., 1990). The fruit quality of 'Alphonso' and 'Langra' mangoes had been improved following ABA (10^{-6} M) treatment through increasing total sugars, sucrose, fructose, carotenoid content and reducing acidity (Palejwala et al., 1988). Advanced fruit softening was also noticed during ripening in 'Robusta Harichal' bananas after being treated with 100 μM ABA through the enhancement of stimulation of PE and cellulase but not for PG activities (Lohani et al., 2004). The exogenous application of ABA (10^{-3} M) to bananas exhibited faster changes in skin colour than untreated fruit (Jiang et al., 2000).

The increased endogenous levels of ABA hastened fruit ripening and softening followed by the improved fruit colour development in 'Everest' strawberries (Jiang and Joyce, 2003). Likewise, 'Everest' strawberries treated with 10^{-4} to 10^{-5} M ABA at 20°C showed reduced fruit firmness coupled with increased the accumulation of anthocynin and phenolics, consequently accelerating red colour development (Jiang and Joyce, 2003). Similarly, the increased anthocynin content in 'Bigarreau Napoleon' cherries have been reported when fruit were reated with 0.1 mM ABA (Hartmann, 1992) and 10^{-4} M ABA (Hartmann, 1991), thus, stimulating reddening on the fruit skin. Increases of total sugars and anthocyanin synthesis was promoted during maturation, with little effect on chlorophyll degradation in litchi treated as dip treatment of 800 mg L^{-1} ABA (Wang et al., 2007). An application of ABA has been reported to promote fruit softening and accelerated fruit ripening in 'Zaoxian' and 'Hayward' kiwifruit and leading to the reduction in endogenous levels of indole-3-acetic acid and gibberellic acid (Chen et al., 2005).

The efficiency of post-harvest application of translation inhibitor cycloheximide (1.0 mg mL^{-1}), which block the effects of ABA on α-amylase expression cyclohexamide has been shown to significantly delay fruit softening, increased total acid and reduced total sugars, sugar:acid ratio, carotenoid content, sucrose and fructose, but maintained higher glucose in 'Alphonso' and 'Langra' mangoes (Palejwala et al., 1988). The effects of ABA and its inhibitors on the activities of fruit softening enzymes (*exo*-, *endo*-PG, PE, and EGase activities) during fruit ripening together with fruit quality changes need further investigations.

2.6.3 Auxins

The effects of exogenous application of indole-3-acetic acid (IAA) on the changes in fruit softening has been extensively studied in different cultivars of climacteric fruit such as mangoes (Kobiler et al., 2001; Wang et al., 2008a; Zhou et al., 1996), peaches (Ohmiya, 2000), pear (Frenkel and Dyck, 1973), and bananas (Lohani et al., 2004; Payasi et al., 2004).

Effects of exogenously applied IAA on ripening in different types of fruits varies depending upon cultivar, concentration, storage condition, and type of synthetic auxin used (Lamikanra, 2002). The exogenous application of 150 mg L^{-1} 2,4-D at 4°C delayed the occurrence of fruit softening and improved SSC and soluble sugar as well as delayed peel chlorophyll levels in 'Tainong' mangoes (Wang et al., 2008a). The exogenous application of lower IAA concentration (0.1 mM) suppressed fruit softening by suppressing the activity of PG, PE and PL enzyme, but the application of higher concentration 2,4-D (1.0 mM) promoted fruit softening in bananas (Lohani et al., 2004; Payasi et al., 2004).

In order to retard fruit softening in 'Barlett' pears, antiauxin called 3-methylene oxindole (3-Meox) has been successfully used (Frenkel, 1975; Frenkel and Haard, 1973). The higher concentration of 3-Meox (10.0 μM) diminished fruit softening as compared to its lower concentration (0.1 - 1.0 μM 3-Meox) (Frenkel, 1975). In addition, the onset of chlorophyll degradation and fruit softening was accelerated in 'Bartlett' pears treated with 0.02 mM PCIB (Frenkel and Haard, 1973). No research work has been reported on influences of auxin and antiauxin on mango fruit softening and warrants to be investigated.

2.6.4 Cytokinins

In mangoes, post-harvest application of 10 mg L^{-1} N-(2-chloro-4-pyridyl)-N′-phenylurea (CPPU) has reported reduced fruit firmness losses and improved skin colour, taste and aroma of the 'Arumanis' mango (Notodimedjo, 2000). However, the exogenous application of 100 mg L^{-1} CPPU in combination with 100 mg L^{-1} GA_3 decreased the sugar content of 'Irwin' mangoes (Sasaki and Utsunomiya, 2002).

Post-harvest treatment of CPPU (10 - 20 mg L^{-1}) applied 2 to 3 WAFB has been reported to reduce fruit firmness losses, increased SSC, as well as decreased TA in 'Hayward' kiwifruit after 125 days of cold storage at 0 ± 0.5°C (Antognozzi et al., 1997). On the other hand, the exogenous application of 20 mg L^{-1} CPPU at 2 WAFB in kiwifruit maintained fruit firmness and decreased SSC during storage time in -0.5°C (Costa et al., 1995). It seems the role of CPPU in the ripening of mangoes is still unclear and needs further investigation.

2.6.5 Gibberellic acid

Post-harvest treatment of 150 mg L^{-1} gibberellic acid (GA_3) has been reported to increase fruit firmness, SSC and TA in 'Srisaket 007' mangoes compared to the untreated fruit (Benjawan et al., 2006). Reddy and Haripriya (2002) have shown that application of 200 mg L^{-1} GA_3 and then being kept in a polyethylene bag and ripened at room temperature delayed colour development, slowed the increase in SSC and sugars, and retained TA in 'Bangalore' and 'Neelam' mangoes.

Pre-harvest GA_3 treatments (50 mg L^{-1}) in combination with post-harvest 500 nL L^{-1} 1-MCP stored at 1°C for 20 days followed by ripening at 20°C for 5 days increased fruit firmness, acetydehyde and SSC, but lowered the colour index of the 'Rojo Brillante' persimmon (Besada et al., 2008). Pre-harvest spray application of GA_3 (30 - 40 mg L^{-1}) to 'Fuyu' persimmon decreased pulp firmness, TA, chlorophyll, phenolic and carotenoid content (Ferri et al., 2002; Ferri et al., 2004). On the other hand, pre-harvest spray application of 250 gallon L^{-1} GA (30 g a.i. $acre^{-1}$ ProGibb®) treatments in combination of 0.05% 'Silwet L-77' as acidifier to maintain the acidity of the solution, did not improve post-harvest quality retention of Florida-grown 'Fallglo' and 'Sunburst' tangerines, 'Minneola' tangelos and 'Marsh' grape fruit in simulated commercial handling and storage (Ritenour et al., 2005; Ritenour and

Stover, 2000). Apparently, the reported research work on the effects of exogenous application of gibberellic acid on fruit ripening, softening, quality of mango fruit is sporadic and inconclusive.

2.6.6 Brassinosteroids (BRs)

Earlier, Vardhini and Rao (2002) reported the exogenous application of 3.0 μM of 28-homo-BL or 24-Epi-BL to tomato pericarp discs elevated the levels of lycopene, lowered chlorophyll and ascorbic acid, increased reducing sugars and total sugars during fruit ripening. Later on, the exogenous application of 1.0 mg L^{-1} BL has shown the changes in PE and PG activity during the early stages of fruit development as well as decreased fruit cracking of 'Nuomoci' litchi compared to untreated fruit (Peng et al., 2004). Exogenous application of BRs (3 mg L^{-1}) 3 weeks after anthesis increased the SSC in yellow passion fruit (Gomes et al., 2006). Similarly, the exogenous application of 5 μL Epi-BL in 'Cabernet Sauvignon' grapes increased SSC during ripening (Symons et al., 2006). Decreases in fruit firmness have been well documented in mango varies in different cultivars, including 'Harumanis' (Ali et al., 1995), 'Alphonso' (Prasanna et al., 2006; Yashoda et al., 2007), 'Keitt' (Roe and Bruemmer, 1981) and 'Dashehari' (Chourasia et al., 2006) due to the changes in the PE, PG and PL. NO research work has been reported on the role of brassinosteroids and its inhibitors on the regulation of fruit ripening, softening and the changes in mango fruit quality.

2.6.7 Nitric oxide (NO)

2.6.7.1 NO on respiration

The effect of NO occurred in both plant and animal mitochondria due to the competition with oxygen, it inhibit the function of cytochrome oxidase, thus reduces the rate of respiration in the cell (Brown and Cooper, 1994; Cleeter et al., 1994; Millar and Day, 1996). Considine et al. (2001) reported that cytochrome chain components play an important role in facilitating the climacteric burst of respiration before the alternative oxidase and uncoupling proteins, which play a role in post-climacteric senescence in mango fruit. The exogenous application of NO (10 - 20 μL L^{-1}) has been reported to reduce the respiration rate in 'Amber Jewel' plum during ripening for 10 days at ambient temperature (Singh et al., 2009). A similarly reduction in respiration rate has been reported in NO-treated 'Fuji' apple fruit during

ripening period (Wang et al., 2008b). The effects of post-harvest applications of NO on the changes of respiration rate in mango fruit during ripening has been reported by Zaharah and Singh (2011a; b; c) which is based on the experimental results presented in Chapter 6, 7, and 8.

2.6.7.2 Regulation of NO on fruit softening

Post-harvest application of NO have been claimed to regulate fruit softening during fruit ripening and during cold storage in climacteric and non-climacteric fruit (Cheng et al., 2009; Flores et al., 2008; Sozzi et al., 2003; Sun et al., 2011; Wang et al., 2008b; Yang et al., 2010; Zhang et al., 2008; Zhu et al., 2006; Zhu et al., 2010b). The exogenous application of NO (5, 10, 15, or 20 μL L^{-1}) retained higher fruit firmness in apple (Wang et al., 2008b), plum (Singh et al., 2009), peach (Flores et al., 2008; Sun et al., 2011; Zhu et al., 2010a; Zhu et al., 2006), and pear (Sozzi et al., 2003) during ripening at ambient temperature (20°C - 25°C) or cold storage (0°C - 5°C). Similarly, 'Xuxiang' kiwifruit and 'Damili' plum either dipped with 1.0 μmol L^{-1} NO or treated with 1.0 mM SNP delayed fruit softening during ripening or cold storage, respectively (Zhang et al., 2008; Zhu et al., 2010b).

The delay and reduction in fruit softening of 'Brazil' banana slices treated with 5 mM SNP has been ascribed to decreased activities of PG and EGase during ripening at 24°C for 5 days (Cheng et al., 2009; Yang et al., 2010). Additionally, Cheng et al. (2009) and Yang et al. (2010) also found the reduction in the activities of PE and *β*-Gal, respectively, in NO-treated of banana slices during ripening under the same ripening conditions and period. Similarly, activity of *endo*-PG in 'Feicheng' peach was suppressed in NO-treated fruit up to 20 days of cold storage (5°C) and the activity increased during later storage period (Zhu et al., 2010a). The activities of fruit softening enzymes such as *exo*-, *endo*-PG, PE, and EGase influenced by NO treatment have not been investigated in mango fruit, except those published by Zaharah and Singh (2011a) which is based on the experimental results presented in Chapter 7.

2.6.7.3 NO on other physico-chemical changes in the fruit

The inhibition of ethylene production by NO greatly influences the post-harvest quality in many climacteric fruit. The extension of post-harvest storage and shelf life

mainly involved the regulation of ethylene (Saltveit, 1999). The post-harvest application of NO have been reported to influence the changes of their physico-chemical attributes in various fruit such as apple (Wang et al., 2008b), jujube (Zhu et al., 2009), kiwifruit (Zhu et al., 2008; Zhu et al., 2010b), litchi (Kramchote et al., 2008), longan (Duan et al., 2007), plum (Singh et al., 2009; Zhang et al., 2008), and peach (Flores et al., 2008; Zhu et al., 2006). The exogenous application of 0.5 mM SNP or 10 μL L^{-1} to 20 μL L^{-1} NO has been reported to retard skin colour development, including improved L* and higher h° value in 'Chakrapad' litchi, 'Amber Jewel' plum and 'Bartlett' pear during ripening and/or cold storage (Kramchote et al., 2008; Singh et al., 2009). Lower total anthocynins content contributed to delay in turning colour of flesh and skin development in NO-treated jujube and 'Damili' plum fruit during ripening (Zhang et al., 2008; Zhu et al., 2009). However, the fruit fumigated with 5 μL L^{-1} or dipped in 1.0 μmol L^{-1} NO, did not influence total carotenoid as well as chlorophyll content in peach fruit (Flores et al., 2008), and jujube (Zhu et al., 2010a) respectively. Exogenous application of NO treatment retained higher TA in apple (Wang et al., 2008b) and plum (Singh et al., 2009) during ripening or cold storage. Post-harvest application of NO (10 μL L^{-1}) in 'Amber Jewel' plum had lower concentration of individual sugars (fructose, glucose, sucrose, and sorbitol) during 5 days ripening at ambient temperature following 5, 6, or 7 weeks of cold storage (Singh et al., 2009). Similarly, 'Feicheng' peach treated with 10 μL L^{-1} NO inhibited the accumulation of fructose and glucose SSC:TA ratio during 4, 8 and 12 days of ripening period, and higher sucrose content during 4 and 8 days of ripening period, lower sorbitol dehydrogenase, sorbitol oxidase, and sucrose phosphate synthase throughout ripening period, without any different on the sorbitol content (Sun et al., 2011). They also reported that the application of NO (10 and 30 μL L^{-1}) suppressed the activities of AI and neutral invertase, SUS in decomposition and in synthesis during ripening period than control fruit (Sun et al., 2011). The effects of post-harvest applications of NO on the physico-chemical changes during ripening or cold storage have not been studied in mango, except those reported by Zaharah and Singh (2011a; b; c) which is based on the experimental results presented in Chapter 6, 7, and 8.

2.7 Storage

Mango is a seasonal tropical fruit unlike many other tropical fruits. The shelf life of mango varies among varieties which are highly depending upon storage conditions, for example low temperature storage, modified atmosphere packaging (MAP) or controlled atmosphere (CA). Only low temperature storage had been reviewed as mentioned in Section 2.7.1.

2.7.1 Low temperature storage

Low temperature storage has been used to extend the shelf life of mango fruit and to provide better fruit quality to the consumer. Mango is a tropical fruit and highly sensitive to chilling temperatures (Medlicott et al., 1990b; Nair, 2002; Nair et al., 2003; 2004a; 2004b; O'Hare and Prasad, 1993; Pesis et al., 1997). Many studies have been conducted to investigate the most suitable storage temperature in different cultivars of mango fruit. Storage temperature of 12 - 13°C for two to three weeks storage is considered the most suitable conditions for mango fruit (Kader, 1992; Kalra and Tandon, 1983; Malik and Singh, 2005; Medlicott et al., 1990b; Mohammed and Brecht, 2002; Thomas and Oke, 1983). The genotype, developmental stage, and season of harvest of mango fruit are predominant factors affecting ripening at low temperature storage (Farooqui et al., 1985; Medlicott et al., 1988; Medlicott et al., 1990b; Seymour et al., 1990). The cultivars such as 'Tommy Atkins', 'Keitt' (Medlicott et al., 1990b), 'Alphonso' (Thomas and Oke, 1983), 'Kensington Pride' (O'Hare and Prasad, 1993), 'Kent' (Veloz et al., 1977), and 'Samar Bahisht' (Farooqui et al., 1985) can be stored for three weeks at 13°C. Tree-ripe mangoes (cv. 'Keitt' and 'Tommy Atkins') can be stored at 5°C for two weeks without CI symptoms plus five days ripening at ambient temperature (20°C) (Bender, 1996; Bender et al., 2000). Fruit harvested in the middle or late season stored better without any sign of CI at 10°C than 12°C (Medlicott et al., 1990b). Mango stored at low temperature storage in combination with pre-storage treatments such as ethrel dip (Nair and Singh, 2003), temperature pre-conditioning (Pesis et al., 1997; Thomas and Oke, 1983), hot-air (McCollum et al., 1993; Pesis et al., 1997), HWT (Jacobi and Wong, 1992), ethanol vapours, and low oxygen (Pesis et al., 1997) can positively modulate the ripening process without any CI symptoms. 'Tommy Atkins' and 'Keitt' mangoes can be safely stored at 9°C for three weeks after pre-conditioning treatment, which is by decreasing the temperature from 20°C to 17°C or 14°C for two days

(Pesis et al., 1997). The susceptibility of mango fruit to CI injury limits the use of low temperature storage to its maximum potential.

Sensory and nutritional quality of ripe mangoes fruit have been reported to be greatly influenced by duration and storage temperature. In 'Kensington Pride' mango fruit stored at 5 °C to 15°C resulted in significant reduction of total aroma volatile compounds (Nair et al., 2003). Mature green or tree-ripe 'Irwin' mangoes produced off-flavours in pulp when the fruit stored at 5°C for 20 or 30 days, respectively, due to presence of aldehydes (Shivashankara et al., 2006). The suppression of typical aroma and flavour was more pronounced in 'Alphonso' mangoes during ripening for 14 days at temperature below 20°C (Thomas, 1975). Whilst, ripe chill-injured 'Alphonso' mangoes had poor flavour and carotenoid development after 30 days at 10°C (Thomas and Oke, 1983).

2.8 Chilling injury

Mango fruit is highly sensitive to chilling injury (CI) when stored below 13°C (Chaplin et al., 1991; Kane et al., 1982; Malik and Singh, 2005; Medlicott et al., 1986; O'Hare and Prasad, 1993). Symptoms of CI in mango fruit are skin discolouration, reduction in carotenoid development, lenticel spotting, flesh browning, pitting or sunken lesions, uneven ripening, insipid flavour and increased susceptibility to decay (Chaplin et al., 1991; Chhatpar et al., 1971; Han et al., 2006; Kane et al., 1982; Medlicott et al., 1990b; Mohammed and Brecht, 2002; Nair and Singh, 2003; Nair et al., 2004b; Pesis et al., 2000; Phakawatmongkol et al., 2004). Development of CI in plant tissues is due to disruption of cell membrane including cellular and subcellular structures as well as the metabolic reactions in mango including ethylene production, increased in respiration rate, interference in energy production, accumulation of toxic compounds (ethanol and acetaldehyde), leading to uneven ripening and poor fruit quality (Chaplin et al., 1991; Chhatpar et al., 1971; Han et al., 2006; Lederman et al., 1997; McCollum et al., 1993; Medlicott et al., 1990b; Nair et al., 2003; 2004a; 2004b; Phakawatmongkol et al., 2004; Zauberman et al., 1988; Zhao et al., 2006). Various factors influence the severity of CI such as cultivar, harvest maturity, storage temperature, duration of exposure, and pre-storage conditions (Medlicott et al., 1990a; Mohammed and Brecht, 2002; Nair et al., 2004a; Nair et al., 2004b; Phakawatmongkol et al., 2004; Wang, 1993). The fruit kept under

chilling temperature at PC stage were more susceptible to CI than fruit at post-climacteric stage (Cheema et al., 1950; Medlicott et al., 1990a; Mohammed and Brecht, 2002).

The development of CI symptoms did not occur during cold storage but only after the fruit was transferred to ambient conditions for ripening. The suppression of PG and β-Gal activities in pulp of fruit kept at chilling temperature resulted in retaining firm texture of chill-injured tissues (Ketsa et al., 1999). Additionally, the cell wall of chilled fruit contained less water- and alkali-soluble pectin but more ammonium oxalate-soluble pectin compared to non-chilled fruit (Ketsa et al., 1999). Mango fruit exposed to pre-storage treatment (0.1 mM methyl salicylate) prior to their storage at 5°C for 35 days reduced the incidence and severity of CI (Han et al., 2006).

The activities of ethylene biosynthesis enzymes (ACS and ACO) are highly affected by CI leading to suppressed ethylene biosynthesis and fruit failure to ripen (Nair et al., 2004b). The post-harvest application of ethrel prior to cold storage at 5°C for 28 days alleviated the CI development in 'Kensington Pride' mangoes (Nair and Singh, 2003), indicating that ethylene was involved in development of CI. Polyamines – a plant hormone which play a roles in cell proliferation and cell differentiation, can be used in reducing CI stress in mango (Malik and Singh, 2005; Malik et al., 2003; Nair et al., 2004a). Nair et al. (2004a) reported that chilled-injured of mango fruit reduced the levels of spermidine and spermine in the skin and pulp, but promoted the accumulation of putrescine in the fruit skin. In order to replenish the depleted spermidine and spermine contents in chilled-injured mango fruit, the pre-storage exogenous application of 0.50 mM spermine effectively alleviated CI in mango (Nair et al., 2004a).

Methyl jasmonate (MJ) has also been reported to play a role in preventing CI in mango (Gonzalez-Aguilar et al., 2001; Gonzalez-Aguilar et al., 2000; Kondo et al., 2005). MJ vapour treatment (10^{-4} M) for 24 h at 25°C reduced CI in fruit stored for 21 days at 7°C followed by 5 days shelf life at 20°C in 'Tommy Atkins' mangoes. The application of MJ induced tolerance of mango fruit to CI by reducing the electrolyte leakage in fruit tissue (Gonzalez-Aguilar et al., 2001; Gonzalez-Aguilar et

al., 2000). Dip application of n-propyl dihydrojasmonate (0.39 mM) for 15 min reduced the CI development in 'Choke Anan' mangoes following storage at 6°C for 12 days (Kondo et al., 2005).

CA or MAP has also been reported to alleviate CI in mango fruit (O'Hare and Prasad, 1993; Pesis et al., 2000; Pesis et al., 1997). CA comprising 5 - 10% CO_2 stored at <10°C reduced CI symptoms in 'Kensington Pride' mangoes, but higher concentrations caused severe injury (O'Hare and Prasad, 1993). 'Tommy Atkins' mango exposed to low O_2 treatments induced higher CO_2 levels and alleviated the development of CI stored at 5°C (Pesis et al., 1997). MAP (~5% CO_2 + ~10% O_2) created by microperforated polyethylene or Xtend® film or kept in CA chambers alleviated CI in mango fruit stored at 12°C for 21 days followed by one week shelf life at 20°C (Pesis et al., 2000). 'Kensington Pride' mangoes fruit coated with carnauba natural wax (0.1%) has been reported to reduce the CI development at 5°C for 28 days (Nair, 2002). Similarly, individual mango fruit (cv. 'Banganpalli' and 'Alphonso') wrapped with shrink film (Cryovac®) alleviated the CI symptoms during storage at 8°C for 30 days followed by normal ripening at ambient temperature (Sudhakar Rao and Shivashankara, 2004).

Recently, low temperature conditioning had been reported to alleviate CI development (Zhao et al., 2006). 'Wacheng' mangoes subjected to cold-shock at 0°C for 4 h followed by storage at 2°C for 12 days showed lower CI index than control. Fruit exposed to cold-shock treatment become tolerant to CI due to increased activities of catalase, ascorbate peroxidase and higher glutathione and phenolic compounds contents during storage. The mechanisms of CI occur in mango possibly due to phenomenon of oxidative stress which related to the failure of antioxidant defence machinery. Pesis et al. (1997) also claimed that pre-conditioning of 'Tommy Atkins' and 'Keitt' mangoes by decreasing the temperature from 20°C to 17°C or 14°C for 2 days facilitated their safe storage at 9°C up to 21 days. Pre-storage of HWT for 3 or 5 min at 55°C or hot-air treatment for 12 h at 38°C induced chilling tolerance in mangoes (Zhu et al., 2003). Similarly, 'Keitt' mangoes pre-conditioned at 38°C for 24 or 48 h prior storage at 5°C for 11 days alleviated CI symptoms upon transfer to 21°C for 9 days (McCollum et al., 1993).

NO has been reported to alleviate CI symptoms during cold storage of fruits. Plum (cv. 'Damili') fruit was dipped in 1.0 mM SNP solution for 3 min exhibited a potential for reducing CI and extending storage life for 120 days at 2°C (85 - 90% RH) (Zhang et al., 2008). Recently, the NO fumigation (10 μL L^{-1}) for 2 h has been reported to alleviate CI symptoms during cold storage at 0°C for 6 weeks followed by ripening for 5 days at 21 ± 1°C in cultivar 'Amber Jewel' of Japanese plum (Singh et al., 2009). The prevention of CI in 'Feicheng' peach fumigated with 15 μL L^{-1} NO as well as NO in combination with intermittent warming following stored at 5°C for 50 days also associated with the reduction in mealiness symptoms due to lower *exo*-PG activity during the early storage period, but increased in the activity of *endo*-PG activity during the later storage period (Zhu et al., 2010a). As discussed above, there are number of strategies that can be adopted to alleviate the adverse effects of CI, but the information on the effects of NO in alleviating CI during cold storage have not been studied in mango, except has been reported by Zaharah and Singh (2011c) which is based on the experimental results presented in Chapter 8.

CHAPTER 3

General materials and methods

3.1 Mango fruit

Hard mature green 'Kensington Pride' mangoes were obtained from different commercial orchards located at Gingin (lat. 31° 21´ S, long. 115° 55´ E), Chittering (lat. 31° 25´S, long. 116° 5´E), Carnarvon (lat. 24° 53´S, long. 113° 40´E) and Dongara (lat. 29.26° 15´S, long. 114.93° 55´E), Western Australia (WA, Figure 3.1). Mango fruit were dipped for 2 min in a fungicide solution (Sportak 0.55 mL L^{-1}), which contained percloraz as an active ingredient (a.i.). The fruit were allowed to dry at 21 ± 1°C, packed into soft-board trays, kept in a refrigerated truck (15 ± 1°C) and transported to the Horticultural Research Laboratory, Curtin University, Perth, WA.

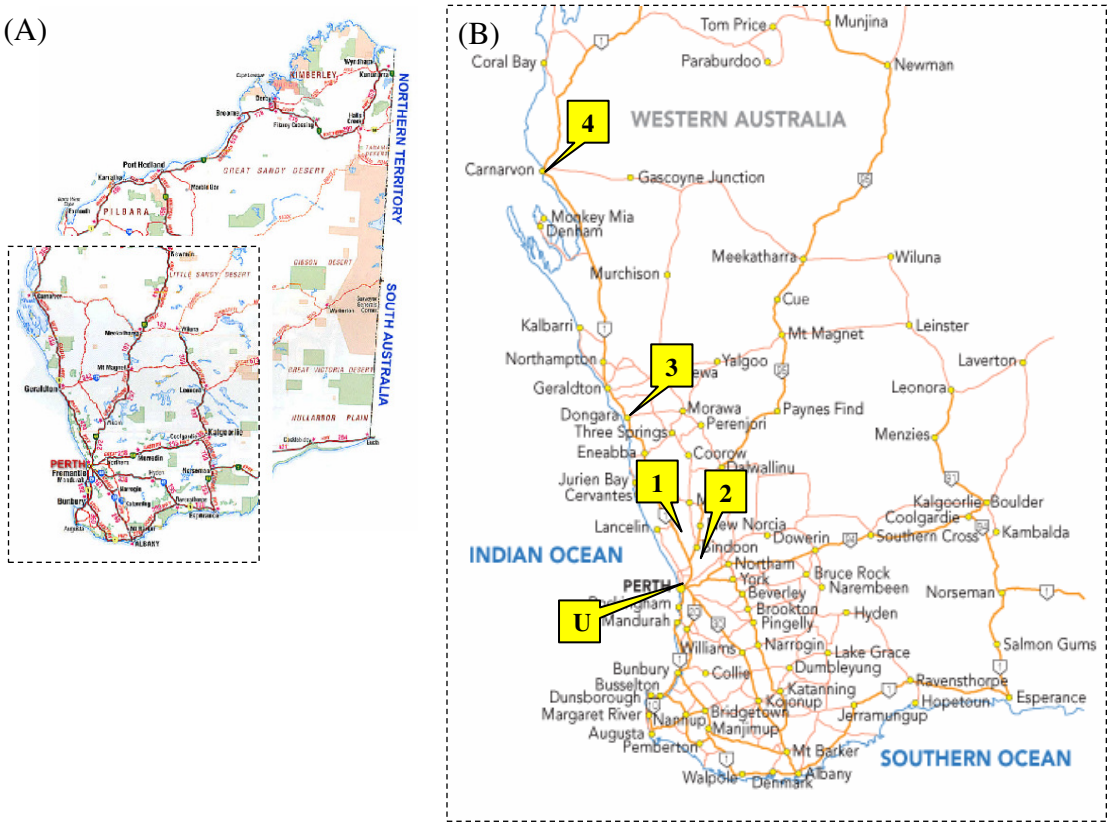

Figure 3.1. Location of orchards in WA (A and B) including Gin Gin (1), Chittering (2), Dongara (3), and Carnarvon (4), from where green mature of 'Kensington Pride' mangoes were obtained and transported to Curtin Horticultural Research Laboratory, Curtin University (U). Sources: (A) About Australia (http://australia-escapes.com/) and (B) Australia Travel & Tourism (http://www.atn.com.au/).

3.2 Ripening conditions

Treated and untreated mangoes were stored and/or allowed to ripen either at ambient temperature (21 ± 1°C, Figure 3.2), cool storage (13 ± 1°C, Figure 3.3) or at cold storage (5 ± 1°C, Figure 3.4). Fruit were considered ripe when they reached the eating soft stage and/or the skin colour was more than 75% yellow, as details mentioned in Section 3.6.1.

3.3 Temperature and relative humidity recording

Tinytag*Plus* Gemini Data Loggers (Gemini Data Loggers, Ltd., UK) interfaced to a personal computer with Tinytag Explorer software version 4.6.95 (Gemini Data Loggers, Ltd., UK) to monitor temperature and relative humidity (RH) during all the experiments was used. The data were recorded at each 15 min interval. The changes of the temperature and relative humidity at ambient temperature (21 ± 1°C, 55.98 ± 14.69% RH), cool storage (13 ± 0.1°C, 95.62 ± 2.01% RH) or cold storage (5 ± 1°C, 93.81 ± 2.02% RH) are shown in Figure 3.2, 3.3 and 3.4, respectively.

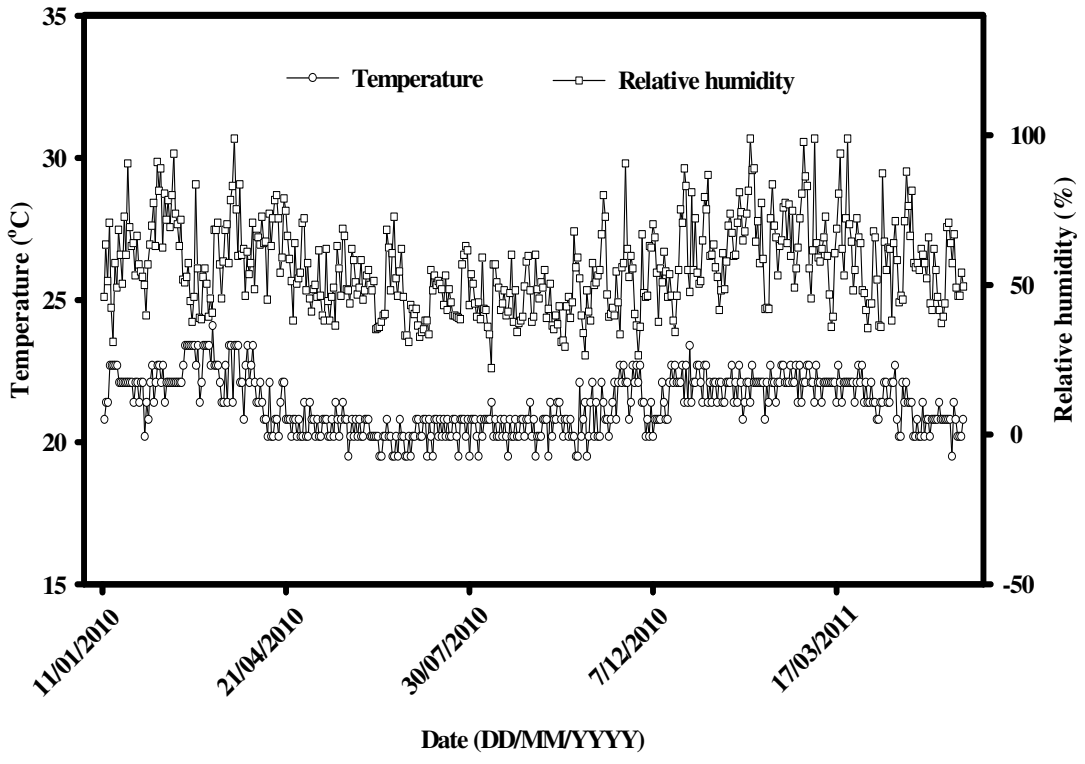

Figure 3.2. Temperature and relative humidity at ambient temperature (21°C) during fruit ripening monitored using Tinytag*Plus* Gemini data loggers.

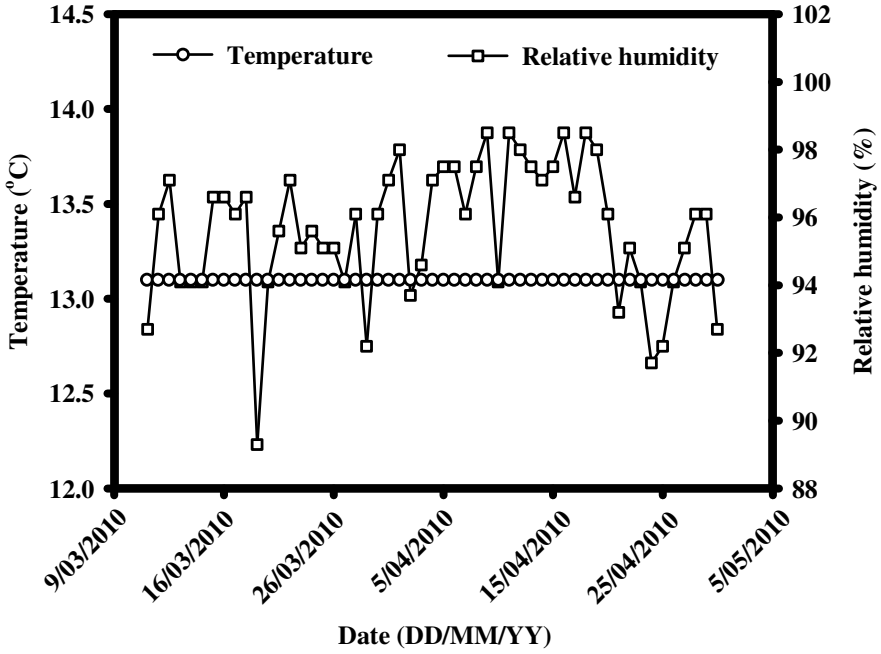

Figure 3.3. Temperature and relative humidity during cool storage at 13°C monitored using Tinytag*Plus* Gemini data loggers.

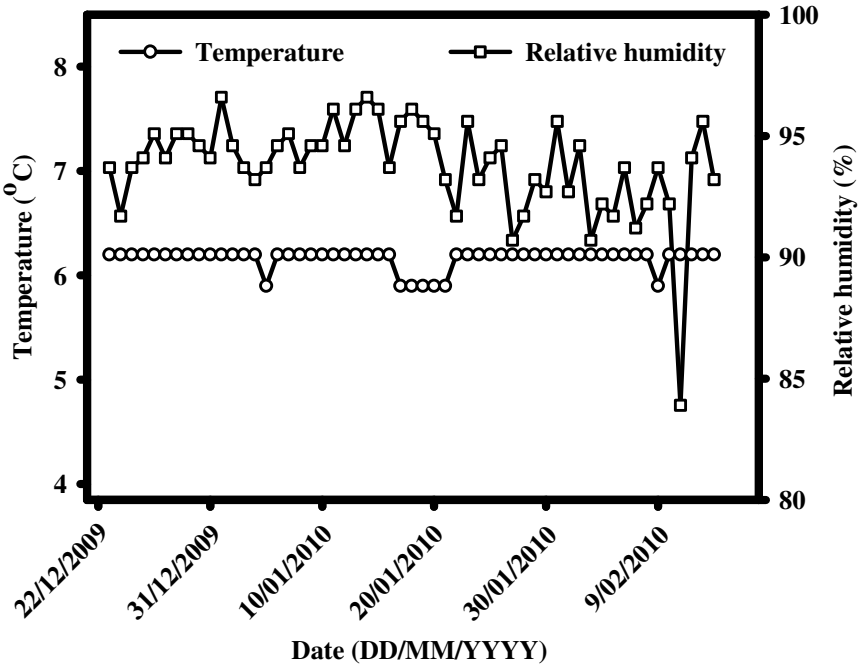

Figure 3.4. Temperature and relative humidity during cold storage at 5°C monitored using Tinytag*Plus* Gemini data loggers.

3.4 Determination of plant hormones

3.4.1 Determination of full name in brassinosteroids (BRs), abscisic acid (ABA) and indole-3-acetic acid (IAA)

3.4.1.1 Extraction of BRs, ABA and IAA

Mango fruit pulp (200 g) was immersed and homogenised in 500.0 mL of cold methanol (-20°C) and distilled water (dH_2O) (80:20, v/v) containing butylated hydroxy toluene (100 mg L^{-1}) to prevent oxidation of plant hormones (Ross et al., 1987). The extraction of plant hormones occurred in a 4°C refrigerator for 12 h. Extracts were then filtered using Whatman No. 1 filter paper and stored at -20°C.

3.4.1.2 Purification and quantification of IAA and ABA

An aliquot of each extract equivalent to 1.0 g fresh weight (FW) was taken and 50 ng of $^{13}C_6$ IAA (Cambridge Isotope Laboratories, MA, USA) and 40 ng of $^{2}H_4$ ABA (National Research Council of Canada, Saskatoon, Canada) were added to each sample. Samples were reduced in volume to less than 1.0 mL under vacuum at 35°C, and taken up in 3 × 3 mL washes 0.4% (v/v) acetic acid in dH_2O. These washes were then passed through a C18-Sep Pak cartridge preconditioned with 10.0 mL of methanol followed by 10.0 mL of 0.4% (v/v) acetic acid in dH_2O. Hormones were then eluted with 15.0 mL of 70% (v/v) methanol in 0.4% (v/v) acetic acid. The elution was reduced to dryness under vacuum at 35°C, taken up in 400 µL of methanol, methylated with 1, 500 µL of 0.2 M (Tri methyl silyl) diazomethane (in diethyl ether), and then dried under a stream of nitrogen (N_2). As a final purification step, the sample was dissolved in 1.0 mL of dH_2O, and then partitioned against 1 × 800 µL, followed by 2 times of 400 µL of diethyl ether. The hormone-containing ether fraction was then divided in two. One half of the sample was reduced to dryness under N_2, and then dissolved in 20 µL of chloroform for ABA methyl ester analysis using a triple quadrupole gas chromatograph-mass spectrometer (GC-MS-MS, 7000A, Agilent Technologies Inc., Denver, Colorado). The second half of the sample was reduced to dryness under N_2 and the IAA-methyl ester was silylated twice, first in 10 µL pyridine and 40 µL *N*,*O*-Bis (trimethylsilyl)trifluoroacetamide (BSTFA, Sigma-Aldrich Pty. Ltd., Castle Hill, Australia) at 80°C for 20 min, then dried under N_2, and secondly again with 40 µL BSTFA at 80°C for 20 min. These samples were then reduced to dryness under N_2 before being dissolved in 20 µL of

chloroform for GC-MS-MS analysis IAA. GC-MS-MS analysis was performed with a Varian 8400 Autosampler and a Varian 3800 GC, coupled to a Varian 1200 triple quadrupole MS. The analysis of IAA and ABA samples were carried out using the method described by Jager et al. (2008) and were calculated as described by Ross et al. (1995). Positive identification of IAA and ABA from mango pulp tissues was achieved by full-scan mass spectrometry.

3.4.2 Determination of ethylene production

The rates of ethylene (C_2H_4) production and respiration in mango fruit during ripening were determined according to Khan (2007). One fruit per replication were sealed in a 1.0 L air-tight jar, fitted with a rubber septum (SubaSeal®, Sigma-Aldrich Co., St. Louis, USA), for 1 h at 20 ± 1°C. A headspace gas sample (1.0 mL) was then injected into a gas chromatograph to estimate the rate of ethylene production. The concentration of ethylene produced by fruit was quantified using a gas chromatograph (6890N Network GC System, Agilent Technology, Palo Alto, CA^2, USA) fitted with a 2 m-long stainless steel column filled with 80/100 mesh size Porapaq-Q (3.18 mm internal diameter, Supelco, Bellefonte, PA, USA) and a flame ionisation detector (FID). N_2 was used as a carrier gas with head space 23.23 psi. The injector, column and detector temperatures were maintained at 100°C, 150°C and 250°C respectively. Ethylene in the gas was identified by comparing its retention time and co-chromatography with authentic standards (0.9 ± 0.1 μL L^{-1} and/or 7.8 μL L^{-1} of ethylene in N_2) certified as β-standard and obtained from BOC Gases, Australia Ltd., Perth, Australia. The chromatogram of ethylene standard peak and mango fruits were shown in Figure 3.5A and 3.5B. The ethylene production rate was expressed as nmol C_2H_4 kg^{-1} h^{-1}. All the estimations were performed twice. The calculation of ethylene production as following formula:

$$C_2H_4 \text{ production } (\mu L\ kg^{-1}\ h^{-1}) = \frac{\text{Concentration of } C_2H_4\ (\mu L\ L^{-1}) \times \text{Vol. of container (L)}}{\text{Fruit weight (kg)} \times \text{Incubation time (h)}}$$

Ethylene production were converted from μL kg^{-1} h^{-1} to nmol kg^{-1} h^{-1} using Ideal Gas Low, $PV = nRT$, where P is pressure (kPa), V is volume (L), n is the number of moles, R = 8.314 (the ideal gas constant) and T is temperature (Kelvin) (Bower et al.,

1998). Data of barometric pressure during the ethylene measurement were collected from Bereau of Meteorology Australia, WA.

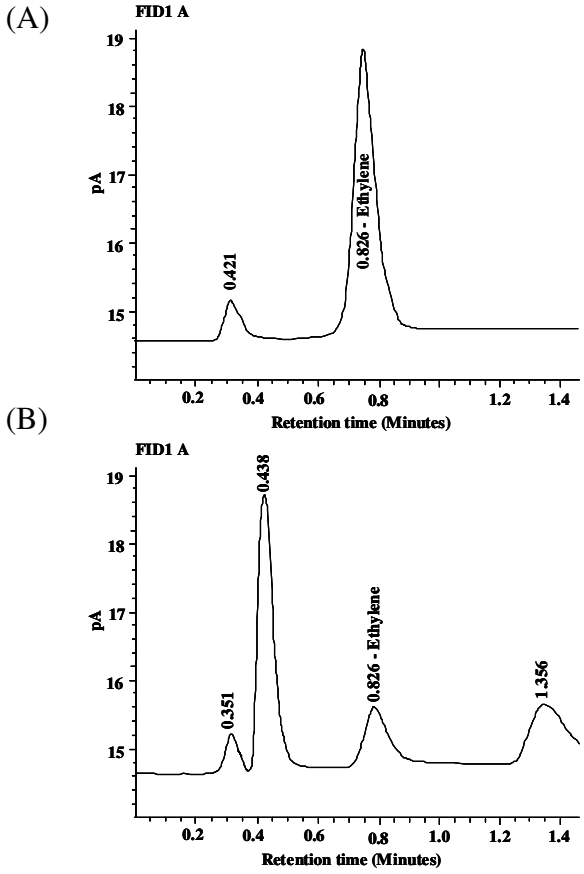

Figure 3.5. GC chromatographic profile of ethylene production of (A) 0.9 ± 0.1 µL L^{-1} ethylene standard and (B) whole mango fruit; pA= peak area.

3.5 Determination of respiration rate

Respiration rate was determined as CO_2 production from mango fruit during ripening according to the method described earlier by Khan and Singh (2008). The headspace gas sample (1.0 mL) was taken through rubber septum (SubaSeal®, Sigma-Aldrich Co., St. Louis, USA) using a syringe from the same jar used for measurement of ethylene production as explained in Section 3.4, and injected into an infrared gas analyzer [Servomex Gas Analyzer, Analyzer series 1450 Food Package Analyzer, Servomex (UK) Ltd., East Sussex, UK]. The respiration rate was calculated on the basis of the peak areas of 1.0 mL gas sample (SCO_2) and CO_2 standard ($StdCO_2$, 8.52 ± 0.17%) (Figure 3.6). The $StdCO_2$ was purchased from BOC Gases, Australia Ltd., Perth, Australia. All the estimations were performed twice.

Respiration rate was calculated following formula and expressed as mL CO_2 kg^{-1} h^{-1}.

$$\text{Respiration rate (mL } CO_2 \text{ kg}^{-1} \text{ h}^{-1}) = \frac{\text{Changes in } CO_2 \text{ concentration (\%)} \times \text{Vol. of container (L)}}{\text{Fruit weight (kg)} \times \text{Incubation time (h)}}$$

Respiration rate were converted from mL CO_2 kg^{-1} h^{-1} to mmol CO_2 kg^{-1} h^{-1} using Ideal Gas Low, $PV = nRT$ as explained in Section 3.4.2. To check the possibility of CO_2 emission from the rubber septum or normal air, a blank injection from the headspace of the empty jar or air was also run under same conditions as mentioned in this section.

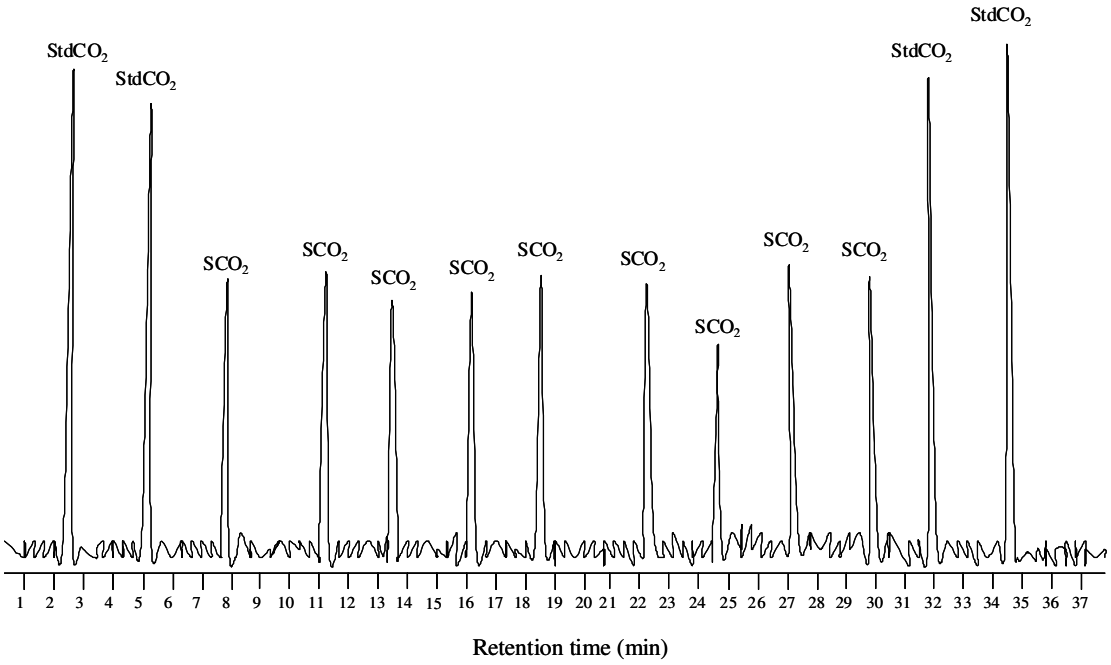

Figure 3.6. Servomex Series 1400 (Sussex, England) chromatographic profile of respiration peak of 8.52 ± 0.17% CO_2 standard ($StdCO_2$) and mango sample peak (SCO_2).

3.6 Fruit quality analysis

3.6.1 Skin colour

3.6.1.1 Visual assessment

The visual assessment of skin colour was recorded daily during fruit ripening period by following a rating scale ranging from 1 to 5 (Figure 3.7) according to the percentage of greenish and yellowish colour as previously described by Dang et al. (2008a).

(1) 100% green (2) 1- 25% yellow (3) 26 - 50% yellow

(4) 51 - 75% yellow (5) 76 - 100% yellow

Figure 3.7. Changes on the skin colour of 'Kensington Pride' mango during ripening period.

3.6.1.2 Fruit colour using a HunterLab ColourFlex

The mango skin colour was also recorded from opposite positions of each fruit in Commission International de L' Eclairage (CIE) units using a ColorFlex 45°/0° spectrophotometer (HunterLab ColorFlex, Hunter Associates Inc., Reston, VA, USA) using the head 15 mm diameter, as L*, a* and b* colour coordinates on the fruit surface (Hunter, 1975) and chroma value (C*) and hue angle (h°) were calculated. Chromaticity L* represents the lightness of the fruit colour, which ranges from 0 (black) to 100 (white). Chromaticity a* indicates the redness (+a*) or greenness (-a*), and chromaticity b* indicates the yellow (+b*) or blue (-b*) colour of fruit skin (Figure 3.8). For colour interpretation, red was at an angle of 0° or 360°, yellow at 90°, green at 180°, and blue at 270°, hue angle (h°) values was calculated as: $h° = \tan^{-1} b*/a*$. Chroma (C*) was defined as the intensity of colour saturation from dull to a vivid colour (low-to-high values, respectively) and was calculated as: $C* = (a*^2 + b*^2)^{1/2}$. Hue angle and chroma value was calculated following the formula mentioned earlier by McGuire (1992).

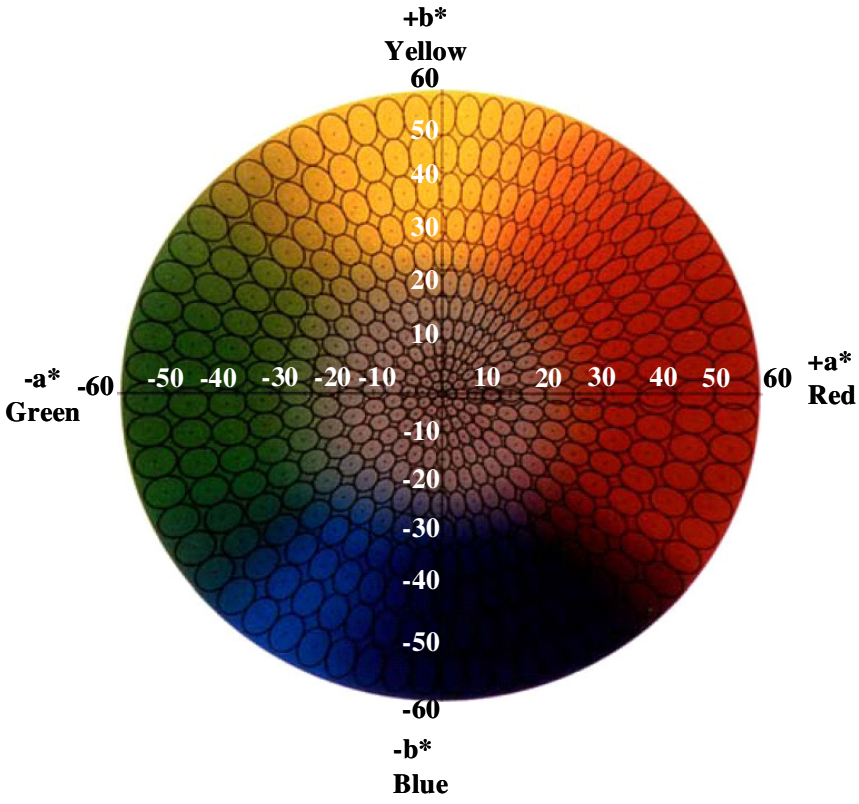

Figure 3.8. Colour chart – Commission International de L' Eclairage (CIE) L*, a* and b* (HunterLab, 1998).

3.6.2 Fruit firmness

3.6.2.1 Subjective fruit firmness

Daily subjective firmness of individual fruit in each replication was recorded using a rating scale 1 to 5 (1 = hard, 2 = sprung, 3 = slightly soft, 4 = eating soft, and 5 = over soft) as described earlier by Dang et al. (2008a).

3.6.2.2 Rheological properties of pulp

Rheological properties of pulp (hardness, springiness, cohesiveness, chewiness, adhesiveness and stiffness) of ripe fruit was also determined using a texture profile analyser (TPA Plus, AMETEK Lloyd Instruments Ltd, Hampshire, UK), equipped with horizontal square base table (15 cm × 15 cm) and interfaced to a personal computer with Nexygen® software following the methods that have been explained earlier by Zaharah and Singh (2011b). A 7/16 inch Magness-Taylor probe, with a 500 N load cell on, punctured to the 5 cm × 2 cm × 2 cm (length, breadth and height) of pulp sample. The pulp sample was placed on the top of the base table and the gap size between the pulp sample and probe was at least 2.0 mm. The crosshead speed, trigger and compression were at 2.0 mm s^{-1}, 0.5 N and 25%, respectively, for all determinations.

The chromatographic profile of rheological properties of mango pulp is shown in Figure 3.9. The rheological properties were defined and expressed as mentioned by Bourne (1978) :

a) *Hardness* (*N*) = maximum force required to compress the sample
b) *Springiness* (*mm*) = Length 2/Length 1 (the ability of the sample to recover its original form after deforming force was removed)
c) *Adhesiveness* (*Nmm*) = area under the abscissa after the first compression,
d) *Cohesiveness* = Area 2/Area 1 (extent to which the sample could be deformed prior to rupture)
e) *Chewiness* (*Nmm*) = Cohesiveness × Hardness × Springiness (work required to masticate the sample before swallowing)
f) *Stiffness* (*kg f* mm^{-1}) = the ratio of the force applied to a structure to the corresponding displacement.

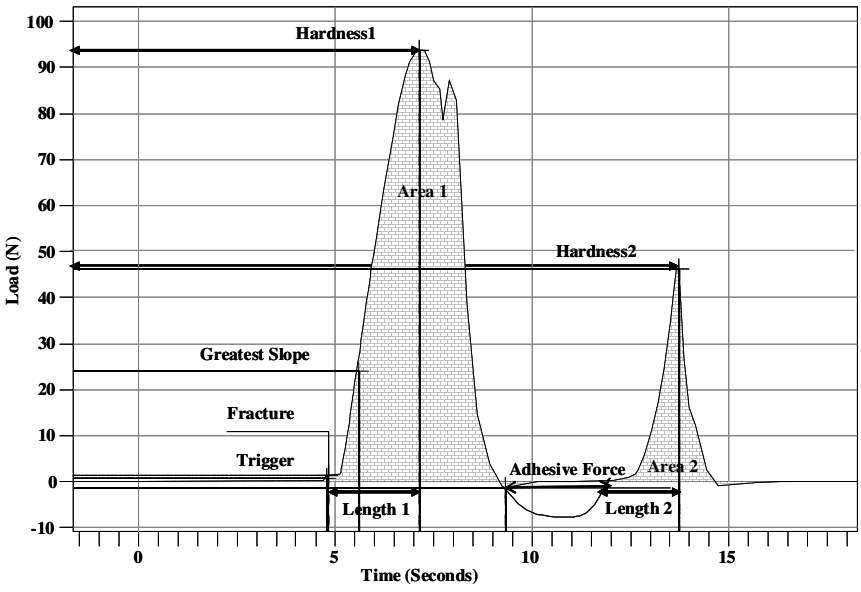

Figure 3.9. Chromatographic profile of rheological properties of 'Kensington Pride' mango pulp using texture profile analyzer (TPA).

3.6.3 Determination of individual sugars and organic acids

3.6.3.1 Chemicals

The details of the individual standard used for determination of sugars (sucrose, D-glucose anhydrous and D-(-)-fructose) and organic acid (citric, ammonium tartrate di-basic, shikimic acid, fumaric acid and DL-malic acid) were obtained and purchased from different companies as listed in Appendix 1.

3.6.3.2 Sample preparation

Pulp (~15 g each) was pooled from inner and outer mesocarp at the middle position of ten full-ripe fruit. A subsample of pulp (1.0 g) was homogenised in 25.0 mL of Milli-Q water which was passed through a purification water system (Millipore, Bedford, MA, USA), using a mini-mixer (DIAX 900, Heidolph Co., Ltd., Schwabach, Germany) for 1 min to extract individual sugars and organic acids. Following centrifugation, using a centrifuge (Eppendorf 5810 R, Hamburg, Germany) at 10, 000 × *g* for 15 min at 15°C, and the supernatant was collected and diluted with Milli-Q water to make up the volume to 50 mL. It was filtered through a syringe using a 0.2 µm nylon filter (Alltech Associates Ltd., Baulkham Hills, Australia) and loaded into the 1.0 mL glass vial prior determination of sugar and organic acid using high-performance liquid chromatography (HPLC) analysis.

3.6.3.3 HPLC conditions

The determination of individual sugars and organic acids were performed using reverse phase-liquid chromatography using a HPLC system (Waters 1525, Milford Corp., MA, USA) fitted to Dual λ Absorbance Detector (Waters 2487, Milford Corp., MA, USA). An aliquot (20 µL) of the extract from mango pulp was injected using an autosampler (Waters 717plus, Milford Corp., MA, USA) maintained at 25°C. Sugars and organic acids were separated on a Bio-Rad Aminex® HPX-87C Fast Carbohydrate column (100 × 7.8 mm) and Bio-Rad Aminex® HPX-87H column (300 × 7.8 mm) (Bio-Rad Laboratories, Inc., Hercules, USA) with a particle size of 9 µm, respectively. The column was preceded by Cation H Bio-Rad Micro-Guard® column (30 × 4.6 mm) (Bio-Rad Laboratories, Inc., Hercules, USA). Both the column and guard column were kept at 60°C and 45°C for sugars and acids, respectively, during the analysis. The organic acids were eluted isocratically (1:1) with degassed water (Solvent A) and 0.05 mM sulphuric acid solution (Solvent B) as mobile phase with the flow rate were kept at 0.3 mL min^{-1}. Meanwhile, the sugars were eluted with degassed water only and the flow rate was kept at 1.0 mL min^{-1}. All compounds of organic acids were detected at 210 nm with dual wavelength UV detector, while the individual sugars were detected with Refractive Index (RI) Detector (Water 2414, Milford Corp., MA, USA). Chromatographic peaks were identified by comparing retention times with those of standards and by spiking samples with pure compounds while quantification was carried out using the external standard method. The data were collected and processed with Breeze® 3.30 software (Waters, Milford Corp., MA, USA).

3.6.3.4 Elution orders and retention times

The elution order, retentions and different wavelengths used for determination of individual sugar and organic acid were shown in Table 3.1 and 3.2. The HPLC chromatograms of individual standard sugars or organic acids compounds, and aliquots samples from 'Kensington Pride' mango pulp were presented in Figure 3.10A and 3.11A, as well as Figure 3.10B and 3.11B, respectively.

Table 3.1. Elution order, retention times and detective wavelength of different sugars standards used for identifying the individual sugars concentration in 'Kensington Pride' mango.

Elution order	Sugars standard compound	Retention Time (min)	Detector
1	Sucrose	2.492	RI
2	Glucose	2.909	RI
3	Fructose	3.989	RI

RI = refractive index

Table 3.2. Elution order, retention times and detective wavelength of different organic acids standards used for identifying the individual sugars concentration in 'Kensington Pride' mango.

Elution order	Organic acids standard compound	Retention time (min)	Detection wavelength (nm)
1	Citric	15.726	210
2	Tartaric	16.613	210
3	Malic	19.091	210
4	Shikimic	23.926	210
5	Fumaric	26.443	210

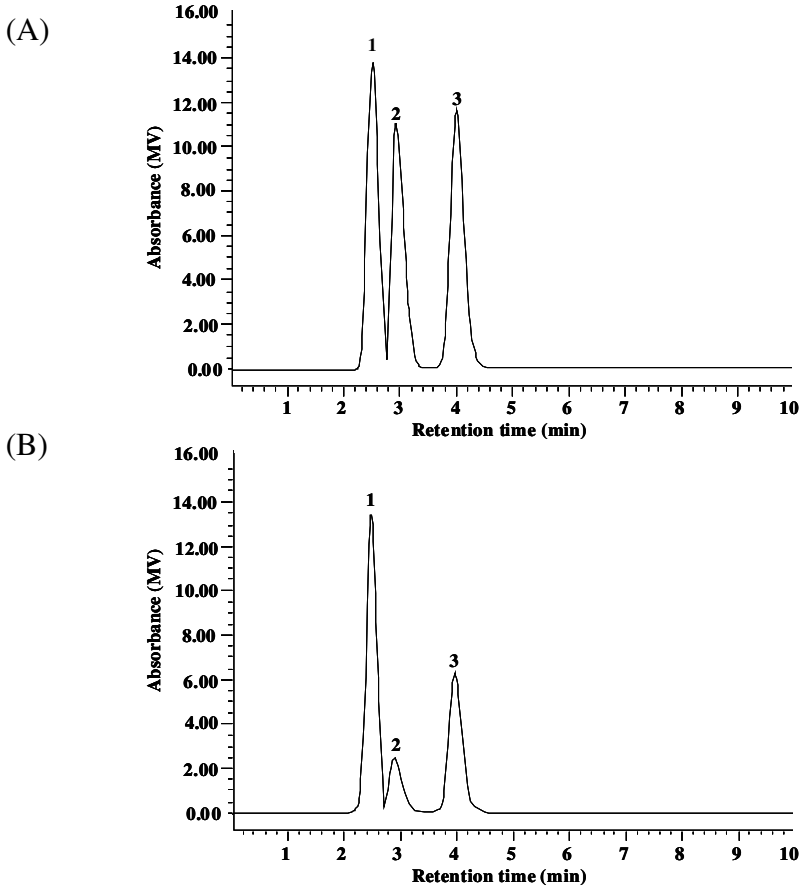

Figure 3.10. HPLC chromatographic profile of individual standard sugars and in the pulp of 'Kensington Pride' mango. (A) Individual peaks of different standard sugars (B) Individual peaks of different sugars from mango pulp. (1) Sucrose, (2) Glucose and (3) Fructose; MV = Millivolt(s).

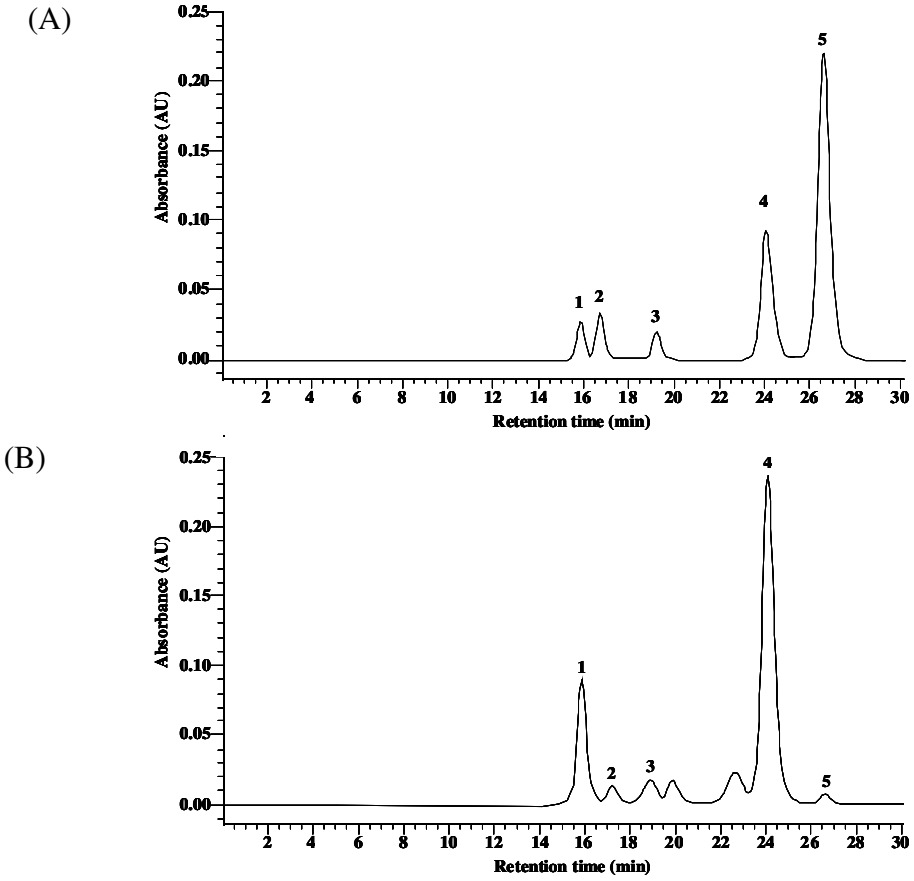

Figure 3.11. HPLC chromatographic profile of individual standard organic acids and in the pulp of 'Kensington Pride' mangoes. (A) Individual peaks of different standard organic acids and (B) Individual peaks of organic acids from mango pulp. (1) Citric acid, (2) Tartaric acid, (3) Malic acid, (4) Shikimic acid and (5) Fumaric acid; AU = Absorbance units.

3.6.3.5 Preparation of standards

Standard solutions of sucrose and fructose were prepared by dissolving 0.5 g of sucrose and D-(-)-fructose in 100.0 mL of Milli-Q water, while for glucose were prepared by dissolving 0.05 g of D-glucose in 100.0 mL of Milli-Q water. The organic acids were prepared by dissolving citric, tartaric and malic acid (0.1 g) and shikimic and fumaric acid (0.01 g) in 100.0 mL of Milli-Q water. Details of sugar and organic acids used are mentioned in Section 3.6.3.1 and shown in Appendix 1. Aliquots of 4, 8, 12, 16 and 20 µL of standard solutions were injected into the HPLC system following the same conditions and gradient as detailed in Section 3.6.3.3.

Standard curves for individual standard were generated using Water Breeze software (Version 3.30) by plotting each peak area against different amounts (mg) of individual sugars or organic acids compounds. The relationship between the peak area of individual standard sugars and organic acids compounds is shown by slope (*a*), intercept (*b*) and *r* values in Table 3.3 and 3.4. Slope (*a*) and intercept (*b*) represent coefficients following the regression equation $y = ax + b$, where x is amount of the individual sugars and organic acids compounds, *y* is peak area and *r* is correlation coefficient of the equation. The *r* values for all individual sugars and organics acids compounds showed very high linearity ($r = 0.99$).

Table 3.3. Analytical characteristic of the standard curves of individual sugars standard.

Elution order	Sugars standard compound	Slope (*a*)	Intercept (*b*)	*r*
1	Sucrose	6.95^{e+003}	$+2.52^{e+003}$	0.99
2	Glucose	6.83^{e+003}	-2.32^{e+002}	0.99
3	Fructose	7.44^{e+003}	-7.49^{e+000}	0.99

Table 3.4. Analytical characteristic of the standard curves of individual organic acids standard.

Elution order	Organic acids standard compound	Slope (*a*)	Intercept (*b*)	*r*
1	Citric	1.68^{e+005}	$+2.42^{e+005}$	0.99
2	Tartaric	1.80^{e+005}	$+5.60^{e+005}$	0.99
3	Malic	1.02^{e+005}	$+3.81^{e+005}$	0.99
4	Shikimic	6.78^{e+006}	$+1.21^{e+006}$	0.99
5	Fumaric	1.67^{e+007}	$+4.52^{e+006}$	0.99

3.6.4 Soluble solids concentration (SSC)

Pulp from the inner and outer mesocarp at the middle of ten full-ripe fruit (~15 g each fruit per treatment) was used to extract juice using a fruit juicer (Model JE8500, Sunbeam Corp. Ltd., Botany, Australia). SSC was determined using an infrared digital refractometer (Atago-Palette PR 101, Atago Co. Ltd., Tokyo, Japan) and expressed as a percentage (%).

3.6.5 Titratable acidity (TA)

Titratable acidity (TA) was estimated from the same extract juice sample as mentioned in Section 3.6.4. Ten mL of freshly extracted juice was diluted with 20.0 mL dH_2O. Aliquot (5.0 mL) was titrated against 0.1 *N* NaOH solutions using phenolphthalein as an indicator to a pink colour end point and expressed as per cent malic acid. TA was calculated by using the following formula:

$$\text{Malic acid (\%)} = \frac{0.0067 \times \text{Vol. of NaOH} \times 30 \times 100}{5 \times 10}$$

Where,

0.0067 = Milli-equivalent weight of malic acid
30 = Total volume (mL)
5 = Extract juice sample (mL)
10 = Volume of aliquot (mL)

3.6.6 SSC:TA ratio

SSC:TA ratio was calculated by dividing SSC (%) with the corresponding TA (%).

3.6.7 Total carotenoid

Total carotenoid were estimated according to the methods described by Lalel (2002) with some modifications as shown in Figure 3.12. Two g of mango pulp was mixed with 50 mg of magnesium carbonate were ground and extracted two times with 20.0 mL of acetone:n-hexane (75:60, v/v). The pool extract was washed with 40.0 mL of 10% NaCl and twice with 40.0 mL of dH_2O to remove the acetone. The hexane extract was measured for its absorbance at 436 nm using spectrophotometer model

6405 UV/VIS (Jenvey Ltd., Essex, England). Total carotenoid concentration was expressed as mg g^{-1} FW of β–carotene from a standard curve β–carotene.

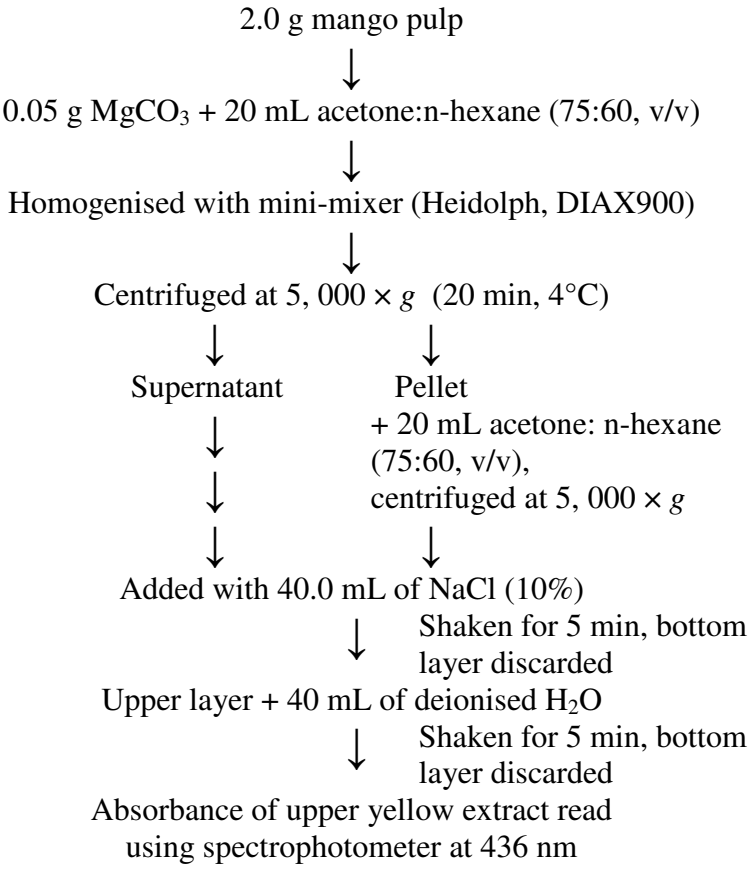

Figure 3.12. Flow chart of extraction and estimation of total carotenoid in pulp of mango fruit.

3.6.8 Determination of total antioxidant

The levels of total antioxidant in mango skin and/or pulp tissue of ripe fruit were determined by using the method of Brand-Williams et al. (1995) and Zaliha (2009), with some modifications (Figure 3.13). Mango pulp (5 g) and skin (5 g) were ground using a mortar and pestle added in the presence of ~200 mg of White Quartz Sand (-50+70 mesh, Sigma Aldrich, USA) in 10.0 mL extraction buffer (2 mM NaF dissolved in 200.0 mL dH_2O and 800.0 mL methanol). The free radical was 2,2-diphenyl-1-picrylhydrazyl (DPPH) as stock solution by dissolving 24.0 mg DPPH in 100.0 mL methanol. Diluted stock solution (1:4, v/v) was prepared as a working solution. The homogenate was centrifuged at 10, 000 × *g* for 20 min (Eppendorf 5810R, Hamburg, Germany). The supernatant (50 µL) was mixed with 950 µL working solution. After 15 min, the absorbance was recorded using a UV-VIS

spectrophotometer (Jenway Spectrophotometers Model 6405, Dunmow, Essex, UK) at 515 nm wavelength. Absorbance lower than 0.6 or higher than 0.7 was repeated using increased or decreased dilution of supernatant. Total antioxidant was estimated using a standard curve of 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) and was expressed as mM Trolox Equivalent Antioxidant Activity (TEAC) 100 g^{-1} FW basis.

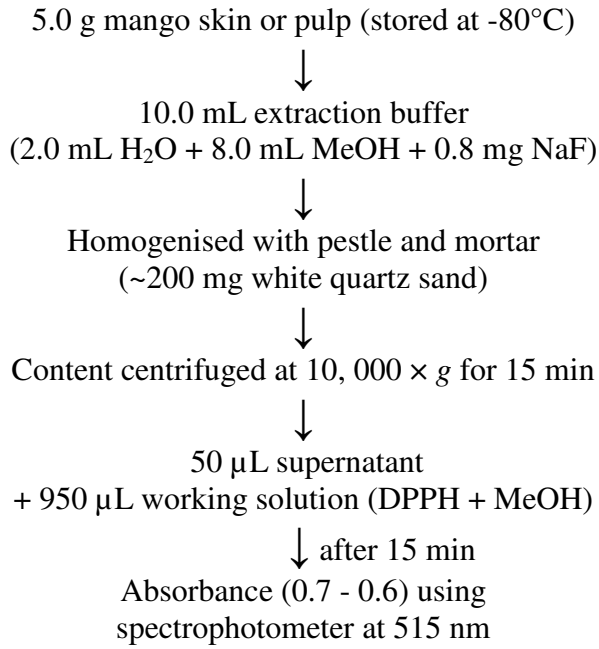

Figure 3.13. Flow chart for determination of total antioxidant in pulp of mango fruit.

3.6.9 Determination of ascorbic acid

The concentrations of ascorbic acid was determined by following the method Malik and Singh (2005) as described in Figure 3.14. Mango pulp (5.0 g) were homogenised in glass mortar and pestle using ~200 mg white quartz sand (-50+70 mesh, Sigma Aldrich, USA) with 20.0 mL of 6% metaphosphoric acid containing 0.18 g of disodium salt ethylenediaminetetraacetate acid (EDTA). The homogenate was centrifuge at 5, 000 × *g* for 20 min (Eppendorf Centrifuge, Hamburg, Germany). The supernatant (400 µL) was mixed with 200 µL of 3% metaphosphoric acid, 1.4 mL dH_2O, and diluted with 200 µL Folin reagent (Folin:dH_2O, 1:5 v/v). Disposable cuvettes (2.0 mL) were used to record the absorbance of the mixed sample after 10 min at 760 nm wavelength using a UV/VIS spectrophotometer (Jenway spectrophotometer Model 6405, Dunmow, Essex, UK). Ascorbic acid concentration

was quantified using a standard curve of L-ascorbic acid and was expressed as mg 100 g^{-1} FW basis.

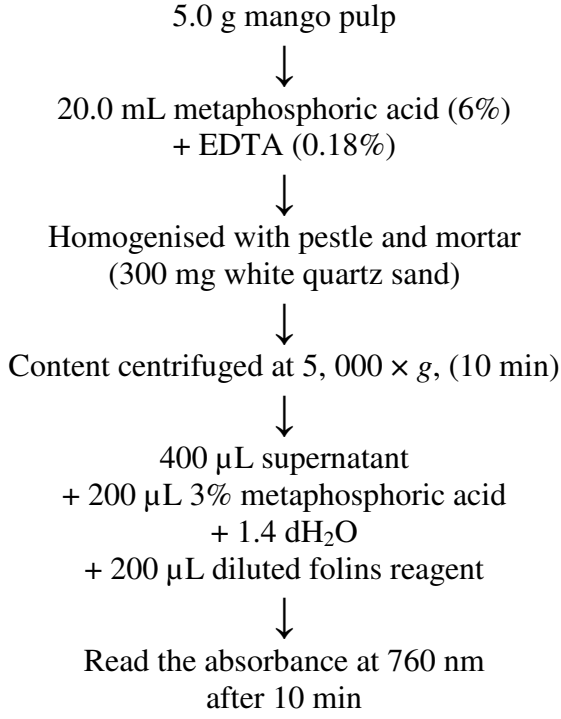

Figure 3.14. Flow chart for determination of ascorbic acid in pulp of mango fruit.

3.7 Determination of 1-aminocyclopropane-1-carboxylic acid (ACC) content and activities of ethylene biosynthesis enzymes

The 1-aminocyclopropane-1-carboxylic acid (ACC) content and the activity of ethylene biosynthesis enzymes such as ACC oxidase (ACO, EC 4.4.17.4) and ACC synthase (ACS, EC 4.4.1.14) were estimated by the following methods explained in Section 3.7.1, 3.7.2 and 3.7.3 respectively.

3.7.1 Determination of ACC content

The pulp of fruit tissue (2.0 g) was homogenized with 10.0 mL dH_2O and 300 mg white quartz sand using a glass pestle and mortar at 2 ± 1°C temperature, followed by centrifugation at 10, 000 × *g* for 20 min at 4°C. The supernatant (0.5 mL) was mixed with 0.1 mL 50 mM $HgCl_2$ and with or without 0.1 mL 100 µM ACC, and the final volume 1.8 mL was made with dH_2O. The reaction tubes were then sealed with a rubber septum (SubaSeal®, Sigma-Aldrich Co., St. Louis, USA). The reaction tubes were placed on ice prior 0.2 mL of 5% NaOCl and saturated NaOH (2:1, v/v) was

injected and then stirred for 5 s. All the sample were kept on ice for 24 min, then were stirred again before 1.0 mL gas sample was taken from headspace and injected into a GC for an estimation of ACC activity. Ethylene production was calculated from the peak area obtained from the tissue extract (0.5 mL) in comparison with the peak obtained for the sample without internal ACC (0.1 mL) as the standard. The determination of the ACC content was extracted from pulp by using methods described in Figure 3.15 (Khan and Singh, 2007; Lizada and Yang, 1979; Tovar et al., 2001).

The ACC content was expressed as pmol g^{-1} FW and calculated using the following formula:

$$A = \frac{B \times C \times 0.01}{D}$$

$$E = \left(\frac{F \times G \times 0.01}{H} \right) - A$$

$$\text{ACC contents} = \frac{0.0000101 \times A \times 10 \times 41320.4}{E \times \text{Sample weight (g)} \times 0.5}$$

Where,

A = Concentration of C_2H_4 in the sample without internal standard (µL)

B = Peak area of C_2H_4 in the sample without internal standard

C = Concentration of standard C_2H_4 used (0.9 µL L^{-1})

D = Peak area of 0.9 µL L^{-1} ethylene standard used

E = Concentration of C_2H_4 in the sample with ACC internal standard (µL)

F = Peak area from the sample with ACC internal standard

G = Concentration of standard C_2H_4 used (7.8 µL L^{-1})

H = Peak area of 8.0 µL L^{-1} ethylene standard used

0.01 = Head space volume (L)

0.0000101 = Amount of ACC in internal standard (g)

10 = Vol. buffer (mL)

0.5 = Extract enzymes used (mL)

41320.4 = Constant to convert µL g^{-1} into pmol g^{-1} at Perth pressure (21°C)

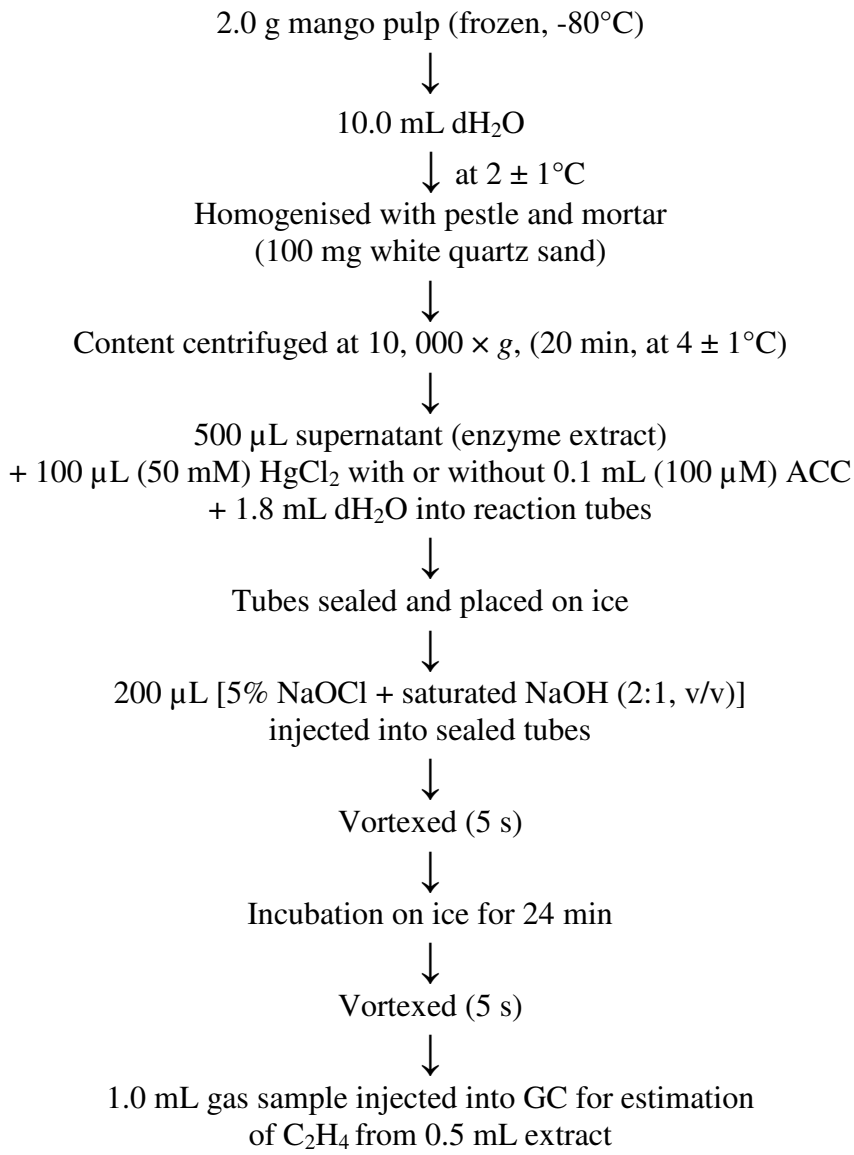

Figure 3.15. Flow chart for extraction and assay of ACC content in pulp tissues of mango fruit.

3.7.2 Determination of ACC oxidase (ACO)

The method described by Gorny and Kader (1996) was used to determine ACO activity from the pulp and with some modifications (Figure 3.16). The frozen pulp tissues (2.5 g) from -80°C freezer were homogenized with pestle and mortar with 5 mL extraction buffer containing 0.1 M Tris-HCl (pH 7.2), 10% (w/v) glycerol and 30 mM sodium ascorbate in the presence of 5% PVPP together with 200 mg of white quartz sand. The extraction was centrifuged at 15, 000 × *g* for 30 min at 4°C. The supernatant (enzyme preparation) was used for enzyme assays. The enzyme was assayed in a 2.0 mL reaction mixture containing 1.8 mL of the enzyme preparation and 0.2 mL standard reaction mixture containing 0.01 M Tris-HCl buffer (pH 7.2), 10% glycerol, 1.0 mM ACC, 0.02 mM $FeSO_4$, 5 mM sodium ascorbate, 1 mM dithiotheritol (DTT) and 20 mM sodium bicarbonate. The reaction tube was sealed with a rubber septum and incubated at 30°C for 1 h. The reaction tube was stirred for 5 s before 1.0 mL of gas was withdrawn from headspace with a syringe and injected into the GC for ACO activity estimation. Ethylene production was calculated from the peak area obtained from the tissue extract (1.8 mL) in comparison with the peak area obtained for the ethylene standard. ACO activity was expressed as nmol C_2H_4 mg^{-1} protein h^{-1} and calculated using following formula:

$$\text{ACO activity} = \frac{I \times C \times 0.01 \times 5 \times 41.32}{D \times \text{Sample weight (g)} \times 1.8 \times \text{Protein amount (mg mL}^{-1}\text{)}}$$

Where,

I = Peak area of ACO sample

C = Concentration of standard C_2H_4 used (0.9 µL L^{-1})

D = Peak area of 0.9 µL L^{-1} ethylene standard used

0.01 = Test tube head space volume (L)

5 = Buffer volume (mL)

41.23 = Constant to convert µL g^{-1} into nmol g^{-1} at Perth pressure (21°C)

1.8 = Vol. of enzyme extract used (mL)

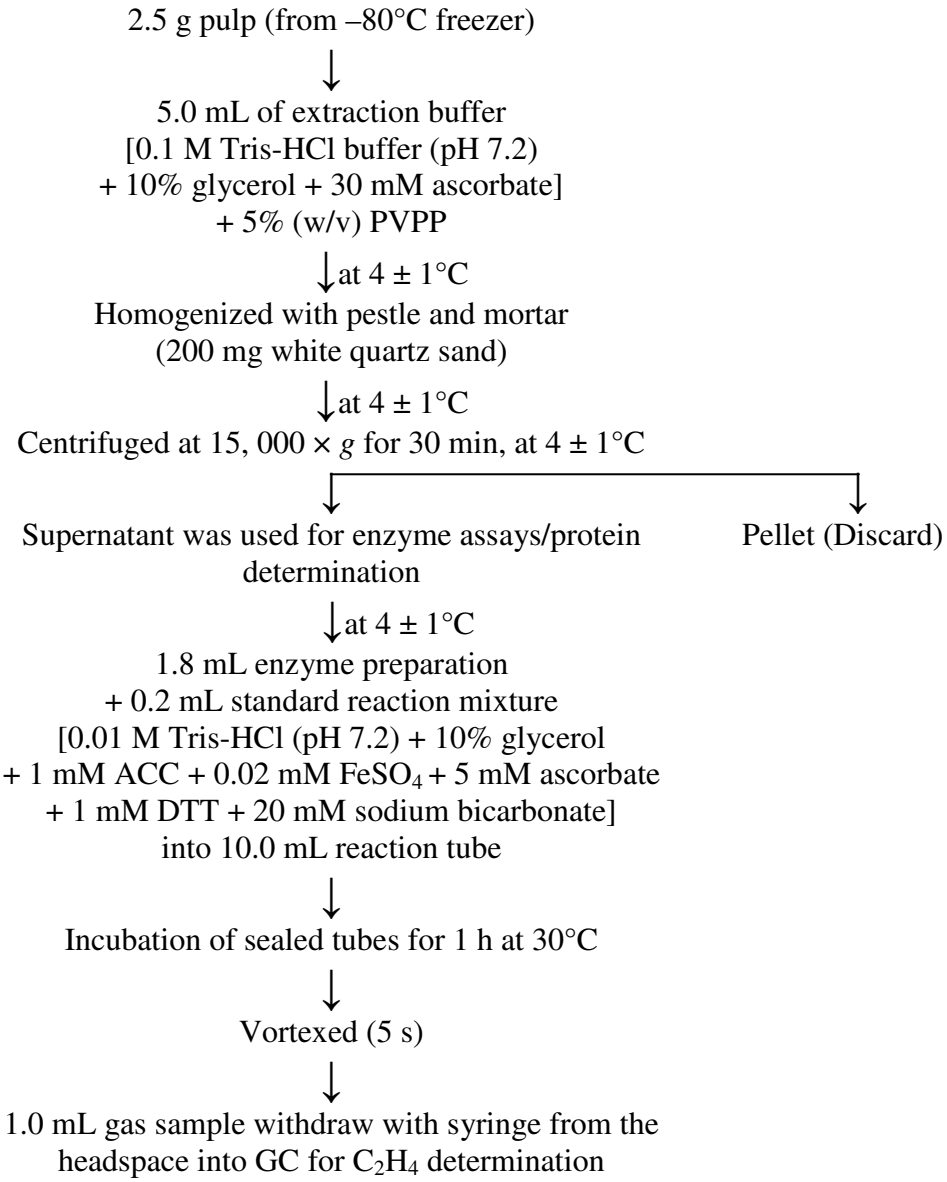

Figure 3.16. Flow chart for extraction and assay of ACO activity in the pulp tissue of mango fruit.

3.7.3 Determination of ACC synthase (ACS)

ACS activity was determined from skin or pulp tissue by following the method of Gorny and Kader (1996), Lizada and Yang (1979), Nair et al. (2004b) and Yip et al. (1991) with some modification (Figure 3.17). Mango pulp (2.5 g) was homogenized with 5.0 mL of potassium phosphate buffer (400 µM, pH 8.5) containing 0.01 mM pyridoxal-5-phosphate (PLP), 1.0 mM EDTA and 0.5% 2-mercaptoethanol and 200 mg of white quartz sand (-50+70 mess, Sigma Aldrich, Australia) at 4 ± 1°C. The extractions were centrifuged at 15, 000 × *g* for 30 min at 4°C. The supernatant was discarded and the pellet was resuspended with 5.0 mL of 20 mM potassium

phosphate (pH 8.5) containing 1 mM EDTA, 1 mM 2-mercaptoethanol, 10 µM PLP and 30% glycerol and then incubated for 30 min at 4 ± 1°C before centrifuged at 15, 000 × *g* for 30 min. Two mL of enzyme extract was mixed with 1.0 mL of 200 µM S-adenosylmethionine (SAM), 10 µM PLP, 50 mM Hepes-KOH buffer (pH 8.5) and 1% Triton X-100 into 10 mL of glass reaction tube. The rubber septum was used to seal the reaction tube, and then the sample was incubated for 1 h at 30°C before being cooled into ice tub. A 100 µL of 50 mM $HgCl_2$ and 300 µL of 5% NaOCl and saturated NaOH (2:1, v/v) were injected using a syringe. The reaction tube was incubated on ice for 2.5 min and a 1.0 mL gas sample was taken from the headspace and injected into the GC for ACS activity estimation. Ethylene production was calculated from the peak area obtained from the tissue extract (2.0 mL) in comparison with the peak obtained for the ethylene standard. ACS was expressed as pmol ACC mg^{-1} protein h^{-1} and calculated following the formula below:

$$\text{ACS activity} = \frac{J \times C \times 0.01 \times 5 \times 41320.4 \times K}{\begin{array}{c} D \times \text{Sample weight (g)} \times 2 \\ \times \text{Protein amount (mg mL}^{-1}\text{)} \times L \times 1 \end{array}}$$

Where,

J = Peak area of ACS

C = Concentration of standard C_2H_4 used (0.9 µL L^{-1})

D = Peak area of 0.9 µL L^{-1} ethylene standard used

K = ACC content in nmol g^{-1}

L = ACC content in nmol

0.01 = Test tube head space volume (L)

1 = Incubation time to convert per h

2 = Amount of enzyme extract used (mL)

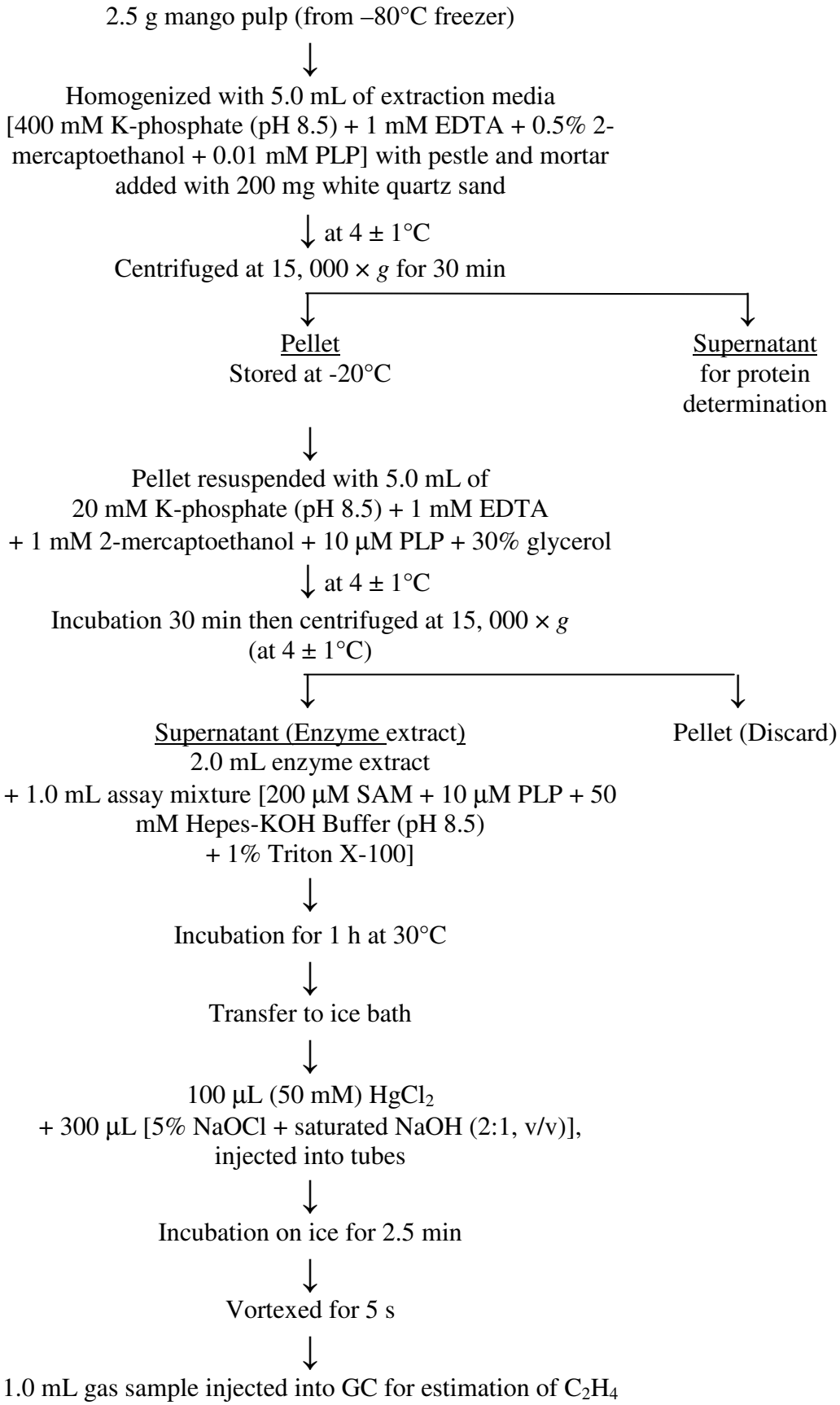

Figure 3.17. Flow chart for extraction and assay of ACS activity in pulp of mango fruit.

3.8 Fruit softening enzymes analysis

Fruit softening enzymes, including *exo*-polygalacturonase (*exo*-PG), *endo*-polygalacturonase (*endo*-PG), pectinesterase (PE) and *endo*-1,4-*β*-D-glucanase (EGase) from fruit pulp tissue by following the methods of Khan and Singh (2007) with some modifications (Section 3.8.1 - 3.8.3).

3.8.1 Extraction of exo-, endo-PG, PE and EGase

The fruit pulp tissue (13.0 g) was homogenized with 13.0 mL cold solution, containing 12% polyethylene-glycol and 0.2% sodium bisulphite ($NaHSO_3$). The supernatant was immediately stored at -80°C freezer for determination of protein content as mentioned details in Section 3.9. Following centrifugation at 4°C for 40 min at 15, 000 × *g*, the pellet was washed with a 13.0 mL aqueous solution of $NaHSO_3$ (0.2%) and re-centrifuged at 4°C for 40 min at 15, 000 × *g*. The pellet was stored at -80°C freezer for determination of *exo*- and *endo*-PG (Section 3.8.2), PE (Section 3.8.3) and EGase (Section 3.8.4). A flowchart of the enzyme extraction is shown in Figure 3.18.

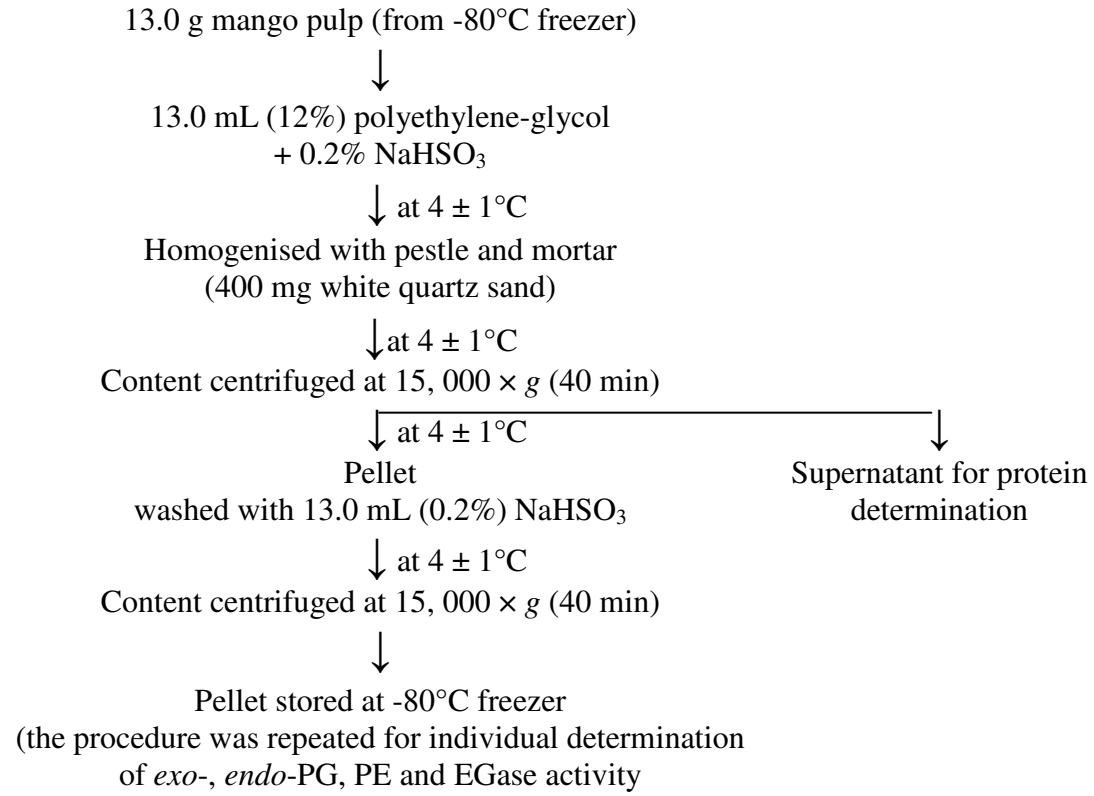

Figure 3.18. Flow chart for extraction and assay of *endo*-, *exo*-PG, PE and EGase activities in pulp of mango fruit.

3.8.2 Determination of exo- and endo-PG activity

3.8.2.1 Preparation of crude enzyme extract for determination of exo- and endo-PG activity

Pellet from detail extraction method mention in Section 3.8.1 was incubated on shaker at 4°C for 1 h in 15.0 mL cold 50 mM sodium acetate (CH_3COONa) buffer (pH 5) containing 0.5 M NaCl (Figure 3.19). Following the centrifugation at 15, 000 × *g* for 15 min at 4°C, supernatant was diluted 1:1 with 50 mM CH_3COONa buffer (pH 5) and was used as a crude enzyme extract for determination of *exo*- and *endo*-PG analysis.

Pellet incubated with cold 15.0 mL (50 mM) CH_3COONa, pH 5
+ 0.5 M NaCl on shaker for 1 h
↓ at 4 ± 1°C
Content centrifuged at 15, 000 × *g* for 15 min
↓ at 4 ± 1°C
Supernatant diluted with 50 mM CH_3COONa buffer, pH 5 (1:1, v/v)
(its crude enzyme extract)

Figure 3.19: Flow chart for crude enzyme extract preparation for *exo*- and *endo*-PG analysis

3.8.2.2 Determination of exo-PG activity

The enzyme extract (0.15 mL) was mixed with 0.15 mL of (0.5%) polygalacturonic acid in 50 mM CH_3COONa buffer (pH 4.4), and the content was incubated at 30°C for 18 h (Figure 3.20). To determine the amount of galacturonic acid released, 2.0 mL of 0.1 M borate buffer (pH 9.0) and 0.3 mL of 1% cyanoacetamide were added to reaction mixture and then boiled for 10 min. The absorbance of the cold solution was read at 274 nm by using a UV-VIS spectrophotometer (Model 6405, Jenway Ltd., Felsted, Dunmow, Essex, England) and was calculated against standard curve of D-galacturonic acid. The activity of e*xo*-PG was expressed as µg galacturonic acid mg protein^{-1} h^{-1} and calculated using the following formula:

$$\textit{Exo}\text{-PG activity} = \frac{\text{Galacturonic acid in sample } (\mu g) \times 2 \times 15}{0.15 \times 13 \times 18 \times \text{Protein amount } (\text{mg mL}^{-1})}$$

Where,

2 = Dilution factor

15 = Vol. of enzyme extract (mL)

0.15 = Vol. of extract used (mL)

13 = Vol. of extraction buffer (mL)

18 = Incubation time (h)

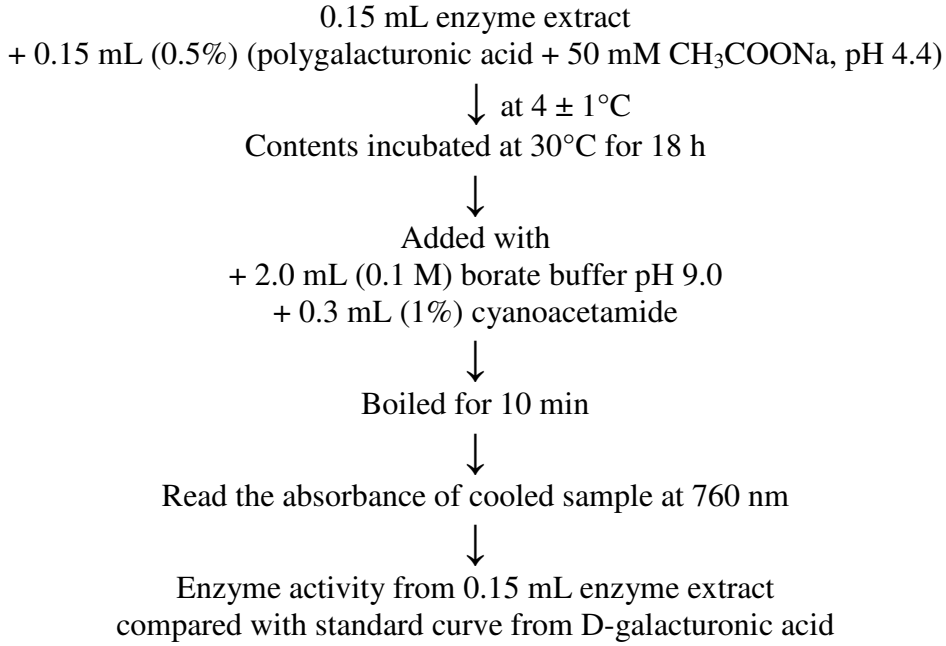

Figure 3.20. Flow chart for determination of *exo*-PG activity in pulp of mango fruit.

3.8.2.3 Determination of endo-PG activity

The activity of *endo*-PG was determined by measuring the viscosity using a Cannon-Fenske viscometer (Size 50, Cannon Instrument Company, PA, USA). Three mL of enzyme extract (Section 3.8.2.1) was mixed to a cold solution containing 4.5 mL of 2% polygalacturonic acid in a 50 mM CH_3COONa buffer (pH 4.4). The viscosity was measured immediately after the reaction mixture was incubated for 18 h at 30°C (Figure 3.21). The viscosity meter was cleaned with acetone after each run and calibrated with dH_2O.

3.0 mL enzyme extract
+ 4.5 mL [(2%) polygalacturonic acid + 50 mM CH_3COONa, pH 4.4]
↓ at 2 ± 1°C
Contents incubated at 30°C for 18 h
↓
Viscosity measured immediately and after 18 h
↓
Enzyme activity from 3.0 mL enzyme extract

Figure 3.21. Flow chart for determination of *endo*-PG activity in pulp of mango fruit.

The activity of *endo*-PG was expressed as Δ viscosity mg protein^{-1} h^{-1} and calculated using the following formula:

$$\textit{Endo}\text{-PG activity} = \frac{[(V_{before} - VdH_2O) - (V_{after} - VdH_2O)] \times 2 \times 15}{3 \times 13 \times 18 \times \text{Protein amount (mg mL}^{-1})}$$

Where,

V_{before} = Viscosity of sample before incubation (s)
V_{after} = Viscosity of sample after incubation (s)
VdH_2O = Viscosity of dH_2O (s)
2 = Dilution factor
15 = Vol. of enzyme extract solution (mL)
3 = Vol. of extract used (mL)
13 = Vol. of extraction buffer (mL)
18 = Incubation time (h)

3.8.3 Determination of PE activity

To determine the activity of PE, the pellet as described in Section 3.8.1 was resuspended in 15 mL cold solution containing NaCl (7.5%, w/v) + EDTA (0.75%, w/v) at pH 6.5 and incubated at 4°C for 10 min (Figure 3.22). Following centrifugation at 15, 000 × *g* for 15 min, a 20 mL of citrus pectin solution (1% w/v) at pH 7.5 was mixed with 5 mL enzyme extract solution. The reaction mixture was titrated against 0.01 *N* NaOH and maintain to pH 7.4, while incubating at 30°C for 10 min. During the titration and incubation time, the reaction mixture was continuously and slowly shaked by hand. The total amount of 0.01 *N* NaOH to

maintain pH 7.4 was used to calculate the PE activity following the formula as mentioned below and the activity was expressed as mM NaOH mg^{-1} protein h^{-1}.

$$\text{PE activity} = \frac{\text{(Vol. of 0.01 } N \text{ NaOH used} \times 0.01) \text{ mM NaOH} \times \text{Vol. of enzyme extracts (mL)}}{\text{Sample weight (g)} \times (20/60) \text{ h} \times \text{Protein content (mg mL}^{-1})}$$

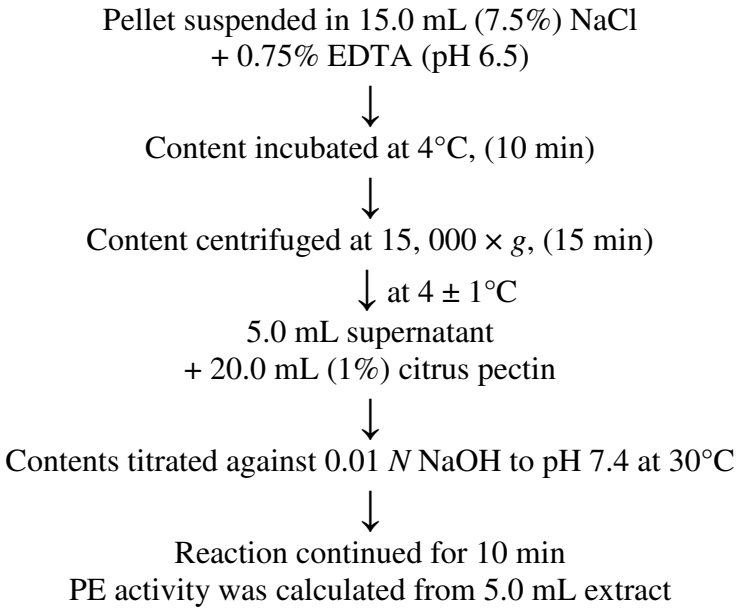

Figure 3.22. Flow chart for determination of PE activity in pulp of mango fruit.

3.8.4 Determination of EGase activity

Determination of EGase activity was performed by stirring the extraction pellet with 15.0 mL of 0.1 M citrate-phosphate buffer (pH 6.0) containing 1.0 M of NaCl for 1 h (Figure 3.23). Following centrifugation at 15, 000 × *g* for 15 min, 3.0 mL of supernatant was mixed with 6.0 mL of 0.2% carboxymethyl cellulose in citrate-phosphate buffer (pH 6.0). The viscosity changes were measured immediately and after 18 h incubated at 30°C using a viscocity meter as mentioned previously in Section 3.8.2.3. The enzyme activity from 3.0 mL enzyme extract was calculated following the formula for calculating the activity of *endo*-PG as mentioned in Section 3.8.2.3 and expressed as Δ viscosity mg $protein^{-1}$ h^{-1}.

Pellet stirred with 15.0 mL (0.1 M) citrate-phosphate buffer, pH 6
+ 1.0 M NaCl for 1 h at 4 ± 1°C

↓

Content centrifuged at 15, 000 × *g* (15 min, at 4 ± 1°C)

↓

3.0 mL supernatant
+ 6.0 mL 0.2% carboxymethyl cellulose in citrate phosphate buffer

↓

Contents incubated at 30°C for 18 h

↓

Viscosity measured immediately and after 18 h incubation

↓

Enzyme activity form 3.0 mL enzyme extract

Figure 3.23. Flow chart for determination of EGase activity in pulp of mango fruit.

3.9 Determination of protein contents

3.9.1 Protein reagent preparation

Total protein content of mango pulp sample was determined following a Bradford method (Bradford, 1976). Coomassie brilliant blue dye G-250 (100.0 mg) was dissolved in 50.0 mL of 95% ethanol and 100.0 mL of 85% phosphoric acid (w/v). The volume was diluted and made up to 1 L with dH_2O. The dye was filtered through Whatman No.1 filter paper (Whatman Intl. Ltd., Maidstone, England) and stored in dark bottles refrigeration.

3.9.2 Determination of protein content in mango sample

Bradford reagent solution (5.0 mL) was added into 100.0 µL of protein sample, took from the extraction sample mentioned in Section 3.8.1, and put in 12 × 100 mm reaction tube. The contents were mixed well using vortex mixer (Heidolph, John Morris Scientific Pty. Ltd., Germany) and the absorbency was taken at 595 nm in 3.0 mL plastic disposable cuvettes using UV-VIS spectrophotometer (Model 6405, Jenway Ltd., Felsted, Dunmow, Essex, England) (Figure 3.24). The reagent blank was prepared by mixing 100 µL of the appropriate buffer with 5.0 mL of protein reagent. The protein concentration was determined by comparing the value with standard graph prepared using bovine serum albumin and was expressed as mg mL^{-1} FW.

100 µL protein sample
↓
Added with 5.0 mL protein reagent
↓ vortex for 5 s
Measure the absorbance after 15 min
↓
Put in 3 mL plastic disposible cuvettes
↓
Read absorbance at 595 nm using a spectrophotometer

Figure 3.24. Flow chart of determination of protein content from pulp tissues of mango fruit.

3.10 Chemicals

Details of all chemical used listed in this chapter are presented in Appendix 1.

3.11 Statistical Analysis

All the experimental data were subjected to one-, two- or three-way analysis of variance (ANOVA) using Statistical Analysis System (SAS) (release 9.1.3, SAS Institute Inc., Cary, NC, USA). Fisher's Least Significant Differences (LSD) were calculated following a significant ($P \leq 0.05$) F-test was used to test the differences between the treatments. All the assumptions of ANOVA were checked to ensure validity of statistical analysis.

CHAPTER 4

Role of brassinosteroids, ethylene, abscisic acid, and indole-3-acetic acid in mango fruit ripening

Summary

Rapid ripening of mango fruit limits its distribution to distant markets. In order to better understand and perhaps manipulate this process, I investigated the role of plant hormones in modulating climacteric ripening of 'Kensington Pride' mango fruits. Changes in endogenous levels of brassinosteroids (BRs), ethylene, abscisic acid (ABA), and indole-3-acetic acid (IAA), the respiration rate, pulp firmness and skin colour were determined at 2 days intervals during an 8 days ripening period at ambient temperature (21 ± 1°C). The effects of exogenously applied epibrassinolide (Epi-BL), S-(+)-*cis, trans*-abscisic acid (ABA) and an inhibitor of ABA biosynthesis, nordihydroguaiaretic acid (NDGA) on fruit-ripening parameters such as respiration, ethylene production, fruit softening and colour were also investigated. Climacteric ethylene production and the respiration peak occurred on the 4th day of ripening, while endogenous level of BRs (castasterone and brassinolide) were present in only trace amounts in fruit pulp throughout the ripening period. However, the exogenous application of Epi-BL (45 and 60 ng g^{-1} FW) advanced the onset of the climacteric peaks of ethylene production and respiration rate by 2 and 1 day(s), respectively, and accelerated fruit colour development and softening during the fruit ripening period. The endogenous level of ABA rose during the climacteric-rise stage on the 2nd day of ripening and peaked on the 4th day of ripening. Exogenous ABA promoted fruit colour development and softening during ripening compared with the control and the trend was reversed in NDGA-treated fruit. The endogenous IAA level in the fruit

This chapter has been accepted for publication in the following articles:
***Zaharah, S.S.**, Zora Singh, Symons, G.M. and Reid, J.B. Role of brassinosteroids, ethylene, abscisic acid, and indole-3-acetic acid in mango fruit ripening. Journal of Plant Growth Regulation. DOI 10.1007/s00344-011-9245-5.*

A small part of this result has also been accepted for publication in the following articles:
***Zaharah, S.S.** and Zora Singh. 2011. Abscisic acid modulates mango fruit ripening. Acta Horticulturae.*
***Zaharah, S.S.** and Zora Singh. 2011. Role of brassinosteroids in mango fruit ripening. Acta Horticulturae.*

pulp was higher during the pre-climacteric (PC) minimum stage and declined during the climacteric and post-climacteric stages. I speculate that higher levels of endogenous IAA in fruit pulp during the PC stage and the accumulation of ABA prior to the climacteric stage might switch on ethylene production that triggers fruit ripening. Whilst exogenous Epi-BL promoted fruit ripening endogenous measurements suggest that changes in BRs levels are unlikely to modulate mango fruit ripening.

4.1 Introduction

Mango (*Mangifera indica* L.) is a climacteric fruit, in which ripening involves a pre-climacteric (PC) minimum, climacteric-rise (CR), climacteric peak and post-climacteric phases (Watada et al., 1984). The ripening process of mango fruit involves numerous biochemical changes including increased respiration, ethylene production, fruit softening, chlorophyll degradation, carotenoid synthesis and several other metabolic activities leading to changes in carbohydrates, organic acids, lipids, phenolics and volatile compounds (Gomez-Lim, 1993; Singh and Singh, 2011). Ethylene plays a pivotal role in regulation of climacteric fruit ripening (Seymour et al., 1993) and the role of ethylene in controlling mango fruit ripening has been well established (Cua and Lizada, 1990; Lalel et al., 2003e; Mann, 1985; Nair and Singh, 2003; Singh and Janes, 2001). Under ambient temperature, 'Kensington Pride' mangoes harvested at the mature green stage exhibit a climacteric ethylene peak during day 3 of ripening with a concomitant increase in respiratory production on the same day (Lalel et al., 2003e). Exogenous application of ethephon increased ethylene production and respiration rate in 'Kensington Pride' mangoes at ambient temperature (21 ± 1°C), whilst, the application of inhibitors of ethylene biosynthesis [e.g. aminoethoxyvinylglycine (AVG)] and action [e.g. 1-methylcyclopropene (1-MCP)], suppressed these processes (Lalel et al., 2003e). Nguyen et al. (2002) also claimed that 'Kensington Pride' mangoes took less days to reach the soft (eating) stage when treated with exogenous ethylene, although they retained the green skin colour.

Abscisic acid (ABA) also plays an important role in fruit ripening (Vendrell and Palomer, 1997). Increased levels of ABA during ripening have been reported in climacteric fruit such as tomato (Ruan et al., 2005), peach (Wu et al., 2003) and plum (Kitamura et al., 1983), although, there are no reports on changes in endogenous

ABA levels during mango fruit during ripening. Exogenous application of ABA enhances fruit ripening in several cultivars of mango such as 'Alphonso', 'Langra' and 'Zihua' (Palejwala et al., 1988; Parikh et al., 1990; Zhou et al., 1996), although the exogenous application of ABA and an inhibitor of its biosynthesis, nordihydroguaiaretic acid (NDGA), in modulating fruit ripening of 'Kensington Pride' mango fruit has not yet been investigated. Exogenous application of ABA and/or NDGA have been reported to regulate the endogenous concentrations of ABA, and consequently influenced fruit ripening in various climacteric fruits such as banana (Lohani et al., 2004), kiwifruit (Chen et al., 2005), and tomato (Zhang et al., 2009b; Zhu et al., 2003).

In climacteric fruit, the level of auxin has been claimed to influence ethylene production through the induction of the activity of 1-aminocyclopropane-1-carboxylic acid (ACC) synthase (ACS) during fruit ripening (Vendrell and Palomer, 1997). A burst of climacteric ethylene resulted in the rapid degradation of endogenous IAA in 'Yulu' peach on the 3rd day of ripening (Wu et al., 2003). Contrarily, endogenous levels of IAA increased during the fruit-beaker and ripe-fruit stages in the *rin* (ripening inhibitor) tomato mutant (Hong and Lee, 1993). However, it is not known how endogenous IAA levels change during the ripening of mango fruit.

Brassinosteroids (BRs) are a group of steroidal plant hormones implicated in numerous plant growth and development processes including cell elongation, cell division, vascular differentiation, reproductive development, pathogen and abiotic tolerance (Clouse, 2002). Recent evidence suggests that BRs are also involved in the ripening of grapes, a non climacteric fruit (Symons et al., 2006). In the climacteric tomato fruit, the exogenous application of BRs has been reported to promote ripening of tomato pericarp discs through increased ethylene production (Vardhini and Rao, 2002). Later on, Montoya et al. (2005) reported higher concentrations of endogenous BRs in tomato fruit during the early stages of development. The intriguing question is therefore whether endogenous BRs levels change during fruit ripening in mango and whether BRs play a key role in climacteric fruit ripening?

I examined the changes in the endogenous level of BRs, ethylene, ABA and IAA changed during the ripening processes of 'Kensington Pride' mango fruit and their exogenous application influenced the rate of ripening in order to further understand the role of plant growth regulators in regulating fruit ripening.

4.2 Materials and methods

In experiment I, changes in the endogenous levels of brassinosteroids (BRs) (catasterone and brassinolide), ethylene, abscisic acid (ABA), and indole-3-acetic acid (IAA) were measured along with fruit firmness and skin colour development during ripening in 'Kensington Pride' mango fruit. In experiments II and III, the effects of exogenous Epibrassinolide (Epi-BL) as well as ABA and nordihydroguaiaretic acid (NDGA) on fruit ripening parameters such as respiration rate, ethylene production, fruit softening, and skin colour development were examined in 'Kensington Pride' mango fruit during ripening at ambient temperature.

4.2.1 Fruit

Hard mature mango fruit (*Mangifera indica* L. cv. 'Kensington Pride'), characterised by green skin and light cream pulp colour, uniform size, free from visual blemishes and diseases were used in all three experiments. In experiments I and II, fruit were obtained from a commercial orchard at Gingin (lat. 115° 55′E, long. 31° 21′S), Western Australia (WA) on 19th and 31st of March, 2008, respectively. In experiment III, hard mature green fruit was obtained from a commercial orchard located at Dongara (lat. 29.26° 15′S and long. 114.93° 55′E), WA on 18th of February, 2009. In experiment I, the mature fruit were firm (92.92 ± 3.71 N) and had a respiration rate of 2.36 ± 0.01 mmol kg^{-1} h^{-1}. In experiment II, the fruit were also firm (102.83 ± 18.48 N) and had a respiration rate of 1.64 ± 0.04 mmol CO_2 kg^{-1} h^{-1}. In experiment III, the fruit were firm (75.6 ± 6.71 N) and had a respiration rate 2.68 ± 0.40 mmol CO_2 kg^{-1} h^{-1}. All the fruit were desapped (following harvest, sap from fruit was allowed to exude from the end of the stalk by physical inversion of the fruit on de-sapping trays to avoid sap burn injury over the skin), fungicide-treated (Sportak 0.55 mL L^{-1} with Prochloraz as an a.i.), air dried, packed in soft-board trays, and transported to Perth, WA, by a refrigerated truck (13°C).

4.2.2 Experiment I: Changes in endogenous level of BRs, ABA, IAA, and ethylene during mango fruit ripening

The mango fruit were kept in soft board trays and allowed to ripen at ambient temperature (21 ± 1°C, 57.18 ± 11.09% RH as detail mentioned in Chapter 3, Section 3.3). Pulp samples from the inner and outer mesocarp were collected at 2 days intervals during the 8 days ripening period. Pulp tissue was immersed in liquid N_2 (-196°C) and stored at -80°C freezer for determination of endogenous levels of BRs, ABA, and IAA. Ethylene production, respiration rate, fruit softness and skin colour development were determined every two days during fruit ripening. The experiment used a completely randomised design, including ripening period as a factor. Ten fruit were used for each of three replicates.

4.2.3 Experiment II: Effects of exogenous application of BRs on mango fruit ripening

Aqueous solution containing different doses (15, 30, 45, and 60 ng g^{-1} FW) of epibrassinolide [Epi-BL; (22R,23R)-2α,3α,22,23-Tetrahydroxy-7-oxa-B-homo-5α-ergostan-6-one] were applied onto the skin of the whole hard mature mango fruit. The Epi-BL was purchased from Sigma-Aldrich Pty. Ltd., Castle Hill, Australia. Untreated fruit served as a control. Following treatment, the fruit were allowed to ripen at ambient temperature (21 ± 1°C, 54.08 ± 9.07% RH as detail mentioned in Chapter 3, Section 3.3). Ethylene production, respiration rate, fruit softness and skin colour development were recorded daily during the ripening period. The time of onset of the climacteric peak and the rate of ethylene production and respiration rate at the climacteric peak were recorded. The experiment used a completely randomised design, including three replicates of ten fruit.

4.2.4 Experiment III: Effects of exogenous application of ABA and NDGA on modulating mango fruit ripening

Hard mature green fruit were dipped for five minutes in an aqueous solution containing different concentrations (0.05, 0.1, and 0.2 mM) of S-(+)-*cis, trans*-abscisic acid (ABA) (Syntake Chemical, Shanghai, China) or 0.05, 0.01, and 0.02 mM of nordihydroguaiaretic acid (NDGA) purchased from Sigma-Aldrich Pty. Ltd., Castle Hill, Australia). 'Tween 20' (0.05%) was used as a surfactant. NDGA is an inhibitor of ABA biosynthesis (Zhang et al., 2009b). Following treatments, the fruit

were allowed to dry and ripen at ambient temperature (21 ± 1°C, 55.2 ± 11.1% RH as explained ealier in Chapter 3, Section 3.3) in soft board trays until reaching the soft (eating) stage (subjective firmness rating score = 4) as previously described by Dang et al. (2008a) and in Chapter 3, Section 3.6.2.1. Control fruit were dipped in water containing 0.05% of 'Tween 20'. Respiration rate, fruit softening and skin colour development were determined daily during the ripening period. The experiment used a completely randomised design, including three replicates of ten fruit.

4.2.5 Extraction, purification and quantification of BRs, ABA, and IAA

4.2.5.1 Extraction of BRs, ABA and IAA

Mango fruit pulp (200 g) was used for the extraction of BRs, ABA and IAA, following the methods described earlier by Ross et al. (1987) as explained in detail in Chapter 3, Section 3.4.1.1.

4.2.5.2 Brassinosteroid (BRs) purification and quantification

An aliquot of each extract equivalent to 50 g FW of pulp was taken and 50 ng of deuterated ($^2H_6$) brassinolide, and castasterone (provided by Dr. Suguru Takatsuto and Prof. Takao Yokota from Japan) were added to each sample as internal standards. Sample purification and gas chromatography-mass spectrometry with selected ion monitoring (GC-MS-SIM) quantification of BRs were performed as described by Symons and Reid (2003). The only exception is that these samples were not subjected to a C18-Sep Pak purification step.

4.2.5.3 IAA and ABA purification and quantification

The purification and quantification of IAA and ABA samples were carried out using the methods described by Jager et al. (2008) and were calculated as described by Ross et al. (1995). Positive identification of IAA and ABA of mango pulp tissues was achieved by full-scan MS and expressed as ng g^{-1} FW. The purification and quantification procedure is explained in detail in Chapter 3, Section 3.4.1.2.

4.2.6 Ethylene production

The rates of ethylene production in mango fruit were estimated during two days interval of ripening period. One mL of headspace gas taken from individual jar sealed for 1 h at 20 ± 1°C was taken and injected into a gas chromatograph (6890N

Network GC System; Agilent Technology, Palo Alto, CA^2, USA). The estimation procedure is explained in detail in Chapter 3, Section 3.4.2 and was expressed as nmol C_2H_4 kg^{-1} h^{-1}. The ethylene production was recorded twice.

4.2.7 Respiration rate

The rate of respiration was determined from the head space gas (1.0 mL) using an infrared gas analyser [Servomex Gas Analyser, Series 1450 Food Package Analyser; Servomex (UK) Ltd., Crowborough, UK] as detailed in Chapter 3, Section 3.5 and were expressed in mmol kg^{-1} h^{-1}. These estimations were repeated twice.

4.2.8 Fruit firmness

Fruit (pulp) firmness was measured every two days during mango fruit ripening in experiment I, using a texture profile analyser (TPA Plus; AMETEK Lloyd Instruments Ltd., Fareham, UK) following a details method as explained in Chapter 3, Section 3.6.2.2. Only the result of mango pulp firmness (hardness) which expressed in Newtons (N) is presented in this chapter. The rate of pulp firmness loss was calculated as percent firmness loss per 2 days interval during the ripening period.

4.2.9 Fruit softness

Fruit softness from the middle of each fruit was recorded daily in experiments II and III using a scale 1 to 5 following a detail rating method as described in Chapter 3, Section 3.6.2.1.

4.2.10 Visual skin colour

The visual fruit skin colour was recorded following methods decribed by Dang et al. (2008a) as detail explained in Chapter 3, Section 3.6.1.1. The data was recorded every two days in the experiment I and daily in experiments II and III during the ripening period.

4.2.11 Statistical analysis

The experimental data were subjected to one- or two-way analysis of variance (ANOVA) using SAS release 9.1 (SAS Institute Inc., Cary, NC, USA). Fisher's Least Significant Differences (LSD) were calculated following a significant ($P \leq$

0.05) F-test. All the assumptions of ANOVA were checked to ensure validity of statistical analysis. Regression analysis was performed using the same programme to find out the relationship between the endogenous level of ABA and IAA, and between ethylene production or respiration rate and the endogenous level of ABA, IAA and respiration, and between the endogenous level of ABA or IAA and fruit softness and skin colour.

4.3 Results

4.3.1 Changes in endogenous level of BRs, ethylene, ABA and IAA during ripening

Castasterone (CAS) and brassinolide (BL) were only detected in fruit pulp in trace amounts during mango fruit ripening, with no clear changes in their accumulation throughout the ripening period (Table 4.1). However the CAS level may be slightly higher on day 8 (< 0.13 ng g^{-1} FW) and BL was only found (at trace levels) after 6 to 8 days of ripening at ambient temperature (Table 4.1).

As expected, a typical climacteric ethylene production peak (8.24 nmol C_2H_4 kg^{-1} h^{-1}) was noticed on day 4 of the ripening period (Figure 4.1A). Ethylene production was substantially lower (in a range of 0.72 to 1.30 nmol C_2H_4 kg^{-1} h^{-1}) during the PC and post-climacteric stages (Figure 4.1A).

The endogenous level of ABA in the pulp tissue significantly ($P \leq 0.001$) increased (2.87-fold) during the CR stage (2 days of ripening) and peaked at the climacteric stage (3.77 fold increase on 4^{th} days of ripening) compared with the PC minimum on day 0 of ripening (Figure 4.1B). The endogenous ABA level in the pulp tissue significantly ($P \leq 0.001$) declined 1.2- to 1.3-fold during the post-climacteric period (after 6 and 8 days of ripening), when compared with the endogenous level of ABA on day 4 of ripening.

The endogenous level of IAA in the pulp tissues was highest (3.30 ng g^{-1} FW, $P \leq 0.001$) at the PC minimum on day 0 of the ripening period and declined substantially during the climacteric and post-climacteric ripening period. The endogenous level of IAA in the pulp tissue was 5.21-fold lower after 8 days of

ripening compared with its concentrations in the pulp tissue during the PC minimum stage (day 0) (Figure 4.1C).

Table 4.1. Changes in endogenous level of BRs (castasterone and brassinolide) in the pulp tissue of 'Kensington Pride' mango during ripening period

Ripening period (d)	Brassinosteroids level (ng g^{-1} FW)	
	Castasterone (CAS)	Brassinolide (BL)
0	Trace < 0.019	ND
2	ND	ND
4	Trace < 0.016	ND
6	Trace <0.009	Possible trace
8	Trace <0.13	Possible trace

Note: ND = not detected in the pulp tissue of 'Kensington Pride' mango

4.3.2 Respiration rate, fruit firmness and skin colour during fruit ripening

The climacteric respiration peak (3.74 mmol CO_2 kg^{-1} h^{-1}) was noticed on the 4th day of ripening and was significantly ($P \leq 0.05$, 1.58-fold) higher compared with the rate at the PC minimum stage on day 0 of ripening (Figure 4.2A). The rate of respiration declined substantially (1.42- and 1.51-fold) during the post-climacteric period (on day 6 and 8 of ripening, respectively) compared with its production during the climacteric respiration peak.

As expected, fruit firmness was highest (92.92 N) at the PC minimum stage on day 0 (Figure 4.2B). The percentage of pulp firmness losses exhibited higher reduction (61.72% and 60.11%) at the climacteric peak stage on day 4 of ripening and on day 6 of ripening, respectively, than at the CR stage on the 2nd day of ripening (36.28%) and post-climacteric stage on the 8th day of ripening (31.64%).

The skin colour was green at the PC minimum and at the CR stages (day 0 and 2 respectively, Figure 4.2C). There was a dramatic increase (2.33-fold) in skin colour development at the climacteric peak stage on day 4 of ripening compared with the colour development at the climacteric minimum stage. The skin colour continued to yellow during the post-climacteric stages (after 6 and 8 days of ripening).

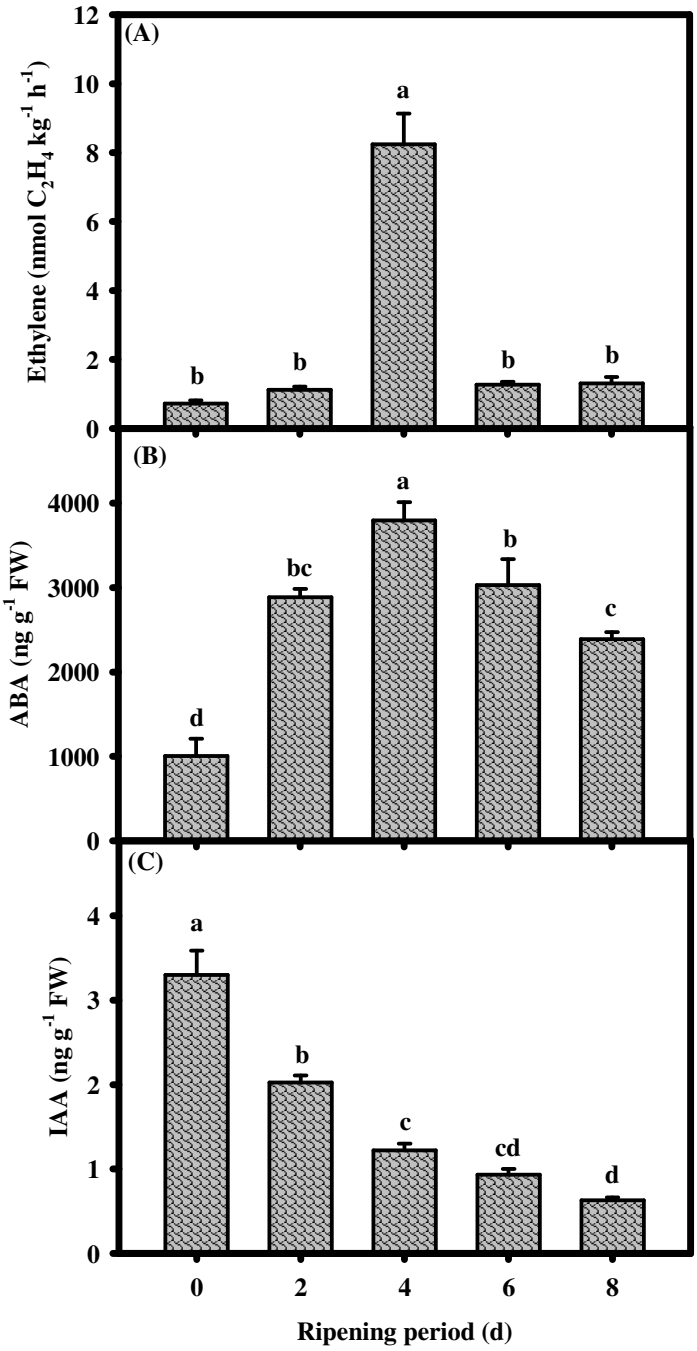

Figure 4.1. Changes in endogenous level of ethylene (A), ABA (B) and IAA (C) in the pulp during fruit ripening period at ambient temperature.
Vertical bars represent S.E. of means followed by the same letters are not significantly different at LSD ($P \leq 0.001$) for ethylene = 0.94, ABA = 631.84 and IAA = 0.45. n = 3 replications.

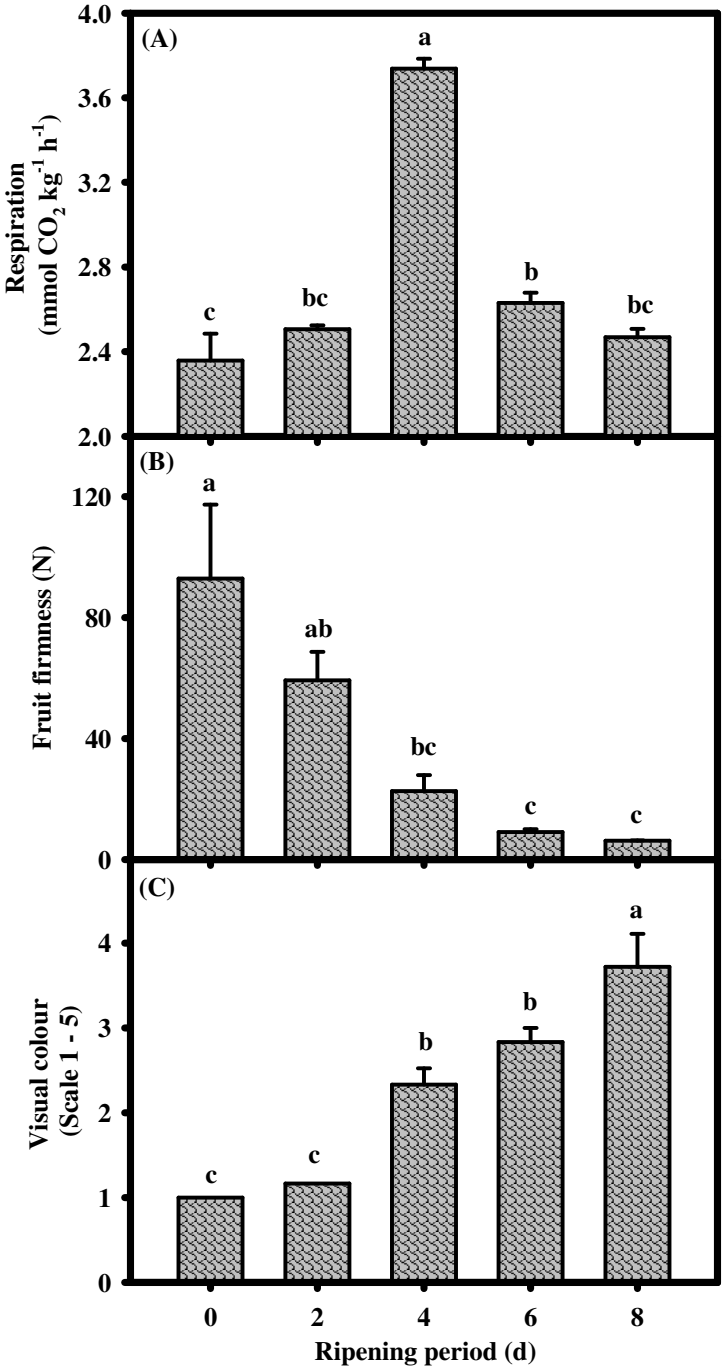

Figure 4.2. Changes in respiration rate (A), fruit firmness (B) and skin colour (C) during fruit ripening period at ambient temperature.
Vertical bars represent S.E. of means followed by the same letters are not significantly different at LSD ($P \leq 0.05$). LSD ($P \leq 0.01$) for respiration rate = 0.16, fruit firmness = 35.75 and skin colour = 0.44. n for respiration rate = 3 replications, fruit firmness = 30 (10 fruit × 3 replications) and skin colour changes = 30 (10 fruit × 3 replications).

4.3.3 Effects of exogenous application of Epi-BL on onset and climacteric peak of ethylene and respiration rate

As expected, mango fruit exhibited climacteric ethylene and respiration peaks during the ripening period. The exogenous application of Epi-BL treatments (45 and 60 ng g^{-1} FW) significantly advanced the onset of the climacteric peak of ethylene production and respiration rate by two and one day respectively (Table 4.2). Both of these treatments also had higher climacteric ethylene production peak (4.81 and 5.74 nmol C_2H_4 kg^{-1} h^{-1}) and respiration rate (4.87 and 5.06 mmol CO_2 kg^{-1} h^{-1}) compared with the control (Table 4.2).

Table 4.2. Changes in climacteric ethylene production and respiration rate during fruit ripening influenced by exogenously applied different doses of Epi-BL.

Epi-BL conc. (ng g^{-1} FW)	Ethylene climacteric		Respiration climacteric	
	Onset (d)	Peak rate (nmol C_2H_4 kg^{-1} h^{-1})	Onset (d)	Peak rate (mmol CO_2 kg^{-1} h^{-1})
0	5.00a	1.58c	5.00a	3.55d
15	5.00a	1.92c	4.00b	4.21c
30	4.00b	3.06b	4.00b	4.45bc
45	3.00c	4.81a	4.00b	4.87ab
60	3.00c	5.74a	4.00b	5.06a
LSD ($P \leq 0.05$)	0.47^{***}	1.05^{***}	0.47^{**}	0.48^{***}

Means followed by the same lowercase within a column are not significantly different at $P \leq 0.05$ with n = 3 replications. ** and *** significantly at $P \leq 0.01$ and 0.001, respectively. Epi-BL = epibrassinolide.

4.3.4 Effect of exogenous application of Epi-BL on fruit softening during ripening

Exogenous applications of Epi-BL promoted fruit softening particularly between day 3 to 7 of the ripening period compared with the control (Figure 4.3A). Fruit softness at the full-ripe stage did not significantly differ between Epi-BL-treated fruit and the control (Figure 4.3A). Averaged over the ripening period, the mean of fruit softness was significantly ($P \leq 0.05$) higher (1.13-, 1.16-, 1.15-, and 1.23-fold) after treated with 15, 30, 45, and 60 ng g^{-1} FW of Epi-BL respectively, compared with the control.

All Epi-BL treatments significantly ($P \leq 0.05$) improved skin colour development between 2 to 7 days of ripening, irrespective to the concentration applied (Figure 4.3B). Averaged over the ripening period, the mean of skin colour development were significantly ($P \leq 0.05$) higher (about 1.3-fold) in all fruit treated with 15, 45 and 60 ng g^{-1} FW of Epi-BL compared with the control.

4.3.5 Effect of exogenous application of ABA and NDGA on fruit softening and skin colour development during ripening

As expected, in ABA-treated fruit, irrespective of the concentration applied, fruit softening was slightly promoted, particularly from day 3 to day 7 of the ripening period, compared with the control and all treatments of NDGA, an ABA biosynthesis inhibitor (Figure 4.3C), whilst the trends were the reverse in NDGA-treated mango fruit. Averaged over the ripening period, the mean of fruit softening was significantly ($P \leq 0.05$) higher (1.06-, 1.11-, and 1.10-fold) after 0.5, 1.0, and 2.0 mM ABA-treatment compared with the control, respectively. Further, averaged over the ripening period, mean fruit softening was significantly ($P \leq 0.05$) lower (1.08-fold) in 0.2 mM NDGA-treated fruit compared with the control.

Fruit treated with 1.0 mM ABA also showed more pronounced skin yellowing after 3 to 7 days of ripening compared with the control (Figure 4.3D). The application of 0.2 mM NDGA inhibited skin colour development from day 1 to 8 of ripening compared with the control (Figure 4.3D). Averaged over the ripening period, the mean of skin colour development was significantly ($P \leq 0.05$) higher (1.08- and 1.11-fold) in 0.5 and 1.0 mM ABA-treated fruit when compared with the control, respectively. The trend of fruit colour development in the 0.05, 0.1, and 0.2 mM of NDGA treated fruit was the reverse.

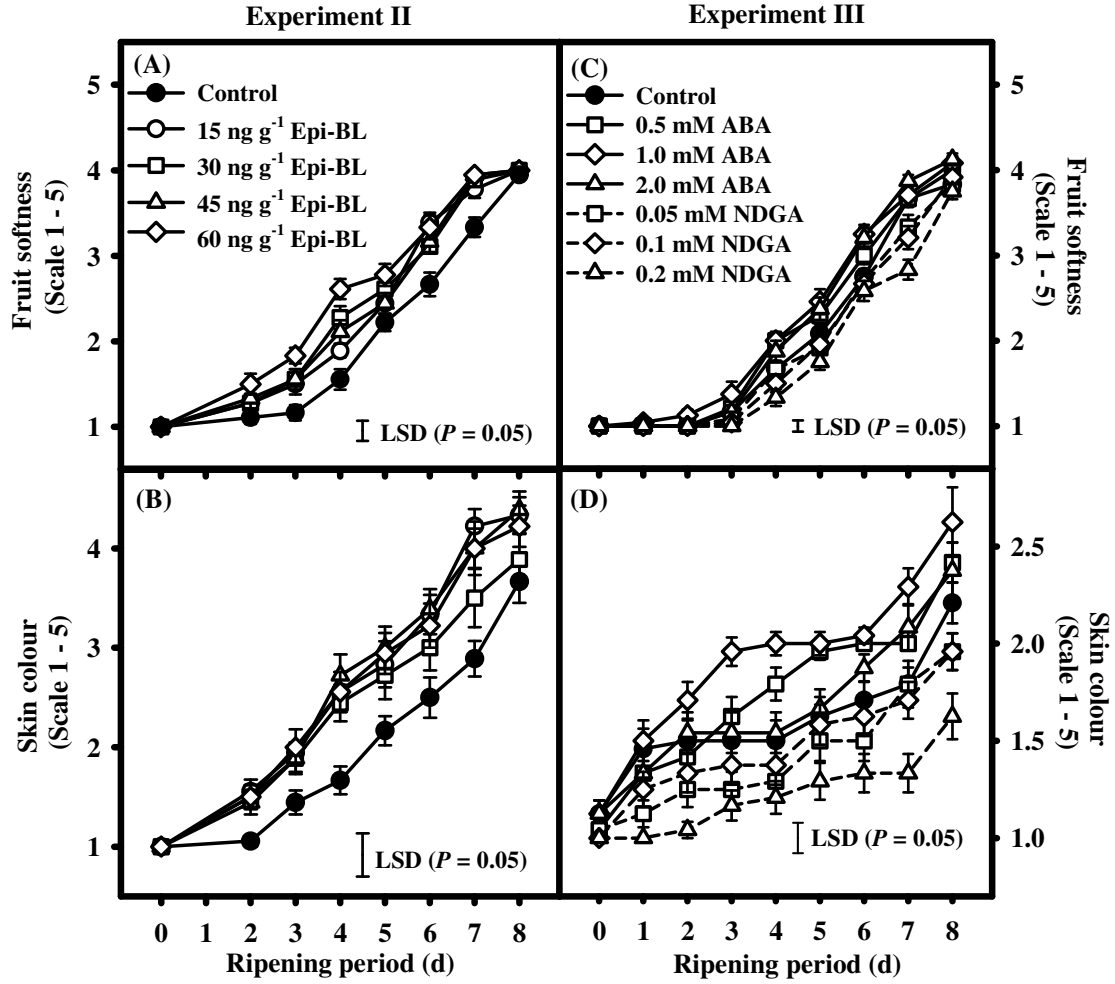

Figure 4.3. Changes in fruit softening and skin colour development influenced by different doses (T) of Epi-BL (A and B) and ABA and its inhibitor (C and D) during ripening (RP) period at ambient temperature.
Vertical bars represent S.E. of mean and are invisible when the values are smaller than the symbol. n = 30 (10 fruit × 3 replications). LSD (* and *** represent significantly different at $P \leq 0.05$ and 0.001) for fruit softness (A): T = 0.10^{***}, RP = 0.12^{***} and T × RP = 0.27^{***} and (C): T = 0.07^{***}, RP = 0.09^{***} and T × RP = 0.23^{*} and skin colour (B): T = 0.17^{***}, RP = 0.22^{***} and T × RP = NS and (D): T = 0.09^{***}, RP = 0.10^{***} and T × RP = 0.27^{*}.

4.4 Discussion

This study is the first time that endogenous levels of a wide range of plant hormones (BRs, IAA, ABA and ethylene) have been investigated simultaneously during mango fruit ripening. Of particular interest is the finding that endogenous levels of the BRs (CAS and BL) were low and did not show pronounced changes during fruit ripening in 'Kensington Pride' mango (Table 4.1). This contrasts with the 13-fold increase in the level of CAS that coincided with the onset of ripening in 'Cabernet Sauvignon' grape (Symons et al., 2006). Grapes also contained much higher CAS levels (> 20 ng g^{-1} FW or > 5 ng $berry^{-1}$) than those found in mango (Table 4.1), along with a consistent increase in the expression of the genes *VvDWF1* and *VvBRI1* that are involved in BRs biosynthesis (Symons et al., 2006). This suggests that changes in endogenous BRs levels may not play a role in the ripening of climacteric fruits like mango, unlike their positive role in the non-climateric fruit, grape.

While the changes in BRs levels were small in mango fruit, the exogenous application of Epi-BL (45 and 60 ng g^{-1} FW) did significantly ($P \leq 0.05$) advance the onset of the climacteric peak of ethylene production and respiration rate by 2 and 1 day(s), respectively (Table 4.2). Both these treatments also increased the climacteric ethylene peak (4.81 and 5.74 nmol C_2H_4 kg^{-1} h^{-1}) and respiration rate (4.87 and 5.06 mmol CO_2 kg^{-1} h^{-1}) compared with the other Epi-BL treatments and the control (Table 4.2). Furthermore, all Epi-BL treatments significantly ($P \leq 0.05$) improved mango skin colour development between 2 to 7 days of fruit ripening (Figure 4.3B). However, these results do not necessarily support a direct role for BRs in mango ripening because it has been suggested that applied BRs may stimulate ethylene biosynthesis (Schlagnhaufer et al., 1984). Brassinosteroids [3.0 μM 28-homobrassinolide (28-homo-BL) or 24-epibrassinolide (24-epi-BL)] have also been reported to stimulate ethylene production in pericarp discs of tomato fruit (Vardhini and Rao, 2002).

It is well established that ethylene plays a pivotal role in regulating the ripening of climacteric fruits, including mango (Brecht and Yahia, 2009; Singh and Singh, 2011). Consistent with previous findings, my results show a climacteric ethylene peak associated with the climacteric respiration peak on the 4^{th} days of ripening (Figure 4.1A and 4.2A), which I suggest triggers ripening of mango fruit.

This increased ethylene production in 'Kensington Pride' mango fruit, probably occurs due to increases in the ACC content as well as the activities of ACS and ACC oxidase (ACO) during the ripening period (Nair et al., 2004b), since ethylene production and respiration is also increased in ethephon-treated and reduced in 1-MCP-treated fruit (Lalel et al., 2003e).

Climacteric ethylene production and the peak in respiration rate observed in mango fruit may be triggered by the higher concentration of ABA detected during the CR phase (day 2, Figure 4.1B). Abscisic acid is known to promote fruit ripening in climacteric fruits (Vendrell and Palomer, 1997). In the current study, the endogenous level of ABA rose on day 2, and peaked on day 4 of the ripening period, before declining in the post-climacteric stage (day 6 and 8, Figure 4.1B). The increase in ABA levels preceded major changes in ethylene levels and there was a significant ($P \leq 0.01$) positive exponential relationship ($r = 0.83$) between the endogenous level of ABA and ethylene production during the ripening period. High endogenous ABA levels have been previously reported to increase ethylene production during ripening of other climacteric fruits such as tomato (Ruan et al., 2005) and peach (Wu et al., 2003), as well as increasing the climacteric respiration rate in plum (Kitamura et al., 1983) and pear (Kochankov et al., 1975). Exogenous application of NDGA has been shown to inhibit biosynthesis of ABA and decrease ethylene production, and consequently retard fruit ripening process in 'Alisa Craig' tomato (Zhang et al., 2009b).

Whilst part of the effect of ABA on mango ripening may be mediated by its proposed effect on ethylene production, a direct role cannot be discounted. For instance, exogenous applications of ABA promoted fruit softening from day 3 of ripening compared with the control and the trend was reversed in NDGA-treated fruit (Figure 4.3C), prior to the climacteric in control fruit. Ethylene has been reported to be involved in increasing the activities of cell wall modifying enzymes such as polygalacturonase (PG) and pectinesterase activity (Brecht and Yahia, 2009; Singh and Singh, 2011). However ABA may also directly affect the activity of fruit softening enzymes, because Zhou et al. (1996) reported that exogenous application of 5 mg L^{-1} ABA increased PG activity and consequently promoted fruit softening in 'Zihua' mango. Likewise, ABA has also been reported to induce the maturation of

'Nam Dok Mai' and 'Nang Klangwan' mangoes (Kondo et al., 2004). In the current study there was a significant ($P \leq 0.01$) positive exponential relationship ($r = 0.77$) between the endogenous level of ABA in pulp tissues and pulp firmness during the ripening period.

The exogenous application of ABA promoted colouration of the skin and the application of its inhibitor, NDGA retarded colour development compared with the control (Figure 4.3D). This change in the skin colour of mangoes may be attributed to the ABA–induced accumulation of carotenoids as reported previously in mango cvs. 'Alphonso', 'Langra' and 'Zihua' (Palejwala et al., 1988; Zhou et al., 1996). In addition to the results obtained in mango, the exogenous application of ABA has also been reported to promote skin colour during fruit development and ripening in cherry (Kondo and Gemma, 1993), persimmon (Nakano et al., 1997) and grape (Koyama et al., 2010). However, it is important to note that in many of these studies (including the current investigation) it is difficult to distinguish between responses that are a direct effect of an exogenous (or applied) hormone and those responses that occur indirectly, and may be mediated by a secondary hormone signal(s).

The endogenous IAA level in the pulp was significantly ($P \leq 0.05$) higher during the initial ripening stage (day 0) and then declined substantially during the ripening period till the fully ripe stage (Figure 4.1C). A similar reduction in the endogenous level of IAA during the ripening period has also been reported in other climacteric fruits such as tomato (Sheng et al., 2000) and kiwifruit (Chen et al., 1999). There was a negative exponential relationship ($r = -0.87$) between the endogenous level of IAA in the pulp and pulp firmness during ripening and a significant positive exponential relationship ($r = 0.96$) between the endogenous level of IAA and skin colour development. Taken together, these results suggest that IAA levels may play a role in controlling these aspects of mango ripening. However, previous reports suggest that pre-harvest application of α-naphthalene acetic acid (NAA) alone or in combination with gibberellic acids (GA_3) promotes skin colour development in 'Keshar' and 'Arumanis' mango during ripening (Notodimedjo, 2000; Wavhal and Athale, 1988). This result raise questions about the importance of the timing of changes in auxin levels in mango fruit ripening and warrants further investigation.

In conclusion, the highest levels of endogenous IAA in the fruit pulp were at the PC minimum stage (day 0), while ABA levels rose before the climacteric peak in ethylene levels and respiration rate. These changes are consistent with ABA promoting ethylene synthesis, leading to climacteric ethylene production and fruit ripening. Exogenous application of Epi-BL or ABA promoted fruit ripening and NDGA an inhibitor of ABA biosynthesis, retarded ripening as measured by fruit softening and colour development. In both cases I speculate that these responses may be, at least in part, mediated by Epi-BL- and ABA-induced increases in ethylene production. In contrast to the control of non-climacteric ripening in grape, BRs levels remain very low and change little during mango ripening. I suggest that BRs may not play a significant role in the climacteric ripening of mango fruit.

CHAPTER 5

Mode of action of ABA in triggering ethylene biosynthesis and softening during ripening in mango fruit

Summary

The role of abscisic acid (ABA) and ethylene in mango (*Mangifera indica* L.) fruit was investigated by determining endogenous ABA concentration and ethylene during ripening, and the effects of exogenous application of ABA [S-(+)-*cis, trans*-abscisic acid] and inhibitor of its biosynthesis - nordihydroguaiaretic acid (NDGA) in regulating ethylene biosynthesis and fruit softening including fruit quality. A positive quadratic relationship between endogenous level of ABA and ethylene production suggests that these hormones may interact to regulate mango (cv. 'Kensington Pride') fruit ripening. In this study, the exogenous application of ABA (1.0 mM) accelerated ethylene biosynthesis with elevation in the activities of ethylene biosynthesis enzymes (ACS and ACO) and ACC content, increased fruit softening, softening enzyme activity (*endo*-PG) and retained higher pectinesterase (PE) activity in the pulp. Whilst, the activities of ethylene biosynthesis and softening enzymes was significantly delayed and/or suppressed in the pulp of NDGA-treated fruit. ABA-treated fruit promoted better quality including increased skin colour development, total sugars and sucrose as well as degradation of total organic acids, citric and fumaric acid than NDGA treatment. These results suggested that ABA is involved in regulating mango fruit ripening and its effects are, at least in part, mediated by changes in ethylene production.

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5.1 Introduction

Mango (*Mangifera indica* L.) is a climacteric fruit, in which the ripening process is thought to be modulated by the changes in the endogenous ethylene levels, and can be manipulated with exogenous ethylene application (Lalel et al., 2003e; Singh and Singh, 2011). Like mango, tomato is also a climacteric fruit (Alexander and Grierson, 2002). However, in *rin* (ripening inhibitor) and *nor* (non-ripening) tomato mutants fruit there is a lack of climacteric peak in respiration and ethylene biosynthesis, and exogenous ethylene does not promote fruit ripening or induce expression of ethylene–regulated ripening genes (Tigchelaar et al., 1978; Yen et al., 1995). Together this suggests that, in addition to general ethylene biosynthesis and signalling, there may be other upstream, ethylene-dependent regulatory factors necessary for climacteric fruit ripening (Tigchelaar et al., 1978; Yen et al., 1995; Zhang et al., 2009b).

Abscisic acid (ABA) has been reported to play a crucial role in fruit maturation and senescence (Giovannoni, 2004; Giovannoni, 2001; Rodrigo et al., 2003; Zhang et al., 2009a; Zhang et al., 2009b). Lower levels of endogenous ABA in unripe fruit and its substantial accumulation during fruit maturation process play a key role in modulating ripening and senescence in climacteric fruit such as peach (Rudnicki et al., 1968; Zhang et al., 2009a), avocado (Adato et al., 1976), tomato (Buta and Spaulding, 1994; Sheng et al., 2008; Zhang et al., 2009b), banana (Lohani et al., 2004), apple (Buesa et al., 1994), and non-climacteric fruit such as grape (Inaba et al., 1976; Zhang et al., 2009a) and orange (Kojima, 1996), suggest that ABA can be considered as another possible regulator of fruit ripening. Additionally, the de-greening stage of ABA-deficient orange mutants commenced later than the wild type suggest a crucial role of ABA in maturation and ripening of orange fruit (Rodrigo et al., 2003). Some earlier reports indicate that endogenous level of ABA increased towards harvest in the fruit skin and pulp of 'Nam Dok Mai', 'Nang Klangwan' (Kondo et al., 2004) and 'Alphonso' mango (Murti and Upreti, 1995). My previous study (Chapter 4) showed that the accumulation of endogenous level of ABA during climacteric-rise (CR) stage might switch on the climacteric peak of endogenous ethylene production during ripening of 'Kensington Pride' mango fruit. However, it is necessary to investigate the relationship between changes in the endogenous levels of ABA and the onset of the rise in ethylene production.

The exogenous application of ABA (10^{-6} M) has been reported to hasten ripening process and induce some structural changes in 'Alphonso' and 'Langra' mango (Palejwala et al., 1988; Parikh et al., 1990). Exogenously applied ABA or its higher endogenous levels have been reported to stimulate ethylene production, and promote ripening in other climacteric fruit such as tomato (Sheng et al., 2008; Zhang et al., 2009b; Zhu et al., 2003), peach (Zhang et al., 2009a) and non-climacteric grape berry (Deytieux et al., 2005; Zhang et al., 2009a). Zhang et al. (2009b) reported that exogenous application of ABA (100 μM) up-regulated of the expression of genes *Le-ACO1* and *Le-ACS2* encoding 1-aminocyclopropane-1-carboxylic acid (ACC) oxidase (ACO, EC 1.14.17.4) and ACC synthase (ACS, EC 4.4.1.14) enzymes, and consequently increased ethylene production and accelerated tomato fruit ripening. In contrast, the exogenous application of 100 μM inhibitors of ABA biosynthesis [fluridone or nordihydroguaiaretic acid (NDGA)], inhibited the expression of both *Le-ACO1* and *Le-ACS2* genes, and delayed tomato fruit ripening (Zhang et al., 2009b). Given the concurrent increase in both ABA and ethylene levels (Chapter 4, Section 4.3.1, Figure 4.1A and 4.1B), it propose that ABA induced ethylene may also play a role in modulating mango fruit ripening. However, to date there have been no research reports published on the interaction between ABA and ethylene during mango fruit ripening.

Mango fruit softening is associated with increased activities of polygalacturonase (PG), *exo*-PG (EC 3.2.1.67), *endo*-PG (EC 3.2.1.15), pectinesterase (PE, EC 3.1.1.11), pectate lyase (PL, EC 4.2.2.2) and *endo*-1,4-*β*-D-glucanase (EGase or cellulase, EC 3.1.1.4) are triggered by ethylene and ripening stage of the fruit (Chourasia et al., 2006; Chourasia et al., 2008; Singh and Singh, 2011). Previously, the exogenous application of ABA (5 mg L^{-1}) has been reported to increase the activity of PG and showed inconsistent effects of its application on the activity of PE during 'Zihua' mango fruit ripening (Zhou et al., 1996). The research work reported on the effects of exogenously applied ABA and its inhibitors to mango in regulating the activities of fruit softening enzymes during ripening is sporadic and inconclusive.

In this study, I have investigated the relationship between changes in the concentrations of endogenous ABA and ethylene production during ripening and the

effects of exogenously applied ABA and its inhibitor in regulating ethylene biosynthesis, activities of fruit softening enzymes and quality in mango fruit.

5.2 Materials and Methods

In experiment I, the changes in the endogenous level of ABA in the pulp and ethylene production in whole fruit during ripening period and their relationship with ripening was investigated. Whilst, in experiment II, the effects of the exogenous application of ABA and inhibitor of its biosynthesis (NDGA) in regulating ethylene biosynthesis, activities of fruit softening enzymes and quality parameters was carried out.

5.2.1 Fruit

In experiment I, hard mature green mango fruit (*Mangifera indica* L. cv. 'Kensington Pride') were obtained from a commercial orchard at Gingin (lat. 31° 21´S and long. 115° 55´E), Western Australia (WA) on 19th of March, 2008. The green mature fruit were firm (120.39 ± 9.71 N) and had a respiration rate of 2.36 ± 0.01 mmol kg^{-1} h^{-1}.

In experiment II, mango fruit (cv. 'Kensington Pride') were obtained from another commercial orchard located at Dongara (lat. 29.26° 15' S and long. 114.93° 55' E) on 26th February, 2009. The fruit was also firm (75.6 ± 6.71 N) and had a respiration rate of 2.68 ± 0.40 mmol CO_2 kg^{-1} h^{-1}.

All the fruit were desapped by allowing the sap to exude from the end of the stalk by physical inversion on the de-sapping trays to avoid sap burn injury over the skin, fungicide-treated (Sportak 0.55 mL L^{-1} with Prochloraz as an a.i.), air dried, packed in soft-board trays, and transported to Perth, WA, by a refrigerated truck. Fruit of uniform size and maturity (hard, green skin and light cream pulp colour), free from visual blemishes and symptoms of diseases were used for both experiments.

5.2.2 Experiment I: Changes in the levels of endogenous ethylene and abscisic acid (ABA) in mango fruit during ripening

The hard green mature mango fruit were kept in soft board trays and allowed to ripen at ambient temperature (21 ± 1°C, 57.18 ± 11.09% RH as detailed in Chapter 3,

Section 3.3). Pulp samples from the inner and outer mesocarp from the middle of the fruit were collected at two days interval during the 8 days of ripening period as similarly mentioned in Chapter 4, Section 4.2.2. Pulp tissue was immersed in liquid N_2 and stored at -80°C freezer for determination of endogenous levels of ABA. During the ripening period, the production of ethylene from the whole fruit was also determined every two days interval. The experiment used a completely randomized design, including ripening period as a factor. Ten fruit were used for each replicate and replicated three times.

5.2.3 Experiment II: Effects of exogenous application of ABA and NDGA on ethylene biosynthesis and fruit softening enzymes in mango fruit during ripening

The mango fruit were dipped for five minutes in an aqueous solution containing 1.0 mM S-(+)-*cis, trans*-abscisic acid (ABA, Syntake Chemical, Shanghai, China) or 0.2 mM nordihydroguaiaretic acid (NDGA) (Sigma-Aldrich Pty. Ltd., Castle Hill, Australia) and 0.05% of 'Tween 20' (Sigma-Aldrich Co., St. Louis, Missouri, USA) as a surfactant. The best concentration of ABA and NDGA were selected based on previous study presented in Chapter 4, Section 4.3.5. Air dried fruit were packed in soft board trays, and allowed to ripen at ambient temperature (21 ± 1°C, 60.02 ± 8.01% RH as detailed in Chapter 3, Section 3.3) until eating soft stage (subjective firmness rating score = 4). Untreated fruit were dipped in an aqueous solution containing only 0.05% of 'Tween 20' (Sigma-Aldrich Co., St. Louis, Missouri, USA). Ethylene production and respiration rate were determined daily during fruit ripening. Samples from pulp tissues were taken and immediately immersed in liquid N_2 and kept at -80°C freezer for estimating the activities of ethylene biosynthesis and fruit softening enzymes. The ACC content, the activities of ethylene biosynthesis enzymes (ACS and ACO) and fruit softening enzymes including *exo*-, *endo*-PG, PE and EGase were determined from the pulp tissues during two days interval of ripening period. Fruit softening, visual skin colour, total and individual sugars as well as organic acids were also determined every two days interval during ripening period. The experiment was laid out as a completely randomized design, including treatments and ripening period as a factor. Ten fruit was used as an experimental unit and replicated three times.

5.2.4 Extraction, purification and quantification of ABA

The endogenous level of ABA from mango pulp were extracted, purified and quantified according to the method outlined by Ross et al. (1987) and Jager et al. (2008) and detail mentioned in Chapter 3, Section 3.4.1.1 and 3.4.1.2. The data were calculated following a formula described by Ross et al. (1995). Positive identification of ABA from mango pulp tissues was achieved by full-scan mass spectrometry.

5.2.5 Ethylene production

During fruit ripening two fruit were randomly selected from each replication and were incubated in a separated airtight jar (1, 000 mL) fitted with rubber septum for 1 h at room temperature (20 ± 1°C, 58.02 ± 7.21% RH as detailed in Chapter 3, Section 3.3). A headspace gas sample (1.0 mL) was then injected into a gas chromatograph (6890N Network GC System; Agilent Technology, Palo Alto, CA^2, USA) to estimate the rate of ethylene production as explained in detail in Chapter 3, Section 3.4.2 and was expressed as nmol C_2H_4 kg^{-1} h^{-1}.

5.2.6 Determination of ACC content, and activity of ACS and ACO in pulp tissue

The ACC content, activities of ACS and ACO from pulp tissue were determined as described by Khan and Singh (2007) with some modification as detailed in Chapter 3, Section 3.7.1 to 3.7.3. The ACC content and the activities of ACS and ACO enzyme were expressed as pmol g^{-1} FW, pmol ACC mg^{-1} protein h^{-1} and nmol C_2H_4 mg^{-1} protein h^{-1}, respectively.

5.2.7 Fruit softness

The softness values of individual mango fruit in each replication were determined manually daily using a subjective 5-point rating scale as described in Chapter 3, Section 3.6.2.1.

5.2.8 Determination of fruit softening enzymes activities in pulp tissue

Activities of *exo*-, *endo*-PG, PE and EGase were determined in pulp tissues following the method as described by Khan and Singh (2007) with some modifications as detailed in Chapter 3, Section 3.8. The *exo*-, *endo*-PG, PE, and EGase enzymes activities were expressed as μg galacturonic acid mg^{-1} protein h^{-1}, Δ

viscosity mg^{-1} protein h^{-1}, mM NaOH mg^{-1} protein h^{-1} and Δ viscosity mg^{-1} protein h^{-1}, respectively.

5.2.9 Protein determination

Protein content in fruit pulp was determined following by Bradford (1976) method using bovine serum albumin (Sigma-Aldrich Pty. Ltd., Castle Hill, Australia) as a standard as explained in Chapter 3, Section 3.9 and was expressed as mg mL^{-1} FW.

5.2.10 Determination of individual sugars and organic acids

All chemicals used for individual sugars determination as detailed in Chapter 3, Section 3.6.3.1. The homogenate mango pulp using a mini-mixer (DIAX 900, Heidolph Co., Ltd., Schwabach, Germany) for 1 min were then centrifuged and filtered as described in Chapter 3, Section 3.6.3.2. Sucrose, glucose and fructose were separated, identified and quantified using the reversed-phase HPLC system (Waters, Milford Corp., MA, USA) fitted with the Fast Carbohydrate Analysis column (Aminex HPX 87C, 100 × 7.8 mm) (Bio-Rad Laboratories, Inc., Hercules, USA) as outlined in Chapter 3, Section 3.6.3.3. The concentrations of sucrose, glucose and fructose were expressed in g 100 g^{-1} FW.

5.2.11 Determination of individual organic acids

All chemicals used for individual sugars determination as detailed in Chapter 3, Section 3.6.3.1. Mango pulp was homogenized using a mini-mixer (DIAX 900, Heidolph Co., Ltd., Schwabach, Germany) as detailed in Chapter 3.6.3.2. Individual organic acids such as citric, tartaric, malic, shikimic, and fumaric acids in mango pulp were separated, identified and quantified using the reversed-phase HPLC system (Waters, Milford Corp., MA, USA) fitted with the Bio-Rad Aminex® HPX-87H column (300 × 7.8 mm) with a particle size of 9 μm (Bio-Rad Laboratories, Inc., Hercules, USA) as explained in Chapter 3, Section 3.6.3.3. The concentration of citric acid were expressed in g 100 g^{-1} FW, whilst tartaric, malic, shikimic, and fumaric acids content were expressed in mg 100 g^{-1} FW.

5.2.12 Statistical analysis

The experimental data were subjected to two-way (treatment × ripening) analysis of variance (ANOVA) using SAS (release 9.1.3, SAS Institute Inc., Cary, NC, USA). Fisher's Least Significant Differences (LSD) were calculated following a significant ($P \leq 0.05$) F-test. All the assumptions of ANOVA were checked to ensure validity of statistical analysis. The relationship between the changes in endogenous level of ABA and ethylene production was calculated from data presented previously in Chapter 4, Section 4.3.1. Pearson correlations were performed between the ethylene production and ethylene biosynthesis enzymes, ethylene production and fruit softening enzyme activities; fruit firmness and ethylene biosynthesis, fruit firmness and fruit softening enzyme activities were performed using the same program at $P \leq 0.05$.

5.3 Results

5.3.1 Experiment I: Changes in the endogenous levels of ABA, ethylene and their relationship during ripening in mango fruit

Mass spectrometry (performed in full-scan mode) of a purified and methylated mango pulp extract yielded a mass spectrum characteristic of authentic ME-ABA, at the known retention time of this compound (Figure 5.1). I have previously shown that, during ripening, the endogenous ABA significantly ($P \leq 0.001$) accumulated (2.87-fold) in the pulp at the CR stage (day 2 of ripening) and peaked at the climacteric stage on the 4th days of ripening compared with its lower level at day 0 of ripening. The climacteric ethylene production peak (8.24 nmol C_2H_4 kg^{-1} h^{-1}) was noticed on day 4 of the ripening period. The accumulation of ABA in the pulp preceeded climacteric peak of ethylene production as presented previously in Chapter 4, Section 4.3.1. There was a significant ($P \leq 0.001$) non-linear (quadratic) relationship (Adj R^2 = 0.63) between endogenous level of ABA in the pulp and ethylene production during fruit ripening (Figure 5.2).

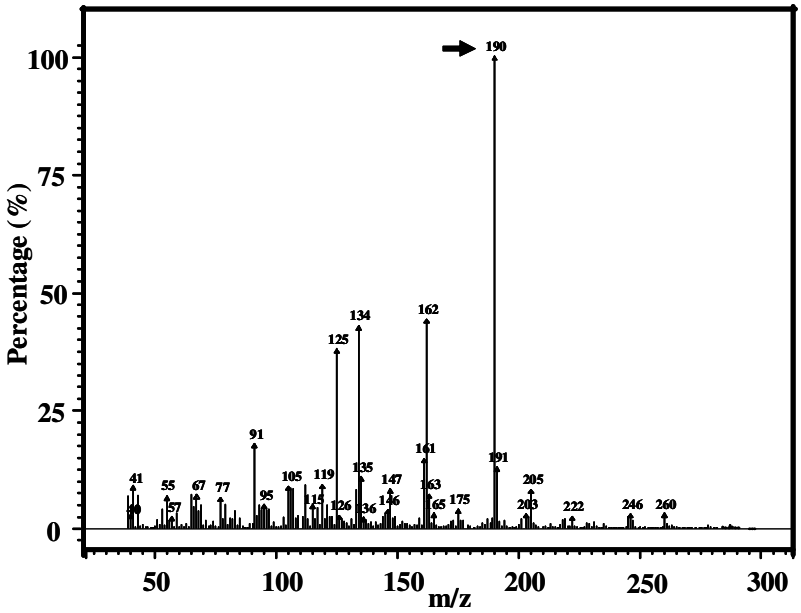

Figure 5.1. Spectrum plot of ABA peak of 'Kensington Pride' mango in the pulp tissue using GC-MS-MS methods performed with a Varian 8400 Autosampler and a Varian 3800 GC, coupled to a Varian 1200 triple quadrupole MS. Black arrow (➞) shows a typical peak of 'Kensington Pride' mango in the pulp tissue.

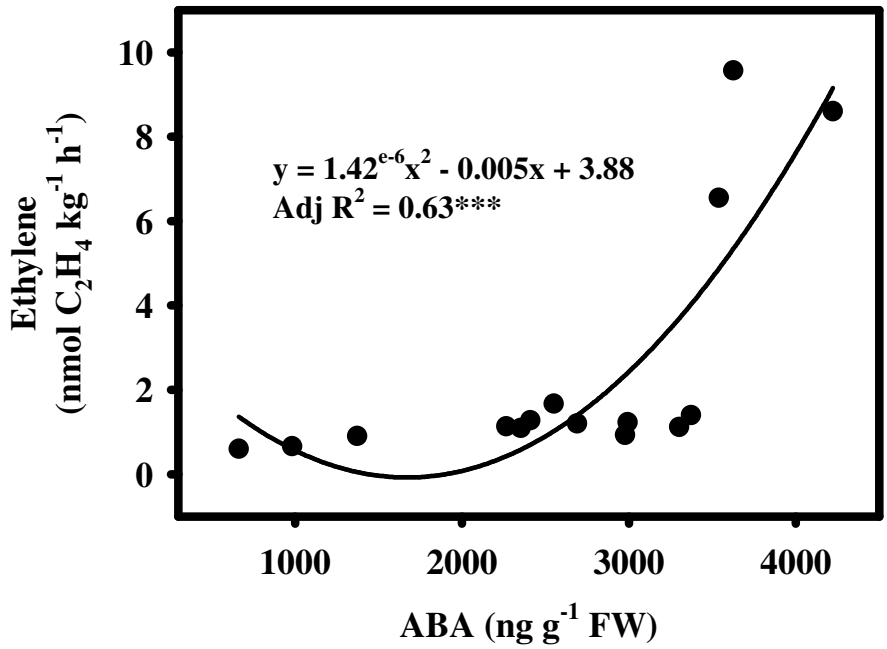

Figure 5.2. Relationship between endogenous level of ABA in pulp tissue and ethylene production from the whole fruit during ripening at ambient temperature.
n = 15 (5 d of ripening × 3 replications). LSD (*** represent significantly different at $P \leq 0.001$).

5.3.2 Experiment II: Effects of exogenous application of ABA and NDGA on ethylene biosynthesis and fruit softening enzymes during ripening in mango fruit

5.3.2.1 Ethylene production

The ABA-treated (1.0 mM) fruit exhibited a climacteric peak of ethylene production that was 1.36-fold higher than in the control fruit (Figure 5.3). NDGA-treated (0.2 mM) fruit showed climacteric peak of ethylene production that was 1.74-fold lower than control fruit (Figure 5.3). When averaged over ripening period, the mean of ethylene production was 1.09-fold higher in the ABA-treated fruit and 1.42-fold lower in NDGA-treated fruit, compared with the control.

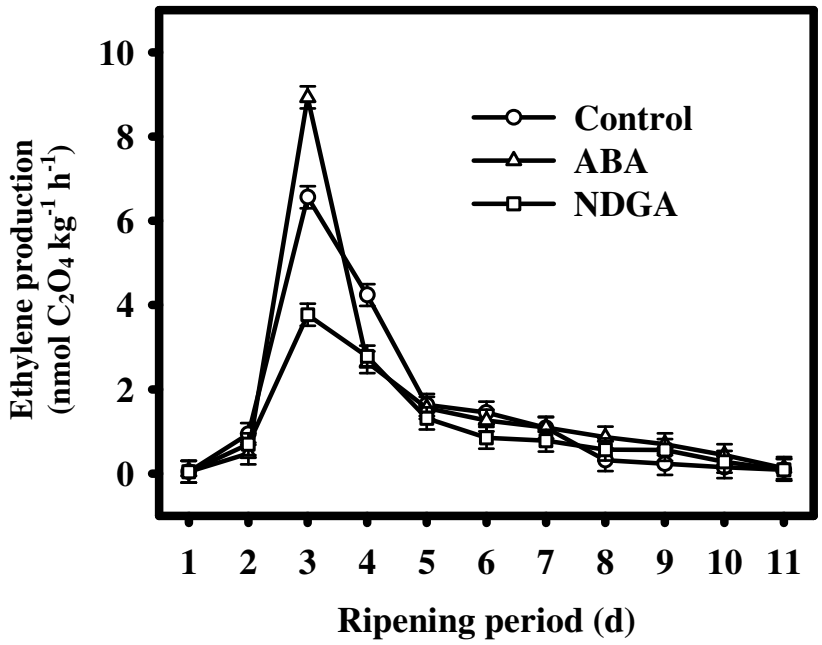

Figure 5.3. Changes in ethylene production as influenced by ABA and its inhibitor biosynthesis (NDGA) treatments (T) during fruit ripening period (RP) at ambient temperature.
Vertical bars represent LSD error means with n = 3 and invisible when the values are smaller than the symbol as well as LSD for the interaction. LSD (**, *** significant at $P \leq 0.01$, 0.001, respectively, for ethylene: T = 0.31**, RP = 0.59***, T × RP = 0.51***.

5.3.2.2 ACC content and activities of ACO, ACS during fruit ripening

ABA-treated fruit exhibited significantly ($P \leq 0.001$) higher ACC contents (1.28 and 1.06 pmol g^{-1} FW, respectively), than NDGA-treated (0.54 and 0.30 pmol g^{-1} FW respectively) and control (0.96 and 0.21 pmol g^{-1} FW respectively) fruits on day 2 and 4 of the ripening period (Figure 5.4A). The application of NDGA was significantly ($P \leq 0.05$) suppressed the levels of ACC in the pulp on day 2 of the ripening period as compared to control, but this effect had diminished by day 4 and beyond (Figure 5.4A).

As was the case for ACC, ABA-treated fruit exhibited significantly higher activities of ACS in the pulp tissue on day 4, 6 and 10 of the ripening period as compared to the control (Figure 5.4B). Similarly, ACS activity was suppressed in NDGA-treated fruit as compared to the control fruit. For instance, the ACS activity in NDGA-treated fruit was significantly ($P \leq 0.001$) reduced compared to the control on day 2 of the ripening period. However, on day 4 to day 10 of ripening the ACS activity did not differ significantly from the control (Figure 5.4B).

ACO activity was significantly reduced in NDGA-treated fruit during day 2 to day 10 of ripening as compared to control (Figure 5.4C). ABA-treated fruit exhibited significantly higher ACO activity as compared to control fruit on day 2, 4, 6, and 10 of ripening (Figure 5.4C). Averaged over 10 days of ripening period, the mean of ACC content, and the activities of the ACS and ACO enzymes in the pulp tissues were significantly higher (2.16-, 2.37-, and 1.35-fold, respectively) in ABA-treated fruits compared to the control.

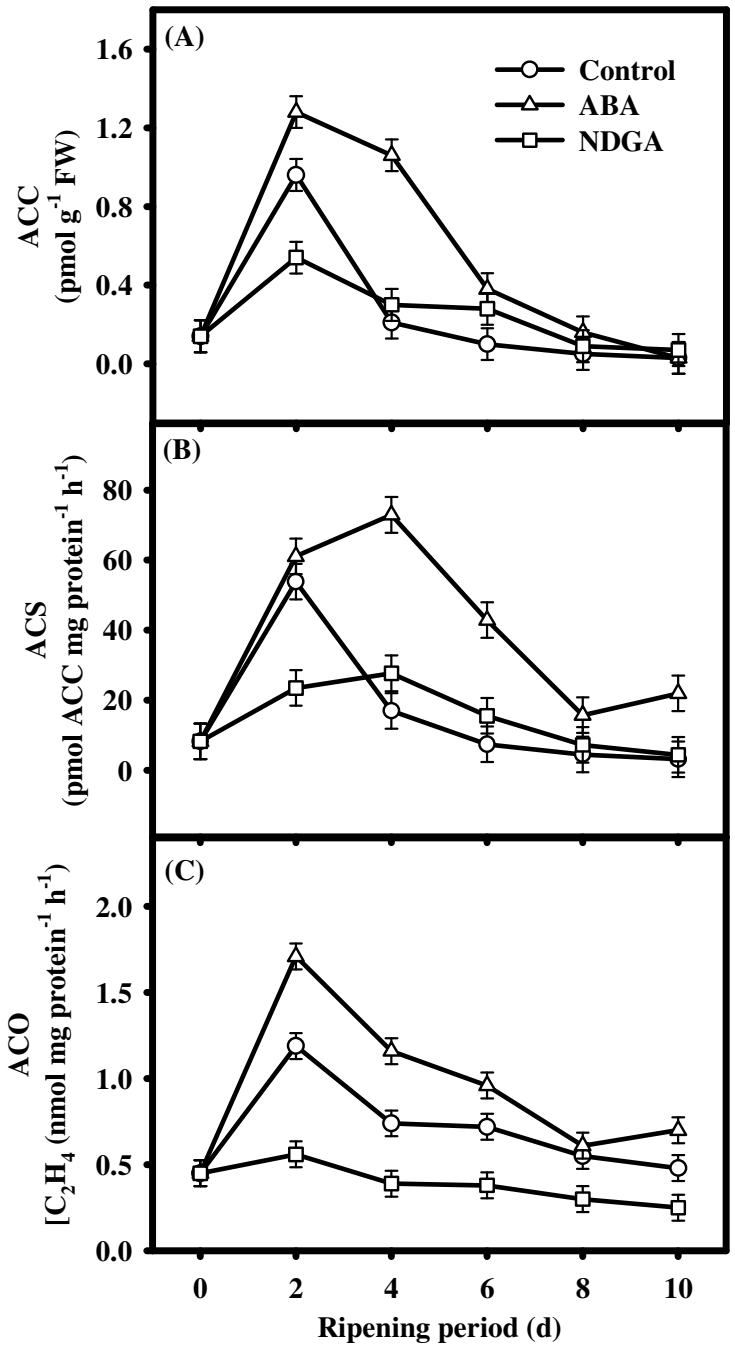

Figure 5.4. Ethylene biosynthesis enzymes, ACC content (A), ACS (B) and ACO enzymes (C) in pulp tissues as influenced by ABA and its inhibitor biosynthesis (NDGA) treatments (T) during fruit ripening period (RP) at ambient temperature.
Vertical bars represent LSD error means with n = 3 and invisible when the values are smaller than the symbol as well as LSD for the interaction. LSD (**, *** significant at $P \leq 0.01$, 0.001, respectively) for ACC content: T = 0.12^{***}, RP = 0.16^{***}, T × RP = 0.28^{***}; ACS: T = 7.19^{***}, RP = 10.17^{***}, T × RP = 17.62^{**} and ACO: T = 0.11^{***}, RP = 0.14^{***}, T × RP = 0.26^{**}.

5.3.2.3 Fruit softness

The exogenous application of ABA promoted fruit softening from day 1 to day 3 during ripening compared to the control (Figure 5.5). The fruit treated with NDGA exhibited delayed fruit softening during fruit ripening period as compared to control and ABA-treated fruit. Averaged all over treatments used, the mean fruit softness was 1.07-fold higher in ABA-treated fruit and 1.20-fold lower in NDGA-treated fruit, compared to the control.

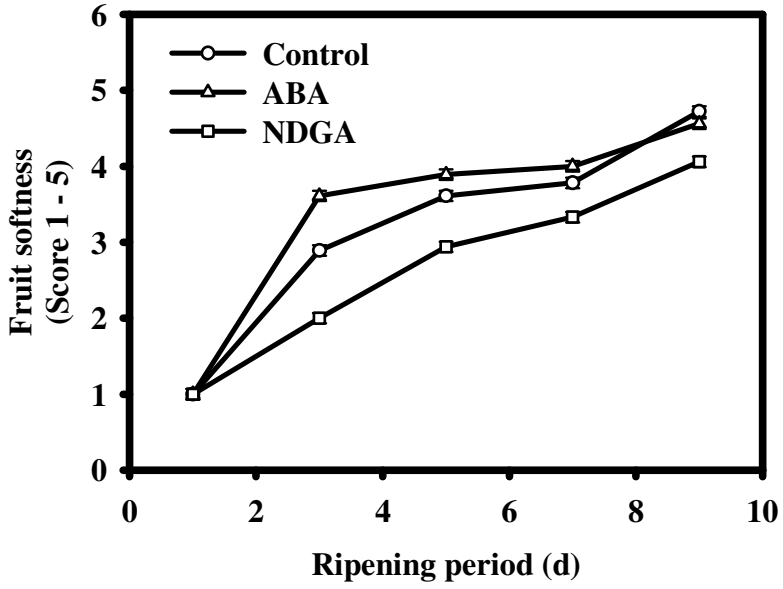

Figure 5.5. Fruit softening as influenced by ABA and its inhibitor biosynthesis (NDGA) treatments (T) during fruit ripening period (RP) at ambient temperature.
Vertical bars represent LSD error means with n = 30 (10 fruit × 3 replications) and invisible when the values are smaller than the symbol as well as LSD for the interaction. LSD (*** significant at $P \leq 0.001$): T = 0.11^{***}, RP = 0.14^{***}, T × RP = 0.24^{***}.

5.3.2.4 Activities of fruit softening enzymes in fruit pulp

ABA or NDGA treatment did not significantly affect *exo*-PG activity in pulp during fruit ripening period compared to control fruit (Table 5.1). When averaged over 10 days of ripening period, the mean activity of *exo*-PG increased in all treatments during ripening period from day 0 to day 4.

In contrast to *exo*-PG, ABA treatment did significantly ($P \leq 0.05$) increased the activity of *endo*-PG in the pulp (2.19-fold) on day 2 of the ripening period, resulting in a peak in activity of this enzyme that was two days earlier than the peak seen in control and NDGA-treated fruit (Table 5.1). After this peak, the activity of *endo*-PG in the pulp of ABA-treated fruit declined rapidly and was infact lower than the control level by day 4 to day 8. In contrast to ABA, NDGA-treatment significantly reduced the activity of *endo*-PG in the pulp during day 2 to day 6 of ripening compared to control (Table 5.1). Averaged of all over treatments used, the mean *endo*-PG activity was significantly ($P \leq 0.05$) 1.15-fold higher in ABA-treated fruit than control, whilst NDGA-treated fruit showed 1.18-fold lower activity as compared to the control.

The activity of PE in the pulp tissue declined over the ripening period irrespective of the treatment applied (Table 5.1). The ABA-treated fruit exhibited lower (1.11-, 1.45-, 1.33-, 1.29-, and 1.17-fold) PE activity in the pulp on day 2, 4, 6, 8, and 10 of the ripening period, respectively, when compared to the control. However, NDGA-treated fruit exhibited 1.31-fold higher PE activity on day 4 of ripening as compared to control fruit and continuously declined with advancements in ripening without showing any different with control on day 6 to day 10 of ripening (Table 5.1). Averaged of all over treatments used, the mean PE activity was significantly lower (0.13 mM NaOH mg protein^{-1} h^{-1}) in ABA-treated than control and NDGA-treated fruit (0.15 and 0.16 mM NaOH mg protein^{-1} h^{-1}, respectively).

The activity of EGase in pulp was not significantly affected by ABA or NDGA treatment during fruit ripening period (Table 5.1). However, averaged over 10 days of ripening period, the mean activity of EGase in all treatments significantly increased on day 2 and 4, stabilised on day 6 and later on declined at ripe stage.

Table 5.1. Changes in the activities of *exo*-PG (µg galacturonic acid mg protein^{-1} h^{-1}), *endo*-PG [Δ viscosity mg protein^{-1} h^{-1}], PE (mM NaOH mg protein^{-1} h^{-1}) and EGase [Δ viscosity mg protein^{-1} h^{-1}] of fruit pulp influenced by ABA and its biosynthesis inhibitor (NDGA) during ripening at ambient temperature.

Parameter	Treatments (T)	Ripening period (d)						Means (T)	LSD ($P \leq 0.05$)
		0	2	4	6	8	10		
Exo-PG	Control	29.12	32.02	35.22	35.23	31.90	23.61	31.18	T = NS
	ABA	29.85	34.26	39.00	37.33	27.91	24.56	32.14	RP = 2.96***
	NDGA	28.69	30.58	32.41	39.35	35.73	23.45	31.70	T × RP = NS
	Mean (RP)	29.22c	32.29b	35.54a	37.31a	31.85bc	23.88d		
Endo-PG	Control	0.66	2.53	3.53	2.48	1.69	1.45	2.06AB	T = 0.51*
	ABA	1.07	5.53	2.06	1.79	1.58	2.18	2.37A	RP = 2.72*
	NDGA	1.00	1.65	2.44	1.85	1.84	1.66	1.74B	T × RP = 1.24***
	Mean (RP)	0.91c	3.23a	2.68a	2.04ab	1.70b	1.76b		
PE	Control	0.26	0.21	0.16	0.12	0.09	0.07	0.15A	T = 0.01***
	ABA	0.26	0.19	0.11	0.09	0.07	0.06	0.13B	RP = 0.02 ***
	NDGA	0.26	0.22	0.21	0.12	0.10	0.07	0.16A	T × RP = NS
	Mean (RP)	0.26a	0.21b	0.16c	0.11d	0.09e	0.07f		
EGase	Control	2.89	5.88	7.35	7.28	5.97	3.94	5.55	T = NS
	ABA	2.89	6.64	7.39	6.97	5.76	4.30	5.66	RP = 0.80***
	NDGA	2.89	4.27	7.14	7.71	7.35	5.26	5.77	T × RP = 1.39*
	Mean (RP)	2.89d	5.60b	7.29a	7.32a	6.36b	4.50c		

Mean values followed by the same upper-case (n = 18, 6 ripening period × 3 treatments) within a column and mean values followed by the same lower-case (n = 9, 3 treatments × 3 replications) within a line are not significantly different at $P \leq 0.05$ by the Least Significant Difference (LSD). *, *** significant different at $P \leq 0.05$, 0.001, respectively and NS = not significant. RP = Ripening Period, T = Treatments, RP × T = Interaction between RP and T, *exo*-PG = *exo*-polygalacturonase, *endo*-PG = *endo*-polygalacturonase, PE = pectin esterase, EGase = *endo*-1,4-*β*-D-glucanase.

5.3.2.5 Changes in concentration of total and individual sugars

ABA-treatment increased the accumulation of both total sugars and sucrose on day 2 of ripening period compared to the control fruit (Table 5.2). In contrast, NDGA-treatment delayed the accumulation of total sugars and sucrose compared to both ABA-treated and control fruits. Despite this delay the concentration of total sugars and sucrose in NDGA-treated fruit eventually surpassed that in ABA-treated fruit, but still do not reach the level recorded in control fruits. ABA or NDGA treatment did not significantly affect the concentration of glucose and fructose during fruit ripening period (Table 5.2). When averaged over ripening period, the mean concentrations of glucose and fructose in all treatments increased significantly up to day 6 of ripening period and declined with advancement of ripening.

5.3.2.6 Changes in concentration of total and individual organic acids

The concentrations of total organic acids, citric acid, malic acid, shikimic acid and fumaric acid (Table 5.3A and 5.3B) in the pulp tissue declined with the advancement of fruit ripening irrespective of the treatment applied. Exogenous application of ABA and NDGA only had a significant effect on the concentrations of total organic acids and citric acid (Table 5.3A). The concentrations of total organic acids and citric acid in the pulp declined at faster rate from day 2 to 4 of ripening in ABA-treated fruit than in the control. In NDGA-treated fruit, the concentrations of total organic acids and citric acid in the pulp declined at slower rate up to day 6 of ripening than in the control. At the ripe stage (day 8 and 10 of ripening), the concentrations of total organic acids and citric acid did not differ significantly among treatments. The concentrations of trace organic acid compound (tartaric acid, malic acid, shikimic acid and fumaric acid) did not significantly vary among treatments during the ripening period (Table 5.3B).

Table 5.2. Changes in the concentration of total sugar, sucrose, glucose and fructose (g 100 g^{-1} FW) of fruit pulp influenced by ABA and its inhibitor (NDGA) during ripening at 21°C.

Parameter	Treatments (T)	Ripening period (d)						Mean (T)	LSD ($P \leq 0.05$)
		0	2	4	6	8	10		
Total sugars	Control	12.80	17.07	20.26	22.53	22.26	22.77	19.62A	T = 1.83**
	ABA	12.80	21.73	21.10	22.89	20.48	19.07	19.68A	RP = 2.58***
	NDGA	12.80	15.67	14.56	18.69	20.38	20.52	17.10B	T × RP = NS
	Mean (RP)	12.80d	18.16c	18.64bc	21.37a	21.04ab	20.79ab		
Sucrose	Control	9.12	13.02	14.89	16.78	17.11	18.09	14.84A	T = 1.48**
	ABA	9.12	16.72	15.86	16.99	16.24	15.13	15.01A	RP = 2.10***
	NDGA	9.12	11.59	10.53	13.78	14.76	16.39	12.69B	T × RP = NS
	Mean (RP)	9.12c	13.78b	13.76b	15.85ab	16.04a	16.53a		
Glucose	Control	0.24	0.42	0.49	0.44	0.34	0.18	0.35	T = NS
	ABA	0.24	0.50	0.57	0.63	0.31	0.16	0.40	RP = 0.07***
	NDGA	0.24	0.42	0.34	0.52	0.53	0.23	0.76	T × RP = NS
	Mean (RP)	0.24d	0.45b	0.47b	0.53a	0.39c	0.19d		
Fructose	Control	3.44	3.63	4.88	5.30	4.82	4.51	4.43	T = NS
	ABA	3.44	4.51	4.67	5.27	4.03	3.79	4.29	RP = 0.68***
	NDGA	3.44	3.66	3.69	4.39	5.09	3.89	4.03	T × RP = NS
	Mean (RP)	3.44d	3.93cd	4.41abc	4.99a	4.65ab	4.06bcd		

Mean values followed by the same upper-case (n = 18, 6 ripening period × 3 treatments) within a column and mean values followed by the same lower-case (n = 9, 3 treatments × 3 replications) within a line are not significantly different at $P \leq 0.05$ by the Least Significant Difference (LSD). **, *** significant different at $P \leq 0.01$, 0.001 and NS = not significant. RP = Ripening period.

Table 5.3A. Changes in the concentration of total organic acids and citric acid (g 100 g^{-1} FW) and tartaric (mg 100 g^{-1} FW) of fruit pulp influenced by ABA and its inhibitor (NDGA) during ripening at 21°C.

Parameter	Treatments (T)	Ripening period (d) 0	2	4	6	8	10	Mean (T)	LSD ($P \leq 0.05$)
Total organic acids	Control	1.41	0.96	0.71	0.32	0.31	0.22	0.62B	T = 0.07***
	ABA	1.41	0.84	0.56	0.39	0.28	0.21	0.66B	RP = 0.10***
	NDGA	1.41	1.17	1.11	0.50	0.34	0.27	0.80A	T × RP = 0.17**
	Mean (RP)	1.41a	0.99b	0.79c	0.40d	0.31de	0.23e		
Citric	Control	1.24	0.86	0.63	0.23	0.23	0.13	0.55B	T = 0.07***
	ABA	1.24	0.74	0.46	0.30	0.20	0.13	0.51B	RP = 0.10***
	NDGA	1.24	1.05	1.01	0.42	0.25	0.19	0.69A	T × RP = 0.18**
	Mean (RP)	1.24a	0.88b	0.70c	0.32d	0.23de	0.15e		
Tartaric	Control	ND	ND	ND	0.85	8.94	22.18	5.33	T = NS
	ABA	ND	ND	0.44	1.91	5.91	25.53	5.63	RP = 2.27***
	NDGA	ND	ND	ND	5.17	10.34	19.27	5.80	T × RP = NS
	Mean (RP)	ND	ND	0.15d	2.64c	8.40b	22.33a		

Mean values followed by the same upper-case (n = 18, 6 ripening period × 3 treatments) within a column and mean values followed by the same lower-case (n = 9, 3 treatments × 3 replications) within a line are not significantly different at $P \leq 0.05$ by the Least Significant Difference (LSD). **, *** significant different at $P \leq 0.01$, 0.001 and NS = not significant. RP = Ripening period, ND = Not detected.

Table 5.3B. Changes in the concentration of total organic acids and citric acid (g 100 g^{-1} FW) as well as tartaric, malic, shikimic and fumaric (mg 100 g^{-1} FW) of fruit pulp as influenced by ABA and its inhibitor (NDGA) during ripening at 21°C.

Parameter	Treatments (T)	Ripening period (d)						Mean (T)	LSD ($P \leq 0.05$)
		0	2	4	6	8	10		
Malic	Control	156.88	82.22	72.75	76.19	65.10	60.63	85.63	T = NS
	ABA	156.88	86.68	82.58	73.52	59.38	57.07	86.02	RP = 15.89***
	NDGA	156.88	102.33	78.68	68.64	63.18	52.71	87.07	T × RP = NS
	Mean (RP)	156.88a	90.41b	78.00 bc	72.79c	62.55cd	56.80d		
Shikimic	Control	17.66	15.97	13.11	12.74	12.43	8.80	13.45	T = NS
	ABA	18.55	15.78	13.38	11.65	10.43	9.06	13.14	RP = 1.46***
	NDGA	18.38	14.08	13.19	11.49	9.60	7.25	12.33	T × RP = NS
	Mean (RP)	18.20a	15.28b	13.23c	11.96cd	10.82d	8.37e		
Fumaric	Control	0.17	0.08	0.05	0.07	0.04	0.03	0.07A	T =0.008**
	ABA	0.17	0.06	0.04	0.06	0.03	0.02	0.06B	RP =0.01***
	NDGA	0.17	0.07	0.06	0.06	0.05	0.05	0.08A	T × RP = NS
	Mean (RP)	0.17a	0.07b	0.05c	0.06bc	0.04d	0.03d		

Mean values followed by the same upper-case (n = 18, 6 ripening period × 3 treatments) within a column and mean values followed by the same lower-case (n = 9, 3 treatments × 3 replications) within a line are not significantly different at $P \leq 0.05$ by the Least Significant Difference (LSD). **, *** significant different at $P \leq 0.01$, 0.001 and NS = not significant. RP = Ripening period.

5.4 Discussion

5.4.1 Endogenous ethylene and ABA during ripening

It is widely accepted that high levels of endogenous ethylene production or exogenous application of ethylene will promote ripening in climacteric fruit (Bapat et al., 2010; Lalel et al., 2003e). Accumulation of endogenous ABA levels have also been implicated in climacteric ripening in avocado (Adato et al., 1976), tomato (Buta and Spaulding, 1994; Sheng et al., 2008; Zhang et al., 2009b), and banana (Lohani et al., 2004). In a previous study my results show that the endogenous levels of ABA accumulated before the climacteric peak in ethylene production during fruit ripening in 'Kensington Pride' mango fruit (Chapter 4, Figure 4.1A and 4.1B). In this chapter, I found that a non-linear (quadratic) relationship between endogenous level of ABA and ethylene production (Figure 5.2), maybe an important hormonal interaction that regulates ripening in the climacteric mango fruits. My experimental results also suggest that ABA, is critical for onset of ripening through its role in triggering the climacteric peak of ethylene production. Ethylene may then play a role in mango fruit ripening at climacteric and post climacteric stages. By way of confirmation, the results also showed that the exogenous application of ABA promoted fruit ripening, whilst the ABA synthesis inhibitor, NDGA, delayed ripening in mango. Similarly, Zhang et al. (2009b) reported the acceleration and retardation of climacteric fruit ripening in tomato fruit with the exogenous application of ABA and fluridone (ABA inhibitor) respectively.

5.4.2 Ethylene biosynthesis

The exogenous application of ABA (1.0 mM) increased ethylene production in 'Kensington Pride' mango during ripening at ambient temperature (21 ± 1°C), whilst the application of NDGA suppressed production of ethylene (Figure 5.3). The increased ethylene biosynthesis in ABA-treated fruit during ripening may be attributed to the increased activities of ACO and ACS (ethylene synthesis) enzymes (Figure 5.4B and 5.4C), as well as higher ACC content in pulp (Figure 5.4A). The reduction in ethylene biosynthesis in NDGA-treated fruit may be ascribed due to the suppression of ACO activity (Figure 5.4C), reduced ACS activity and lowered ACC content in the pulp during ripening (Figure 5.4A and 5.4B). The ACO activity in NDGA-treated fruit did increase slightly on day 2 compared to day 0 and this increase may have been enough to allow the conversion of ACC to ethylene (Figure

5.3, 5.4A and 5.4C). Earlier, Suzuki et al. (2005) also reported that similar lower ACO activity is adequate to convert ACC to ethylene in broccoli following high temperature treatment.

The promotion in ACS and ACO activities during ripening in ABA-treated fruit (Figure 5.4B and 5.4C), may be due to an ABA – induced increase in expression of genes encoding for both ACS and ACO enzymes. However, the suppression in ACS and ACO activities in the pulp during ripening in NDGA-treated fruit (Figure 5.4B and 5.4C) may be associated with down-regulation of expression of these enzymes. If this is in fact the case it would be similar to previous reports in tomato (Zhang et al., 2009b), where the inhibition of ethylene production and delayed ripening in NDGA-treated tomato was reported to be due to the suppression of *LeACS2* and *LeACO1* (ethylene biosynthesis gene) expression. It is unlikely that NDGA would affect the ethylene pathway directly, as it is a known inhibitor of the 9-cis-epoxycarotenoid dioxygenase (NCED) enzyme and has been reported to block ABA biosynthesis (Zhang et al., 2009b). The effects of NDGA on ethylene synthesis are therefore likely to be mediated via the NDGA-induced changes in endogenous ABA levels. Further study is required to elucidate the precise role of ABA in regulating the changes in ethylene biosynthesis gene expression.

In the current study, the exogenous application of ABA was effective in promoting ethylene biosynthesis at climacteric stage of ripening (Figure 5.3). The climacteric ethylene peak occurred just two days after ABA treatment, which suggest that ABA could be able to directly promote ethylene biosynthesis. Contrarily, treating mango fruit with NDGA (which we would expect to reduce both NCED activity and ABA levels) caused a suppression of ethylene biosynthesis at climacteric stage of ripening (Figure 5.4A – 5.4C). NGDA is a known inhibitor of ABA biosynthesis in plants, its activity may be limited to a short period of time after its exogenous application (Zhang et al., 2009b). There were significant ($P \leq 0.001$) positive correlations ($r = 0.74$, $r = 0.62$ and $r = 0.75$) between ACC content, ACS, ACO activities with ethylene production, respectively.

5.4.3 Fruit softening and activities of fruit softening enzymes

Fruit softening increases with the advancement of ripening due to the disintegration of cell walls as well as transformation and solubilization of cell wall polymers such as pectin, cellulose and hemicelluloses (Singh and Singh, 2011). In the current study, fruit softening was more pronounced in ABA-treated fruit compared to the control (Figure 5.5). This response may have been a direct consequence of the ABA or could, at least in part, be due to increased ethylene production brought about by ABA treatment (Figure 5.3). ABA treatment promoted the *endo*-PG activity and reduced PE activity in pulp tissue during ripening (Table 5.1). In untreated fruits, softening substantially increased on the second day of ripening, which may due to higher accumulation of endogenous ABA level (Chapter 4, Figure 4.1B) and this was further pronounced when ABA was applied (Figure 5.5). Similarly, Parikh et al. (1990) reported that softening in ABA-treated mango fruit is associated with loss of structural changes and cellular integrity which contributed to increase in starch degradation during ripening of 'Alphonso' mango. Fruit softening was delayed in NDGA-treated fruit (Figure 5.5), possibly due to directly reduced levels of ABA and/or due to suppression in ethylene production (Figure 5.3), ACC content, activities of ACS and ACO (Figure 5.4A – 5.4C), consequently reduced and delayed the activities of softening-related enzymes such as *endo*-PG, and retained higher PE enzymes (Table 5.1). Increased fruit softening in ABA-treated fruit has previously been reported in kiwifruit (Chen et al., 1999; 2005), and strawberry during ripening (Jiang and Joyce, 2003).

PG is responsible for degrading the (1-4)-linked galacturonic acid residues, and has been reported in mango fruit during ripening (Abu-Sarra and Abu-Goukh, 1992). Our results show that ABA-treated fruit exhibited higher activity of *endo*-PG on the second day of ripening, with NDGA-treated fruit suppressed and exhibited small peak on day 4 of ripening (Table 5.1). The increased activities of *endo*-PG in ABA-treated fruit, may be due to increased endogenous level of ABA and/or due to increased ethylene biosynthesis (Figure 5.3 and 5.4A – 5.4C) coincides with rapid fruit softening in mango fruit during ripening (Figure 5.5). In NDGA-treated fruit *endo*-PG activity is decreased (Table 5.1), and fruit softening is delayed (Figure 5.5), There was a significant ($P \leq 0.01$) positive linear relationship ($r = 0.46$) between the activity of *endo*-PG and fruit softening during ripening. The *exo*-PG enzyme is also

thought to play a key role in mango fruit softening (Ali et al., 2004), however in our study ABA treatment did not dramatically effect the activity of *exo*-PG.

PE activity in the pulp of 'Kensington Pride' mango fruit progressively declined in ABA-treated fruit with advancement of fruit ripening and the activity was higher in NDGA-treated fruit (Table 5.1). The activities of PE have also been reported to decrease in various mango cultivars such as 'Kitcher' and 'Dr Knight' with progression of fruit ripening (Abu-Sarra and Abu-Goukh, 1992). It is also well known that PE catalyses the de-esterification of pectin into pectate and methanol. ABA or NDGA treatment resulted in lower or higher PE activity probably due to interaction between pectate with free calcium to form looser or stronger cell wall in the pulp of mango fruit, leading to increased or decreased fruit softening, respectively, during ripening at ambient temperature. Similarly, the activity of PE was found higher in apple fruit on harvest day and it declined during ripening and softening (Goulao et al., 2007). The application of ABA (5 mg L^{-1} or 100 μM) has also been reported to decrease or stimulate PE activity in 'Zihua' mango (Zhou et al., 1996) and 'Robusta Harichal' banana (Lohani et al., 2004), consequently promoted fruit softening, respectively. There was significant ($P \leq 0.001$) negative correlation (r = -0.82) between fruit softening and the activity of PE during ripening period. My results are consistant with the finding reported by Roe and Bruemmer (1981) during ripening period of 'Keitt' mangoes. ABA or NDGA treatment did not significantly affect the EGase and *exo*-PG activity in pulp of 'Kensington Pride' mango fruit during ripening (Table 5.1). But the ripening period show significantly affected the activity of EGase and *exo*-PG in mango fruit. Similarly, Chourasia et al. (2008) claimed that promotion of EGase activity was closely related with the later stages of fruit ripening contributed to pulp softening in 'Dashehari' mango.

5.4.4 Total sugars and organics acids

The exogenous application of ABA advanced the accumulation of total sugars and sucrose, whilst, NDGA treatment delayed their accumulation during fruit ripening as compared to control (Table 5.2). The faster and slower rates of sugars accumulation during ripening in ABA and NDGA-treated fruit, respectively, suggest that ABA is involved in promoting their catabolism, possibly by increasing carrier-mediated transport across tonoplast and plasma membrane, thus increased degradation of

starch and organic acids as reported earlier by Kobashi et al. (1999). Sucrose seems to be more highly influenced by the exogenous application of ABA and its inhibitor than other individual sugars (glucose and fructose) (Table 5.2). It can be argued that ABA treatment might increase activity of sucrose synthase (SUS) and the expression of *SUS* gene, leading to increased accumulation of sucrose during ripening period in tomato (Bastias et al., 2011). Similarly, Palejwala et al. (1988) reported that exogenous application of ABA (10^{-6} M) increased the concentration of sugars in 'Langra' mango compared to the control fruit. The reduction in the concentration of total sugar and sucrose in ABA-treated fruit on day 8 and 10 of ripening related to the control fruit, may be due to the advanced sugar accumulation at early ripening stage (Table 5.2) and also inter-conversion of starch to respiratory process instead of sugar at the ripe stage (data not shown).

The exogenous application of ABA significantly ($P \leq 0.05$) accelerated, whilst, NDGA-treatment delayed degradation of total organic acids and citric acid, during mango ripening, related to the control fruit (Table 5.3A). A decrease in total organic acids in ABA-treated mango has been reported previously in 'Langra' mango (Palejwala et al., 1988). ABA and NDGA may have influenced the rate of reduction of organic acids concentration during ripening by either directly or indirectly affecting the expression of citrate synthase in mitochondrial (*mCIS*) and glioxisomal (*gCIS*) as well as isocitrate dehydrogenase in mitochondrial (*mICDH*) in treated fruit, as has been reported earlier in tomato (Bastias et al., 2011). My results show that the final concentrations of organic acids in ripe fruit did not significantly vary among treatments (Table 5.3A and 5.3B), which may be due to a reduction in the respiratory process (data not shown), as also reported earlier in 'Tegan Blue' plum treated with 1-MCP by Khan et al. (2009).

In conclusion, there is a significant non-linear (quadratic) relationship between endogenous level of ABA in the pulp and ethylene production during fruit ripening period. The application of exogenous ABA to mango fruits (1) promotes the activities of ethylene biosynthesis enzymes (ACS, ACO) and ACC content, and consequently accelerates the climacteric peak of ethylene production and fruit ripening, (2) promotes mango fruit softening by regulating the activity of softening enzymes including *endo*-PG and PE activity, and (3) increases the concentration of

total sugars and sucrose and decreased the concentration of total organic acids and citric acid. In contrast, the treatment of mango fruit with the ABA biosynthesis inhibitor, NDGA, suppressed and/or delayed above ripening processes and consequently delayed mango fruit ripening. Together these results show that ABA interacts with ethylene to regulate ripening of the climacteric mango fruit.

CHAPTER 6

Post-harvest fumigation with nitric oxide at the pre-climacteric and climacteric-rise stages influences ripening and quality in mango fruit

Summary

Mango fruit ripen very quickly, which limits their distribution to distant markets. Mature mango (*Mangifera indica* cv. 'Kensington Pride') fruit harvested at the pre-climacteric (PC) and climacteric-rise (CR) stages of ripening were fumigated with different concentrations (0, 5, 10, 20, or 40 μL L^{-1}) of nitric oxide (NO) to investigate the effects of this free radical gas on fruit ripening and quality. Fumigation with NO at 20 μL L^{-1} or 40 μL L^{-1} was more effective at delaying and/or suppressing the CR in ethylene production and reducing the rate of respiration when applied to fruit at the PC stage than to fruit at the CR stage. NO fumigation retarded the development of fruit colour with lower chromicity L*, a*, b*, and C* values and higher h° values compared to the control untreated. Higher concentrations of NO (i.e. 20 μL L^{-1} or 40 μL L^{-1}) were more effective in retarding fruit softening during ripening. The pulp of ripe NO-fumigated fruit exhibited improved rheological properties (i.e. cohesiveness, springiness, and chewiness) and increased shikimic acid contents, but reduced concentrations of total sugars and fructose compared to non-fumigated, ripe fruit. In conclusion, NO fumigation at 20 μL L^{-1} or 40 μL L^{-1} was more effective when applied early, at the PC stage, than later during the CR stage.

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6.1 Introduction

The fruit of mango (*Mangifera indica* L.) are climacteric in nature, whereby sudden and substantial rises in respiration and ethylene production accompany their ripening. Typically, the ripening of climacteric fruit is divided into a pre-climacteric (PC) minimum, a climacteric-rise (CR), a climacteric peak, and a post-climacteric phases (Watada et al., 1984). The ripening process in mango fruit involves numerous other changes including flesh softening, the development of pigments, and metabolic activities that change the levels of carbohydrates, organic acids, lipids, phenolics, volatile compounds, and structural polysaccharides (Brecht and Yahia, 2009).

Ripe mango fruit exhibited improved skin colour, flavour, texture, and higher soluble solids concentrations (SSC) when harvested at the PC stage than those harvested at the post-climacteric stage (Lakshminarayana, 1975; Medlicott et al., 1988; Thanaraj et al., 2009). Mango fruit exhibit different responses to exogenously applied chemicals at the different stages of ripening. The highest rates of respiration (4.38 mmol kg^{-1} h^{-1}) and ethylene production (41.48 nmol kg^{-1} h^{-1}) were observed on day 2 and 3 of ripening, respectively, in 'Kensington Pride' mango fruit harvested at the hard mature-green stage, compared to fruit harvested at the sprung-green or ripe stages (Lalel et al., 2003d). Exogenously applied ethylene, at 1.0 mL L^{-1} initiated more rapid ripening, with lower fruit firmness, higher SSC, and improved skin colour in mature and semi-mature 'Tommy Atkins' mango fruit than in untreated fruit (Medlicott et al., 1988). On the other hand, the exogenous application of 30 nL L^{-1} or 120 nL L^{-1} 1-methylcyclopropene (1-MCP) to 'Tommy Atkins' mango fruit at the S2 stage (50% red-coloured skin) delayed and reduced the climacteric peak of respiration, the loss of fruit firmness, and higher titratable acidity (TA) compared to control fruit; while, the application of 1-MCP at the S3 stage (75% red-coloured skin) stage did not significantly affect these fruit ripening parameters (Alves et al., 2004).

Nitric oxide (NO) is a highly reactive, free-radical gas that acts as a multi-functional signalling molecule and antagonises the production of ethylene in various physiological processes during fruit ripening (Leshem and Wills, 1998; Leshem et al., 1998; Manjunatha et al., 2010). Post-harvest applications of NO have been reported to delay fruit ripening through causing a reduction in the biosynthesis of

ethylene in a range of climacteric fruit such as banana, plum, and cold-stored 'Kensington Pride' mango (Cheng et al., 2009a; Singh et al., 2009a; Zaharah and Singh, 2011c). Exogenous application of NO has been reported to delay and/or to down-regulate expression of the 1-amino-cyclopropane carboxylic acid (ACC) oxidase (ACO) genes *LeACO1*, *LeACOH2*, and *LeACO4* at different stages of tomato fruit maturation and ripening such as the mature-green (MG), break colour (BC), pink (PI), and full-red (FR) stages, while expression of the ACC synthase (ACS) genes *LeACS2*, *LeACS4, LeACS6*, *LeACS6A*, and *LeACS6B* did not differ in NO-treated and untreated fruit at the MG and BC stages (Eum et al., 2009).

There have been no reports on the effects of NO fumigation at the different climacteric stages on the regulation of ripening in mango fruit. I hypothesised that NO fumigation, applied at the PC stage could be more effective in delaying the ripening of mango fruit than NO applied at the CR stage. We have therefore, investigated the effects of NO fumigation at the PC and CR stages on the rate of ethylene production, the respiration rate, fruit softening, colour development, and fruit quality in 'Kensington Pride' mango fruit stored at ambient temperature.

6.2 Materials and Methods

6.2.1 Plant materials

Mango (*Mangifera indica* L. 'Kensington Pride') fruit of uniform size and free from visible blemishes and symptoms of diseases were obtained from a commercial grower located in Carnarvon, Western Australia (WA) (lat. 24° 52′S; long. 113° 39′E). Fruits were harvested at the PC and CR stages. All fruit for these experiments were desapped, fungicide-treated (0.55 mL L^{-1} Sportak®, a.i. Prochloraz, purchased from Bayer CropScience, Bundaberg, Australia) air dried, packed in soft-board trays, and transported to Perth, WA, by refrigerated truck (at 13°C). Mature fruit at the PC stage (rate of ethylene production = 0.06 ± 0.01 nmol C_2H_4 kg^{-1} h^{-1} and rate of respiration = 1.64 ± 0.04 mmol CO_2 kg^{-1} h^{-1}) or at the CR stage (rate of ethylene production = 1.66 ± 0.05 nmol C_2H_4 kg^{-1} h^{-1} and rate of respiration = 2.91 ± 0.02 mmol CO_2 kg^{-1} h^{-1}) were used.

6.2.2 NO fumigation and experimental design

Ten fruit per replication were fumigated with different concentrations of NO (0, 5, 10, 20, or 40 μL L^{-1}; BOC Gases Ltd., Sydney, Australia) in 67 L sealed plastic containers for 2 h at ambient temperature (21 ± 1°C, 57.01 ± 10.01% RH as detailed in Chapter 3, Section 3.3) following previously published methods (Zaharah and Singh, 2011c). NO has been reported to be sufficiently stable at low concentrations and for short treatment times to be applied in normal air (Soegiarto *et al*., 2003). All fruit were therefore fumigated with NO in containers in normal air, with no depletion of O_2 (Singh et al., 2009). During NO fumigation (1.5 h), the average concentrations of CO_2 in the headspace of the treatment containers injected with 0, 5, 10, 20, or 40 μL L^{-1} NO were 2.21%, 2.22%, 2.16%, 1.93%, and 2.19% for PC treated-fruit, and 2.86%, 2.86%, 2.78%, 2.49% and 2.83% for CR treated-fruit, respectively.

Following the NO treatments, fruit were allowed to ripen at ambient temperature (21 ± 1°C, 56.03 ± 9.7% RH as mentioned in Chapter 3, Section 3.3) in soft-board trays until attaining the full-ripe stage (subjective firmness rating score = 4). The rates of ethylene production and respiration, fruit softness, and skin colour were measured each day during the fruit ripening period. Fruit skin colour parameters, SSC, TA, the SSC:TA ratio, the concentrations of individual sugars, organic acids, total carotenoid, and ascorbic acid were determined at the full-ripe stage. The experiment was arranged in a completely randomised design and included three replications. Ten fruit were treated as a single experimental unit (i.e. n = 30 per treatment; ten fruit × three replications).

6.2.3 Determination of ethylene production

Ethylene production from the mango fruit during ripening was determined daily as detailed in Chapter 3, Section 3.4.2. Ethylene was estimated using a gas chromatograph (6890N Network GC system; Agilent Technology, Palo Alto, CA^2, USA) fitted with a 2 m-long stainless steel column (Porapaq-Q, 3.18 mm, 80/100 mesh size; Supelco, Bellefonte, PA, USA) and a flame ionisation detector (FID). The ethylene production rate was expressed as nmol C_2H_4 kg^{-1} h^{-1}.

6.2.4 Determination of respiration rate

The rate of respiration by measuring the concentrations of CO_2 were determined using an infrared gas analyzer [Servomex Gas Analyzer, Analyzer series 1450 Food Package Analyzer, Servomex (UK) Ltd., East Sussex, UK] as mentioned in detail in Chapter 3, Section 3.5 and expressed as mmol CO_2 kg^{-1} h^{-1}.

6.2.5 Fruit softness

Daily subjective softness of individual fruit in each replication was recorded using a rating scale 1 to 5 as described earlier in Chapter 3, Section 3.6.2.1 and following the methods by Dang et al. (2008a).

6.2.6 Rheological properties of fruit pulp

Rheological properties of pulp (hardness, springiness, cohesiveness, chewiness, adhesiveness and stiffness) of ripe fruit was also determined using a texture analyser (TPA Plus, AMETEK Lloyd Instruments Ltd., Hampshire, UK) fitted with a 7/16 inch Magness-Taylor probe as detailed in Chapter 3, Section 3.6.2.2. The rheological properties of mango pulp were expressed in Newtons (N) for fruit hardness, millimetre (mm) for springiness, Newton millimetres (Nmm) for chewiness and adhesiveness, and kilogram force per millimetre (kg f mm^{-1}) for stiffness. No specific units were used for measurements of cohesiveness.

6.2.7 Visual skin colour

The visual assessment of skin colour was recorded daily during fruit ripening period by following a rating scale ranging from 1 to 5 according to the percentage of green and yellow colour as described in Chapter 3, Section 3.6.1.1.

6.2.8 Visual skin colour and its chromaticity L, a*, b*, C*, and h°*

Fruit skin colour (10 fruit per replicate) was recorded from opposite positions of each fruit in Commission Internationale de L´ Eclariage (CIE) units using a HunterLab ColorFlex 45°/0° Spectrophotometer (HunterLab ColorFlex, Hunter Associates Inc, Reston, VA, USA), using the head 15 mm diameter as outlined in Chapter 3, Section 3.6.1.2.

6.2.9 Soluble solids concentration (SSC), titratable acidity (TA) and SSC:TA ratio

Soluble solids concentration (SSC) was determined using an infrared digital refractometer (Atago-Palette PR 101, Atago Co. Ltd., Itabashi-Ku, Tokyo, Japan) as procedure outlined in Chapter 3, Section 3.6.4. TA was determined by titrating fruit juice against 0.1 *N* NaOH and expressed as percentage of citric acid as detail described in Chapter 3, Section 3.6.5. SSC:TA ratio was calculated by dividing the percentage of SSC with the TA as mentioned in Chapter 3, Section 3.6.6.

6.2.10 Determination of individual sugars and organic acids

The individual sugars and organic acids were extracted from the pulp of full-ripe fruit (~1 g) and quantified using RP-HPLC system (Waters, Milford, MA, USA) as previously outlined in Chapter 3, Section 3.6.3. The concentration of sucrose, glucose, fructose and citric acid were expressed as g 100 g^{-1} FW, whilst tartaric, malic, shikimic and fumaric were expressed as mg 100 g^{-1} FW basis.

6.2.11 Determination of total carotenoid

Total carotenoid from the pulp of full-ripe mango fruit were determined according to the methods previously described by Lalel et al. (2003d) as explained detail in Chapter 3, Section 3.6.7 and expressed as mg kg^{-1} FW basis.

6.2.12 Ascorbic acid

Ascorbic acid was determined using a UV-VIS spectrophotometer (Jenway Spectrophotometer Model 6405, Dunmow, Essex, UK) according to the method previously described by Malik and Singh (2005) and outlined in Chapter 3, Section 3.6.9. Ascorbic acid concentration was quantified using a standard curve of L-ascorbic acid and was expressed as mg 100 g^{-1} FW basis.

6.3 Statistical analysis

The experimental data were subjected to three-way (i.e. NO-treatment × climacteric stage × ripening period) or two-way (i.e. NO-treatment × climacteric stage) analysis of variance (ANOVA) using SAS Release 9.1 (SAS Institute Inc., Cary, NC, USA). The effects of NO fumigation on the rates of ethylene production and respiration, and on fruit firmness and visual skin colour during the fruit ripening period were assessed

using three-way ANOVA. All the other fruit quality parameters of full-ripe fruit were assessed using two-way ANOVA. Fisher's Least Significant Difference (LSD) values were calculated following a significant ($P \leq 0.05$) F-test. All assumptions of ANOVA were checked to ensure the validity of the statistical analysis.

6.4 Results

6.4.1 Ethylene

NO fumigation applied at the PC or at the CR stage had significantly ($P \leq 0.001$) suppressed and delayed the climacteric ethylene peak during fruit ripening period (Figure 6.1A and 6.1B). Climacteric ethylene peak was suppressed (1.63-, 3.79-, 4.82-, and 3.12-fold) in fruit fumigated with 5, 10, 20, and 40 μL L^{-1} NO at the PC stage respectively than untreated fruit during ripening period (Figure 6.1A). All NO treatments applied at the CR stage resulted in 2.11-, 3.34-, 3.58-, and 3.25-fold reduction climacteric ethylene peak during fruit ripening than control fruit, respectively (Figure 6.1B). The suppression in climacteric ethylene production during fruit ripening was more pronounced with increased of NO concentration applied regardless of climacteric stage applied. Averaged over all NO concentrations tested, the mean ethylene production in fruit was lower (0.91 nmol C_2H_4 kg^{-1} h^{-1}) when NO was applied at the PC than at the CR stage (1.32 nmol C_2H_4 kg^{-1} h^{-1}). The interaction between NO fumigation treatments applied at the PC or at the CR stages and ripening period was significant ($P \leq 0.001$) for ethylene production during fruit ripening.

6.4.2 Respiration rate

All the NO fumigation treatments applied at the PC stage significantly ($P \leq 0.05$) suppressed respiratory climacteric peak (1.24-, 1.26-, 1.24-, and 1.46-fold) than untreated fruit during ripening period (Figure 6.2A). All NO fumigation treatments except 5 μL L^{-1} applied at the CR stage also significantly ($P \leq 0.05$) suppressed the respiratory climacteric peak (1.04-, 1.09-, and 1.14-fold) than untreated fruit during ripening (Figure 6.2B). The suppression in climacteric respiration peak during fruit ripening was more pronounced with increased concentration of NO applied at the PC stage. Averaged over all NO concentrations tested, the mean respiration rate was lower (2.33 mmol CO_2 kg^{-1} h^{-1}) in NO-fumigated fruit at the PC stage than the CR stage (2.77 mmol CO_2 kg^{-1} h^{-1}). The interaction between NO fumigation treatments

and ripening period for respiration rate was found significant ($P \leq 0.01$) when NO was applied at the PC stage only.

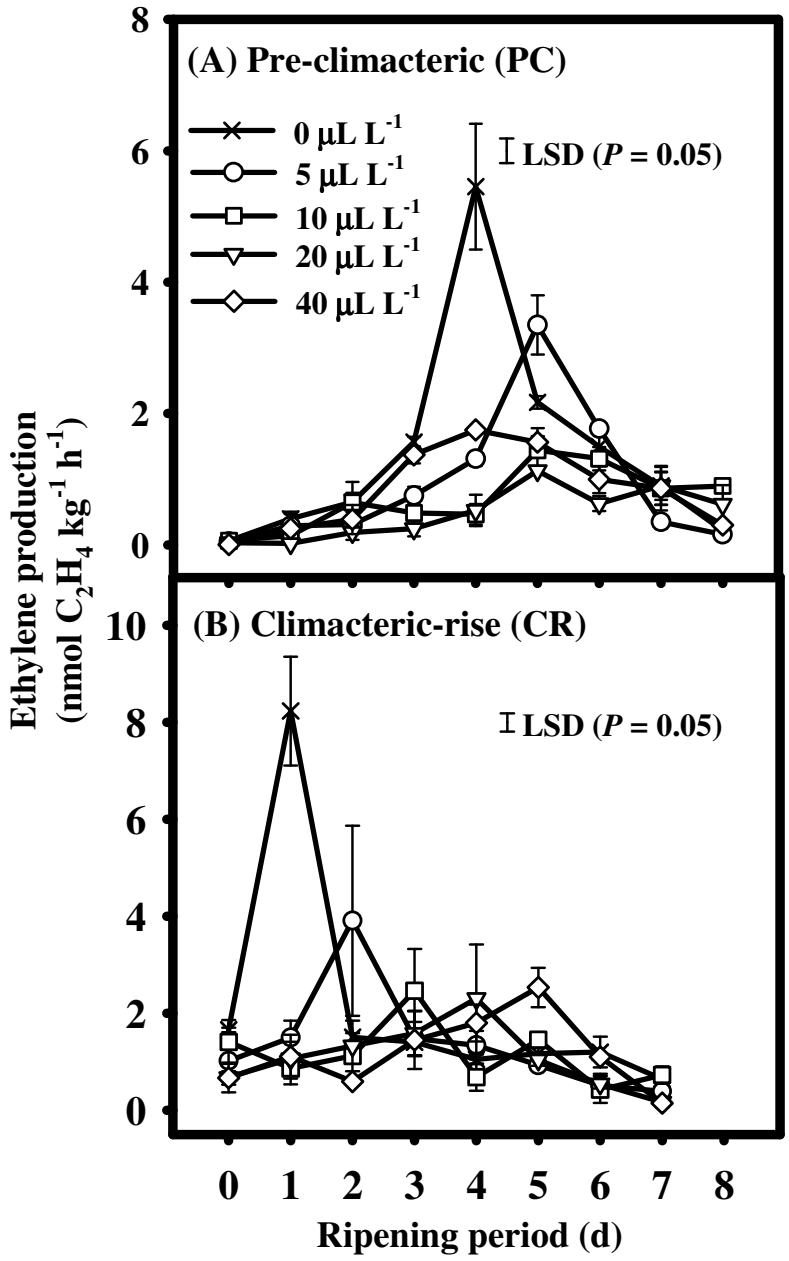

Figure 6.1. Changes in the rate of ethylene production during mango fruit ripening (RP) as influenced by different concentrations of NO fumigation (T) applied at ambient temperature at either of two climacteric stages (CS) to (A) pre-climacteric (PC) fruit or (B) climacteric-rise fruit (CR).

All values (datum points) are means (n = 6; 2 fruit × 3 replications) ± S.E. bars (only visible when larger than the sizes of the symbols used). LSD (***, **, * represents significantly different at $P \leq 0.001$, 0.01, 0.05 and NS indicate non significant differences at $P \leq 0.05$) for T = 0.15^{***}, RP = 0.19^{***}, CS = 0.10^{***}, T × RP = 0.43^{***}; T × CS = 0.22^{***}, RP × CS = 0.27^{***} and T × RP × CS = 0.61^{***}.

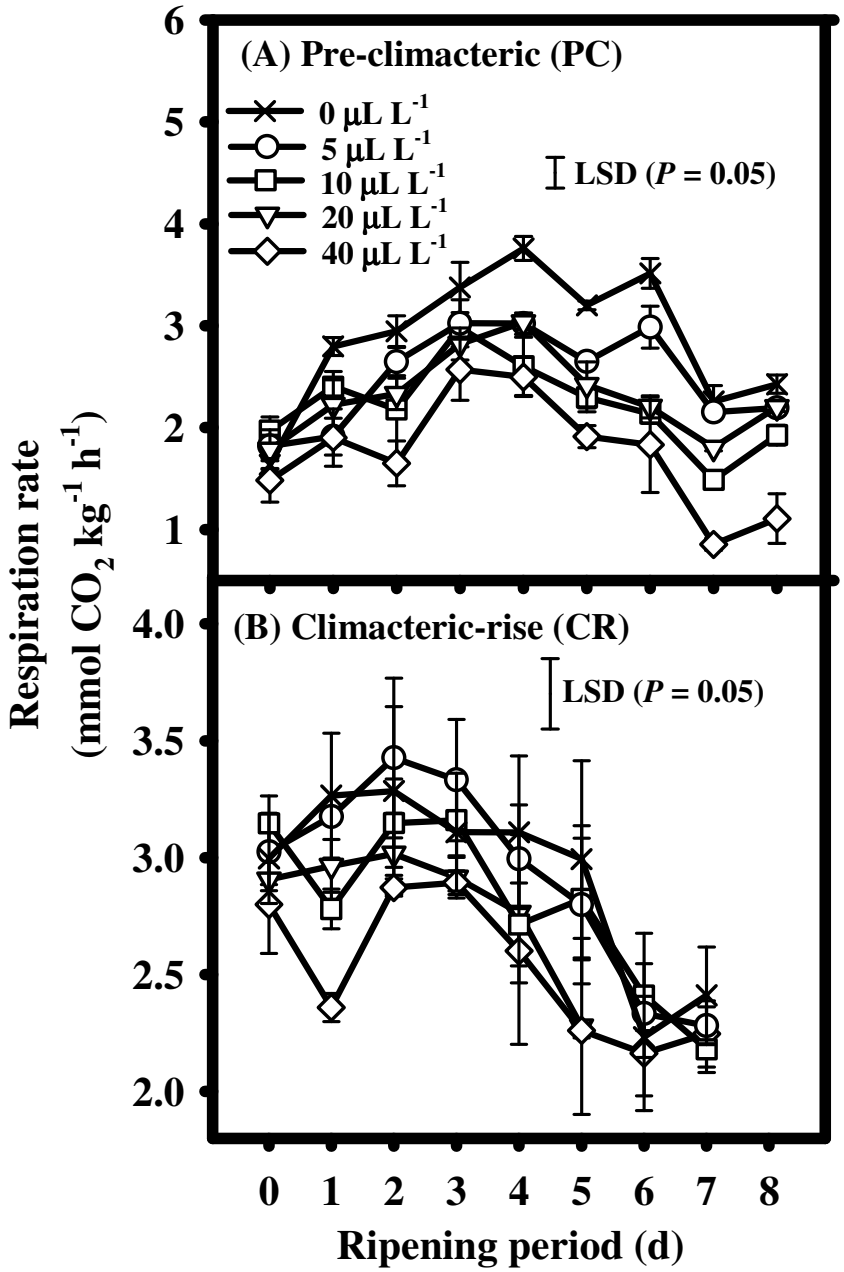

Figure 6.2. Changes in the rate of respiration during mango fruit ripening (RP) as influenced by different concentration of NO fumigation (T) applied at ambient temperature at either of two climacteric stages (CS) to (A) pre-climacteric fruit (PC) or (B) climacteric-rise (CR) fruit.

All values (datum points) are means (n = 6; 2 fruit × 3 replications) ± S.E. bars (only visible when larger than the sizes of the symbols used). LSD (***, **, * represents significantly different at $P \leq 0.001$, 0.01, 0.05 and NS indicate non significant differences at $P \leq 0.05$) for T = 0.10^{***}, RP = 0.13^{***}, CS = 0.06^{***}, T × RP = NS, T × CS = 0.14^{***}, RP × CS = 0.18^{***} and T × RP × CS = NS.

6.4.3 Fruit softness

All NO fumigation treatments applied at the PC or at the CR stage retarded fruit softening during fruit ripening period (Figure 6.3A and 6.3B). Reduction in fruit softening rate during ripening period was more pronounced with increased concentration of NO applied at both stages (Figure 6.3A and 6.3B). Fruit fumigated with NO (20 μL L^{-1} and 40 μL L^{-1}) at the PC stage attained eating soft stage (score 4) two days later than control and all other treatments except 10 μL L^{-1} (Figure 6.3A). Whilst, the fruit fumigated with 20 μL L^{-1} and 40 μL L^{-1} NO at the CR stage resulted in eating soft stage one day later than control and all other treatments (Figure 6.3B). Averaged over all NO concentrations tested, the mean fruit softness score was lower (2.19) in NO-fumigated fruit at the PC stage than at the CR stage (2.81). The interaction between NO fumigation treatments and during ripening period for fruit softening was found significant ($P \leq 0.001$) when NO was applied at the PC stage only.

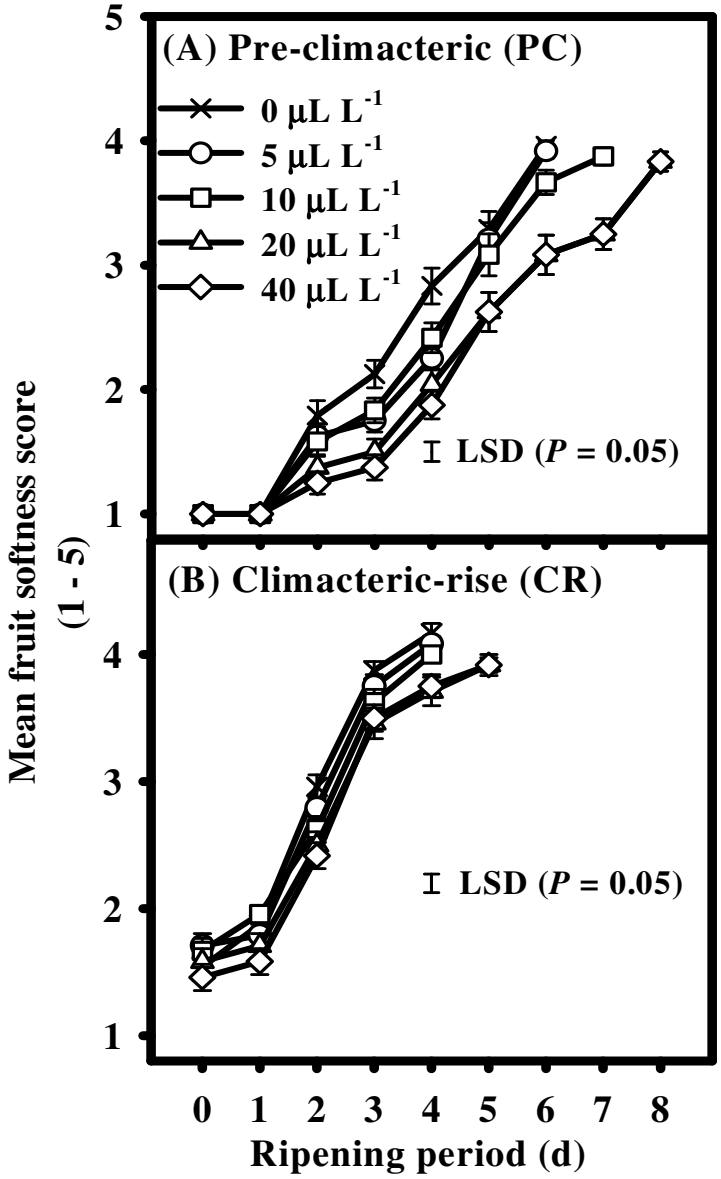

Figure 6.3. Changes in fruit softness (based on arbitrary manual/visual scores of 1 – 5 during mango fruit ripening (RP) as influenced by different concentration of NO (T) applied at ambient temperature at either of two climacteric stages (CS) to (A) pre-climacteric (PC) fruit or (B) climacteric-rise fruit (CR).

All values (datum points) are means (n = 30; ten fruit × three replications) ± S.E. bars (only visible when larger than the size of the symbols used). LSD (***, **, * represents significantly different at $P \leq 0.001$, 0.01, 0.05 and NS indicate non significant differences at $P \leq 0.05$) for T = 0.07^{***}, RP = 0.08^{***}, CS = 0.04^{***}, T × RP = 0.19^{***}, T × CS = NS, RP × CS = 0.12^{***} and T × RP × CS = NS.

6.4.4 Rheological properties of pulp

NO fumigation treatments applied at the PC or at the CR stage had significantly influenced the rheological properties of pulp of full-ripe fruit including cohesiveness, springiness, chewiness, adhesiveness, and stiffness (Table 6.1). Pulp of full-ripe fruit fumigated with NO at the PC or at the CR stage exhibited higher cohesiveness, springiness, chewiness and stiffness as compared to control. Averaged over all NO concentrations tested, mean springiness, chewiness, and adhesiveness of fruit pulp was lower when NO was applied at the CR stage than at the PC stage and the trend was reverse for pulp stiffness. The interactions between NO fumigation and climacteric stage were found to be significant ($P \leq 0.001$) for cohesiveness, springiness and chewiness of pulp at full-ripe fruit (Table 6.1).

6.4.5 Skin colour

All NO fumigation treatments applied at the PC or at the CR stage have retarded skin colour development during fruit ripening period (Figure 6.4A and 6.4B). The suppression in colour development was more pronounced at higher concentration of NO applied at the PC or at the CR. At eating soft stage, NO treatments (20 μL L^{-1} and 40 μL L^{-1}) exhibited lower skin colour development applied at the PC (1.03- and 1.06-fold, respectively) (Figure 6.4A) and at the CR stage (1.14- and 1.20-fold, respectively) (Figure 6.4B), as compared to untreated fruit.

All NO fumigation treatments applied at the PC or CR stage (except 5 μL L^{-1}) exhibited lower chromaticity L*, a*, b*, and C* values at the full-ripe stage (Table 6.2). All NO fumigation treatments applied at the PC stage, but only 20 μL L^{-1} or 40 μL L^{-1} applied at CR stage resulted in higher h° values at the full-ripe stage. Averaged over all NO concentrations tested, the mean chromaticity b* and C* values of fruit skins were higher when NO was applied at the PC stage than at the CR stage, and the trend was reversed for chromaticity L* values of the skin of full-ripe fruit (Table 6.2). Fruit treated with 20 μL L^{-1} or 40 μL L^{-1} NO treatments at the PC stage rather than the CR stage showed a greener skin colour (Figure 6.5). The interactions between NO fumigation treatments and climacteric stage was found significant for chromaticity L* only.

Table 6.1. Effects of various NO fumigation treatments applied to mango fruit at the pre-climacteric (PC) or climacteric-rise (CR) stages of ripening on the rheological properties of the pulp of full-ripe mango fruit.

Climacteric stage	NO conc. ($\mu L\ L^{-1}$)	HD (N)	CH (-)	SP (mm)	CHE (Nmm)	AD (Nmm)	ST (kg f mm^{-1})
PC	0	8.45	0.045b	1.64c	0.69b	0.31b	0.28b
	5	8.39	0.046b	1.69bc	0.69b	0.33ab	0.28b
	10	8.70	0.052b	1.75bc	0.81b	0.39ab	0.29ab
	20	9.06	0.054b	1.85b	0.97b	0.31b	0.32a
	40	8.87	0.093a	2.75a	2.34a	0.47a	0.30ab
	Mean	8.70	0.06	1.94A	1.10	0.36B	0.29B
CR	0	8.04	0.052b	1.54b	0.68b	0.33b	0.25b
	5	9.62	0.066a	1.91a	1.27ab	0.58a	0.38a
	10	8.13	0.058ab	1.80a	0.85ab	0.66a	0.30ab
	20	7.93	0.065a	1.82a	1.39a	0.57a	0.35ab
	40	10.34	0.061a	1.90a	1.36a	0.65a	0.36a
	Mean	8.81	0.06	1.80B	1.11	0.56A	0.33A
LSD of means at $P \leq 0.05$ and significance for a two-factor ANOVA							
Climacteric stage (CS)		NS	NS	0.07^{***}	NS	0.08^{***}	0.03^{*}
NO-fumigation (T)		NS	0.006^{***}	0.11^{***}	0.38^{***}	0.13^{**}	0.05^{*}
CS × T		NS	0.02^{***}	0.44^{***}	1.53^{***}	NS	NS

Mean values followed by the same lower-case (n = 30; ten fruit × three replications) or upper-case (n = 150; 30 fruit × five NO concentrations) letters within a column are not significantly different at $P \leq 0.05$ by the Least Significant Difference (LSD) test. *, **, *** Significantly different at $P \leq 0.05$, 0.01, or 0.001, respectively and NS = not significant. HD = hardness, CH = cohesiveness, SP = springiness, CHE = chewiness, AD = adhesiveness, ST = stiffness and (-) = without unit.

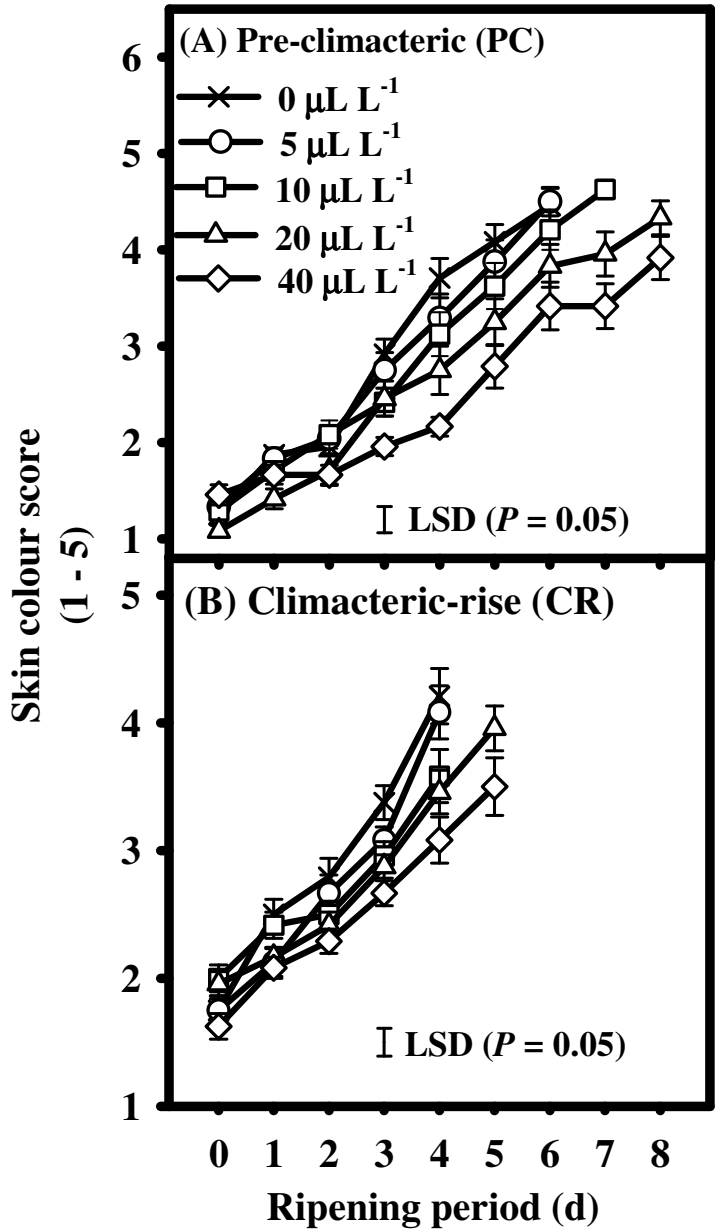

Figure 6.4. Changes in skin colour (based on arbitrary manual/visual scores of 1 – 5 during mango fruit ripening as influenced by different concentration of NO fumigation (T) applied at ambient temperature either of two climacteric stages (CS) to (A) pre-climacteric fruit (PC) or (B) climacteric-rise fruit (CR).

All values (datum points) are means (n = 30; ten fruit × three replications) ± S.E. bars (only visible when larger than the size of the symbols used). LSD (***, **, * represents significantly different at $P \leq 0.001$, 0.01, 0.05 and NS indicate non significant differences at $P \leq 0.05$) for T = 0.11^{***}, RP = 0.14^{***}, CS = 0.07^{***}, T × RP = 0.31^{***}, T × CS = NS, RP × CS = NS and T × RP × CS = NS.

Figure 6.5. Effects of different concentration of NO fumigation applied at (A) pre-climacteric and (B) climacteric rise stage on skin colour development.

Table 6.2. Effects of various NO fumigation treatments applied to mango fruit at the pre-climacteric (PC) or climacteric-rise (CR) stages of ripening on the chromaticity L*, a*, b*, C*, and h° values of skin of full-ripe mango fruit.

Climacteric stage	NO conc. (μL L^{-1})	L*	a*	b*	C*	h°
PC	0	60.60a	10.12a	51.63a	52.71a	78.89c
	5	59.94a	8.97ab	49.39ab	52.02ab	80.71bc
	10	59.55a	8.49ab	49.02b	49.81bc	83.38ab
	20	60.15a	9.13ab	49.21ab	49.31c	83.63ab
	40	57.87b	6.80b	46.99b	47.60c	84.94a
	Mean	59.62B	8.70	49.25A	50.29A	82.30
CR	0	68.96a	10.90a	49.28a	50.25a	77.56d
	5	68.18a	9.39a	48.77ab	48.57ab	79.38cd
	10	66.49b	6.57b	47.66bc	47.76b	81.39cd
	20	64.65c	6.79b	47.19c	47.62b	83.17ab
	40	63.71c	5.38b	44.17d	44.99c	85.29a
	Mean	66.40A	7.81	47.41B	47.84B	81.36
LSD of means at $P \leq 0.05$ and significance for a two-factor ANOVA						
Climacteric stage (CS)		0.66***	NS	0.91***	0.96***	NS
NO-fumigation (T)		1.05***	1.63***	1.43***	1.53***	1.94***
CS × T		1.48***	NS	NS	NS	NS

Mean values followed by the same lower-case (n = 30; ten fruit × three replications) or upper-case (n = 150; 30 fruit × five NO conc.) letters within a column are not significantly different at $P \leq 0.05$ by the Least Significant Difference (LSD) test. *, **, *** Significantly different at $P \leq 0.05$, 0.01, or 0.001, respectively and NS = not significant.

6.4.6 SSC, TA and SSC:TA ratio

NO treatments applied at the PC or at the CR stage did not significantly influence the SSC in full-ripe fruit (Table 6.3). However, NO fumigation applied at the PC or at the CR stage significantly ($P \leq 0.05$ and $P \leq 0.01$) influenced the TA and SSC:TA ratio in pulp of full-ripe fruit without any consistent trends. Averaged over all NO concentrations tested, mean of SSC and SSC:TA ratio were significantly higher (16.39% and 47.06) in the fruit fumigated with NO at the CR than at the PC stage (14.49% and 21.99), respectively, and the trend was reversed for TA. The interactions between NO fumigation and climacteric stage was found significant ($P \leq 0.001$) for TA in the pulp of full-ripe fruit only (Table 6.3).

6.4.7 Changes in concentration of total and individual sugars

All NO treatments applied at the PC or at the CR stage significantly reduced the concentration of total sugars and fructose in the pulp of full-ripe fruit (Table 6.3). Fruit treated with NO (10, 20, and 40 μL L^{-1}) at the PC stage significantly exhibited lower concentration of total sugars (1.38-, 1.40-, and 1.42-fold) and fructose (1.44-, 1.57-, and 1.52-fold) in the pulp of full-ripe fruit as compared to nil treatment (Table 6.3). NO fumigation treatments applied at the CR stage had significantly ($P \leq 0.05$) lower (1.43-fold) concentration of total sugars in the pulp of full-ripe fruit when treated with 40 μL L^{-1} NO as compared to control. Whilst, fruit fumigated with 10 μL L^{-1} and 40 μL L^{-1} NO applied at the CR stage had significantly ($P \leq 0.05$) lower (1.24- and 1.28-fold) concentration of fructose in the pulp of full-ripe fruits as compared to control, respectively (Table 6.3). Averaged over all NO concentrations tested, mean concentration of total sugars, glucose and fructose were significantly higher (15.07 g 100 g^{-1} FW, 0.77 and 5.48 mg 100 g^{-1} FW) in the fruit fumigated with NO at the CR than at the PC stage (11.01 g 100 g^{-1} FW, 0.37, and 3.00 mg 100 g^{-1} FW), respectively.

Table 6.3. Effects of various NO fumigation treatments applied to mango fruit at the pre-climacteric (PC) or climacteric-rise (CR) stages of ripening on SSC, TA, SSC:TA ratio, and the concentrations of total sugars, sucrose, glucose, and fructose in the pulp of full-ripe mango fruit.

Climacteric stage	NO conc. (μL L^{-1})	SSC (%)	TA (%)	SSC:TA ratio	Total sugars (*g 100 g^{-1} FW*)	Sucrose (*g 100 g^{-1} FW*)	Glucose (*g 100 g^{-1} FW*)	Fructose (*g 100 g^{-1} FW*)
PC	0	15.23	0.77ab	19.97b	13.63a	9.21	0.44	3.98a
	5	15.03	0.80a	18.96b	12.19ab	8.70	0.37	3.12b
	10	14.7	0.60bc	24.44ab	9.89b	6.69	0.43	2.77bc
	20	13.57	0.51c	27.02a	9.75b	6.87	0.33	2.54c
	40	13.93	0.72ab	19.57b	9.59b	6.70	0.28	2.62bc
	Mean	14.49B	0.68A	21.99B	11.01B	7.64	0.37B	3.00B
CR	0	16. 27	0.36ab	45.51bc	17.81a	10.58	0.90	6.33a
	5	16.17	0.37ab	43.30bc	16.39ab	10.09	0.78	5.52ab
	10	16.67	0.29b	57.60a	14.96ab	9.03	0.81	5.12b
	20	16.47	0.44a	37.69c	13.78ab	7.92	0.39	5.47ab
	40	16.30	0.32b	51.21ab	12.42b	6.49	0.99	4.94b
	Mean	16.39A	0.36B	47.06A	15.07A	8.82	0.77 A	5.48A
LSD of means at $P \leq 0.05$ and levels of significance for a two-factor ANOVA								
Climacteric stage (CS)		0.043^{***}	0.06^{***}	4.00^{***}	1.54^{***}	NS	0.68^{**}	0.36^{***}
NO-fumigation (T)		NS	0.09^{*}	6.06^{*}	2.44^{**}	NS	NS	0.58^{***}
CS × T		NS	0.13^{**}	NS	NS	NS	NS	NS

Means values followed by the same lower-case (n = 3) or upper-case (n = 15) letters within a column are not significantly different at $P \leq 0.05$ by the Least Significant Difference (LSD) test. *, **, *** Significantly different at $P \leq 0.05$, 0.01, or 0.001, respectively and NS = not significant.

6.4.8 Changes in concentration of total and individual organic acids

Amongst the organic acids determined from the pulp of full-ripe fruit, the NO fumigation treatments applied at both stages has only significantly affected the concentrations of shikimic acid (Table 6.4). Fruit fumigated with NO (20 μL L^{-1} and 5 μL L^{-1}) applied at the PC or at the CR stage exhibited significantly higher (1.44- and 1.56-fold) concentration of shikimic acid in the pulp of full-ripe fruit as compared to untreated fruit, respectively (Table 6.4). Averaged over all NO concentrations tested, mean concentration of total organic acids, citric, tartaric, malic, shikimic and fumaric were significantly higher (1.9-, 2.3-, 4.16-, 1.16-, 1.60-, and 2.11-fold, respectively) in the pulp of full-ripe fruit when NO applied at the PC stage as compared to its fumigation at the CR stage, respectively (Table 6.4). The interactions between NO fumigation treatments and climacteric stages were found significant ($P \leq 0.001$) for the concentration of shikimic acid in the pulp of full-ripe fruit only.

6.4.9 Total carotenoid and ascorbic acid

All NO treatments applied at the PC or at the CR stage did not significantly affect the concentrations of total carotenoid and ascorbic acid in the pulp of full-ripe fruit (Table 6.4). Averaged over all NO concentrations tested, the mean concentration of total carotenoid in the pulp of full-ripe fruit was significantly lower when NO was applied at the PC stage (38.29 mg kg^{-1} FW) rather than at the CR stage (71.22 mg kg^{-1} FW) (Table 6.4). No significant interactions were found between NO fumigation treatments and climacteric stages on total carotenoid content and ascorbic acid in pulp of full-ripe fruit.

Table 6.4. Effects of various NO fumigation treatments applied to mango fruit at the pre-climacteric (PC) or climacteric-rise (CR) stages of ripening on the concentrations of total acids, citric acid, tartaric, malic, shikimic, and fumaric, total carotenoid, and ascorbic acid in the pulp of full-ripe mango fruit.

Climacteric stage	NO conc. ($\mu L\ L^{-1}$)	Total acids (*g 100 g^{-1} FW*)	Citric (*g 100 g^{-1} FW*)	Tartaric (*mg 100 g^{-1} FW*)	Malic (*mg 100 g^{-1} FW*)	Shikimic (*mg 100 g^{-1} FW*)	Fumaric (*mg 100 g^{-1} FW*)	Total carotenoid (*mg kg^{-1} FW*)	Ascorbic acid (*mg 100 g^{-1} FW*)
PC	0	0.92	0.65	19.98	216.20	34.26b	0.19	40.44	21.84
	5	0.92	0.67	14.56	203.93	33.87b	0.16	33.98	19.85
	10	0.97	0.71	20.51	200.50	31.50b	0.22	45.59	22.39
	20	0.82	0.55	14.15	205.49	49.39a	0.16	39.28	20.24
	40	1.15	0.88	24.92	205.09	38.70b	0.19	32.14	20.97
	Mean	0.95A	0.69A	18.83A	206.24A	37.54A	0.19A	38.29B	21.06
CR	0	0.46	0.25	6.00	181.05	18.43b	0.10	71.83	21.16
	5	0.56	0.36	4.97	172.53	28.73a	0.08	61.00	21.31
	10	0.45	0.23	5.49	195.86	21.40ab	0.10	68.42	19.54
	20	0.56	0.36	4.02	170.53	20.29ab	0.08	75.61	20.33
	40	0.49	0.30	2.18	168.02	28.30ab	0.08	76.24	19.89
	Mean	0.50B	0.30B	4.53B	177.55B	23.43B	0.09B	71.22A	20.45
LSD of means at $P \leq 0.05$ and levels of significance for a two-factor ANOVA									
Climacteric stage (CS)		0.09^{***}	0.09^{***}	4.05^{***}	22.07^{**}	3.89^{***}	0.03^{***}	4.54^{***}	NS
NO fumigation (T)		NS	NS	NS	NS	6.14^{*}	NS	NS	NS
CS × T		NS	NS	NS	NS	8.69^{**}	NS	NS	NS

Mean values followed by the same lower-case (n = 3) or upper-case (n = 15) letters within a column are not significantly different at $P \leq 0.05$ by the Least Significant Difference (LSD) test. *, **, *** Significantly different at $P \leq 0.05$, 0.01, or 0.001, respectively and NS = not significant.

6.5 Discussion

6.5.1 Ethylene production

Postharvest NO fumigation at the PC or CR stages of mango fruit significantly ($P \leq 0.001$) suppressed and/or delayed the climacteric peak in ethylene production during the fruit ripening period (Figure 6.1A and 6.1B). This response was more pronounced at increasing concentrations of NO. Lower level of ethylene production in NO-fumigated fruit may reflects the reduced activities of ACS and/or ACO, as previously reported in strawberry (Zhu and Zhou, 2007), in banana (Cheng et al., 2009), and in tomato (Eum et al., 2009). Recently, Manjunatha et al. (2010) proposed that NO bound to ACO, forming a binary ACO-NO complex, which was chelated by 1-aminocyclopropane-1-carboxylic acid (ACC) to produce a stable ternary ACC-ACO-NO complex, which led to reduced ethylene production. The down-regulation of *MA-ACS1* and *MA-ACO1* gene transcription has also been reported to be associated with reduced ethylene production in NO-fumigated banana fruit (Cheng et al., 2009). Similarly, NO fumigation has been reported to reduce ethylene production during the ripening period in various fruit such as cold-stored 'Kensington Pride' mango (Zaharah and Singh, 2011c), plum (Singh et al., 2009), peach (Flores et al., 2008; Zhu et al., 2006), and pear (Sozzi et al., 2003).

When averaged over all NO concentrations tested, the mean level of ethylene produced in fruit was lower when NO was applied at the PC stage rather than at CR stage. This might be due to the fact that fruit harvested at the the PC stage had higher endogenous concentrations of NO than fruit at the CR stage. This warrants further investigation. The endogenous concentrations of NO in unripe avocado and banana fruit have been reported to be approximately ten-fold and four-fold higher than in ripe fruit, respectively (Leshem et al., 2000). 'Kensington Pride' mango fruit harvested at the hard mature green stage exhibited higher climacteric ethylene production during fruit ripening at ambient temperature as compared to the fruit harvest at the sprung mature green, half-ripe and ripe stage (Lalel et al., 2003d). The reduction in ethylene production in tomato fruit was more pronounced when NO was applied at the MG or BC stages compared to NO fumigation at the PI or FR stages. In the case of tomato, down-regulation of the *LeACO1*, *LeACOH2*, and *LeACO4* genes, led to delayed ripening (Eum et al., 2009). The effect of NO fumigation on

regulation of the expression of those genes involved in ethylene biosynthesis during mango fruit ripening also warrants further investigation.

6.5.2 Respiration rate

NO fumigation reduced the rates of respiration in mango fruit during ripening, although the effect was more pronounced when NO was applied at the PC stage rather than at the CR stage (Figure 6.2A and 6.2B). Differences in the reduction of the rates of respiration in fruit fumigated with NO at the PC stage rather than at the CR stage corresponded to the reductions in the rates of endogenous ethylene production (Figure 6.1A). For example, respiration by 'Kensington Pride' mango fruit at the PC stage typically increased in response to exposure to ethylene and decreased following treatment with inhibitors of ethylene biosynthesis (i.e. AVG) or ethylene actions [i.e. 1-MCP; Lalel et al. (2003e)]. NO fumigation may be more effective at reducing the activity of cytochrome-chain components of respiration early during the ripening period, before the climacteric burst of respiration, rather than later at the PC stage. Cytochrome chain components have been reported to play an important role in facilitating the climacteric burst of respiration prior to alternative oxidase and uncoupling proteins, which play a role in post-climacteric senescence in mango fruit (Considine et al., 2001). Millar and Day (1996) claimed that NO can inhibit cytochrome oxidase, but not alternative oxidase, in isolated soybean cotyledon mitochondria. Suppression of the rates of respiration in NO-treated fruit during ripening has also been reported in 'Kensington Pride' mango (Zaharah and Singh, 2011c), plum (Singh et al., 2009), peach (Flores et al., 2008), strawberry (Zhu and Zhou, 2007), and tomato (Zhang et al., 2005).

6.5.3 Fruit softness and rheological properties of fruit pulp

NO treatments applied at both climacteric stages delayed fruit softening during ripening period at ambient temperature (Figure 6.3A and 6.3B). In addition, most of the NO fumigation treatments increased cohesiveness, springiness, chewiness, adhesiveness, and stiffness of the pulp of ripe fruit (Table 6.1). The reduction in fruit softening may be attributed to the suppression of ethylene production by NO, which in turn, reduced the activities of polygalacturonase (PG), and *endo*-1,4-β-D-glucanase (EGase) that are involved in fruit softening in 'Kensington Pride' mango (Zaharah and Singh, 2011a). Similarly, Khan and Singh (2008) reported that higher

activities of fruit softening enzymes such as EGase, *endo*- and *exo*-PG were associated with increased, climacteric, ethylene production during plum fruit ripening. Moreover, treatment with ethylene or 1-MCP at the mature unripe stage (i.e. the PC stage) has also been reported to hasten or delay ripening/softening in 'Dashehari' mango fruit, respectively, through regulating the expression of genes such as an expansion gene [*MiEXPA1*; Sane et al. (2005)], a pectate lyase gene [*MiPel1*; Chourasia et al. (2006)], and a cellulase gene [*MiCel1*; Chourasia et al. (2008)]. Previously, NO treatments have been reported to retard fruit softening during fruit storage and ripening in 'Kensington Pride' mango (Zaharah and Singh, 2011c), kiwifruit (Zhu et al., 2010b), plum (Singh et al., 2009), banana slices (Cheng et al., 2009), and peach (Zhu et al., 2006).

6.5.4 Visual skin colour and chromaticity L, a*, b*, C*, and h°*

NO fumigation applied at the PC or at the CR stage retarded fruit skin colour development including visual colour, chromaticity L*, a*, b*, C*, and maintained higher h° during ripening (Figure 6.4A, 6.4B and Table 6.2). NO treatments (20 µL L^{-1} and 40 µL L^{-1}) applied at the PC stage showed a greener skin colour rather than the CR stage (Figure 6.4B and 6.5). This may be attributed to the suppression of ethylene production in NO-fumigated fruit reducing the degeneration of chlorophyll. Ethylene is known to improve the colouration of mango skin by accelerating the degradation of chlorophyll and synthesis of other pigments such as carotenoid (Brecht and Yahia, 2009). The exogenous application of ethrel (500 mg L^{-1}) has been reported to improve skin colour of 'Neelum' mango during the ripening period (Kulkarni et al., 2004). Reduced skin colour development in the NO-treated fruit has also been reported in tomato (Eum et al., 2009) and plum (Singh et al., 2009) through the reduction of accumulation of lycopene and anthocyanins, respectively.

6.5.5 SSC, TA, and SSC:TA ratio

When averaged over all tested NO concentrations, the mean SSC and SSC:TA ratio in full-ripe stage were found to be higher in the fruit fumigated with NO at the CR stage than at the PC stage, while TA showed a reverse trend (Table 6.3). These results may be associated with delayed and suppressed ethylene production and respiration rate due to NO treatment. The higher SSC:TA ratio in the pulp of full-ripe fruit which were treated with NO at the CR than at the PC stage may be due to the

higher SSC and lower TA. Previously, NO treatment has been reported to delay the increase in SSC during fruit ripening in peach (Zhu et al., 2006), kiwifruit (Zhu et al., 2008; Zhu et al., 2010b) and jujube (Zhu et al., 2009). In addition, the reduction of TA in NO-treated fruit has also been reported in plum during cold storage (0°C) (Singh et al., 2009). An increase in the SSC:TA ratio during ripening of kiwifruit has been associated with NO treatment (Zhu et al., 2008).

6.5.6 Total and individual sugars

The reduction in the concentration of total sugars and fructose in full-ripe fruit due to NO fumigation at the PC or at the CR stage may be attributed to reduced conversion of starch into sugars in the pulp, similarly also reported in slices of banana fruit (Cheng et al., 2009). Averaged over NO treatments, higher concentrations of total sugars, sucrose, glucose and fructose were more pronounced when NO was applied at CR than PC stage (Table 6.3). It may be attributed to the higher activity of amylase in the mango fruit harvested at the post-climcteric stage and suppressed rate of respiration during ripening (Figure 6.2B), contributing to the accumulation of sugars in full-ripe fruit (Dick et al., 2009). Previously, Singh et al. (2009) reported that NO fumigation significantly decreased fructose, glucose, sucrose, and sorbitol levels in 'Japanese' plum compared to non-fumigated fruit after cold storage and ripening.

6.5.7 Total and individual organic acids

Citric and malic acids were present in greatest amount in ripe 'Kensington Pride' mango fruit (Table 6.4). Amongst different organic acids determined, only the concentrations of shikimic acid in the pulp of ripe fruit were significantly affected with the NO fumigation treatments applied at the PC or at the CR stage. Averaged over all NO treatments tested, the mean concentration of total organic acids, citric, tartaric, malic, shikimic, and fumaric were significantly higher in the pulp of the full-ripe fruit, when NO was applied at the PC stage than at the CR stage (Table 6.4). NO fumigation and the mango fruit maturity stage at harvest seem to be influencing the organic acid metabolism in mango fruit during ripening.

6.5.8 Total carotenoid and ascorbic acid

All NO treatments applied at the PC or at the CR stage did not significantly affect the concentrations of total carotenoid and ascorbic acid in the pulp of full-ripe fruit

(Table 6.4). Likewise, NO-treated peach fruit did not exhibit any significant change in the concentration of total carotenoid during ripening at 20°C (Flores et al., 2008). Averaged over all NO concentrations tested, the mean concentration of total carotenoid in the pulp was significantly lower when NO was applied at the PC stage rather than at the CR stage (Table 6.4). It may be attributed to the climacteric stage of the mango fruit at the harvest time. However, Lakshminarayana (1975) reported that the fruit harvested at the medium ripe or ripe (post-climacteric stage) exhibited lower levels of carotenoid in 'Haden', 'Kent', and 'Irwin' mangoes than those harvested at the PC stage following ripening at 25°C.

In conclusion, post-harvest fumigation with NO at 20 μL L^{-1} or 40 μL L^{-1}, applied to mango fruit at the PC stage, was more effective in suppressing ethylene production and the rate of respiration, and in retarding skin colour development and fruit softening during ripening at ambient temperatures as well as increasing the rheological properties (springiness) of pulp, TA, total and individual organic acids level in full-ripe fruit, than NO fumigation at the CR stage.

CHAPTER 7

Mode of action of nitric oxide in inhibiting ethylene biosynthesis and fruit softening during ripening and cool storage of 'Kensington Pride' mango

Summary

The mode of action of nitric oxide (NO) in inhibiting ethylene biosynthesis and fruit softening during ripening and cool storage of mango fruit was investigated. Hard mature green mango (*Mangifera indica* L. cv. 'Kensington Pride') fruit were fumigated with 20 μL L^{-1} NO for 2 h at 21°C and allowed to ripen at 21 ± 1°C for 10 days, or stored at 13 ± 1°C for 21 days. During ripening and cool storage, ethylene production and respiration rate from whole fruit were determined daily. The 1-aminocyclopropane-1-carboxylic acid (ACC) content, activities of ACC synthase (ACS), ACC oxidase (ACO), and fruit softening enzymes such as pectinesterase (PE), *endo*-1,4-*β*-D-glucanase (EGase), *exo*- and *endo*-polygalacturonase (*exo*-PG, *endo*-PG) as well as firmness and rheological properties of pulp were determined at two and seven day intervals during ripening and cool storage, respectively. NO fumigation inhibited ethylene biosynthesis and respiration rate, and maintained stiffness. NO-fumigated fruit during cool storage exhibited reduced ACC content through inhibiting the activities of both ACS and ACO in the fruit pulp. NO-fumigated fruit showed decreased activities of *exo*-, *endo*-PG, EGase, but maintained higher PE activity in pulp tissues during ripening and cool storage. In conclusion, NO fumigation inhibited ethylene biosynthesis through inhibition of ACS and ACO activities leading to reduced ACC content in the fruit pulp which consequently, reduced the activities of fruit softening enzymes during ripening and cool storage.

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7.1 Introduction

Mango fruit ripening involves numerous biochemical changes including increased respiration, ethylene production, fruit softening, development of pigments, changes in carbohydrates, organic acids, lipids, phenolics, volatile compounds and structural polysaccharides (Gomez-Lim, 1997; Lalel et al., 2003e). Ethylene, a ripening hormone, is directly involved in the onset of ripening in climacteric fruits such as mango (Lelievre et al., 1997). Exogenous application of ethephon has also been reported to increase ethylene production, whilst aminoethoxyvinylglycine (AVG) and 1-Methylcyclpropene (1-MCP) treatments suppress its production during ripening, and these treatments consequently hastened and delayed ripening, respectively (Lalel et al., 2003e).

Nitric oxide (NO) is an important signalling molecule known to inhibit ethylene production during ripening and/or storage. As a result of NO treatment, fruit ripening is delayed in various fruits, including mango (Leshem, 1996; Leshem and Wills, 1998; Leshem et al., 1998; Manjunatha et al., 2010; Zaharah and Singh, 2011c). Previously, reduced ethylene production during ripening in NO-fumigated fruit has been reported due to binding of NO with 1-aminocyclopropane-1-carboxylic acid (ACC) and ACC oxidase (ACO, EC 1.14.17.4) to form a stable ternary complex, thus limiting ethylene production (Tierney et al., 2005). Other research also suggests that reduction in ethylene production in NO-fumigated fruit is caused by its inhibitory effects on the activity of ACC synthase (ACS, EC 4.4.1.14) and/or ACO (Cheng et al., 2009; Liu et al., 2007; Zhu et al., 2006; Zhu and Zhou, 2007). Zhu et al. (2006) reported reduced ethylene biosynthesis in NO-treated peach was mainly due to decreased activity of ACO and accumulation of ACC and 1-malonylaminocyclopropane-1-carboxylic acid (MACC) without any significant effects on ACS activity. NO fumigation also reduced ethylene biosynthesis in banana due to decreased activity of ACO, but with higher activity of ACS and ACC content (Cheng et al., 2009). The inhibition in ethylene biosynthesis in NO-fumigated tomato fruit was due to decreased and delayed expression of ACO genes (Eum et al., 2009). In contrast, NO fumigation of strawberry decreased the activity of ACS, but not ACO (Zhu and Zhou, 2007). NO-treated tomato fruit exhibited reduced ethylene biosynthesis and the expression of *LeACO1*, *LeACOH2* and *LeACO4* (Eum et al., 2009) and blocked the expression of *MA-ACS1* and *MA-ACO1* genes in banana slices

(Cheng et al., 2009) during ripening at ambient temperature (24°C) and cool storage (15°C), respectively. Recently, Zaharah and Singh (2011c) proposed that possibly, either ACO or ACS inhibition may explain the mechanism of action of NO in inhibiting ethylene biosynthesis in mango fruit.

Fruit softening in mango fruit is associated with increased activities of cell wall hydrolysis enzymes during ripening (Ali et al., 2004; Ketsa and Daengkanit, 1999) and cold storage (Roe and Bruemmer, 1981), and is triggered by ethylene (Medlicott et al., 1987). The role of several softening enzymes has been investigated in mango fruit, softening including polygalacturonase (PG), *exo*-PG (EC 3.2.1.67), *endo*-PG (EC 3.2.1.15), pectinesterase (PE, EC 3.1.1.11), and *endo*-1,4-*β*-D-glucanase (EGase, EC 3.1.1.4), pectin lyase (PL, EC 4.2.2.2), and *β*-galactosidase (*β*-Gal, EC 3.2.1.23) (Abu-Sarra and Abu-Goukh, 1992; Ali et al., 2004; Chourasia et al., 2006; Chourasia et al., 2008; Lazan et al., 1986; Mitcham and McDonald, 1992; Roe and Bruemmer, 1981). Previously, exogenous application of 1.0 μL L^{-1} 1-MCP decreased ethylene production and activities of PG and PE during ripening of 'Chousa', 'Dashehari' and 'Langra' mango, and consequently, fruit softening delayed (Singh and Neelam, 2008). NO-treated as well as NO in combination with intermittent warming treated peach inhibited *exo*-PG activity and preserved higher *endo*-PG activity, but retained higher PE and EGase during cold storage at 5°C than control fruit (Zhu et al., 2010a). In banana, NO-treated fruit had decreased PG, EGase as well as PE (Cheng et al., 2009), or *β*-Gal during ripening period (Yang et al., 2010).

NO-fumigated mango fruit exhibited reduced ethylene biosynthesis and softening during ripening following 2- and 4-week storage at chilling temperature (5°C) (Zaharah and Singh, 2011c). However, no information is available on how NO inhibits ethylene biosynthesis and fruit softening during ripening and cold storage of mango fruit. In this research, I hypothesised that NO fumigation reduced the activity of ACS and/or ACO, which consequently down regulate ACC content leading to the inhibition of ethylene biosynthesis and downstream the fruit softening enzymes activities. We investigated the effects of NO on inhibition of ethylene biosynthesis and fruit softening including the activities of ethylene biosynthetic enzymes such as ACS and ACO, and ACC content, as well as fruit softening enzymes including PE,

exo-, *endo*-PG and EGase in the pulp of mango fruit during ripening and cold storage.

7.2 Materials and methods

The effects of postharvest NO fumigation on ethylene biosynthesis and the activities of fruit softening enzymes during fruit ripening at ambient temperature (21 ± 1°C) was investigated in Experiment I, and in Experiment II these changes was investigated during cool storage at 13 ± 1°C.

7.2.1 Plant materials

Hard mature green 'Kensington Pride' mangoes (*Mangifera indica* L.) were obtained from a commercial orchard at Chittering (lat. 31° 25´S and long. 116° 5´E), Western Australia. The fruit used for the experiment I were firm (186.77 ± 8.88 N), had the rate of ethylene production (0.03 ± 0.002 nmol C_2H_4 kg^{-1} h^{-1}) and respiration (0.12 ± 0.05 mmol CO_2 kg^{-1} h^{-1}). In experiment II, the fruit used were firm (154.39 ± 3.59 N), had the rate of ethylene production (0.60 ± 0.32 nmol C_2H_4 kg^{-1} h^{-1}) and respiration (1.56 ± 0.11 mmol CO_2 kg^{-1} h^{-1}). Fruit of uniform size, free from visual blemishes and symptoms of diseases were used in both experiments.

7.2.2 NO fumigation

Mango fruit were fumigated with NO (20 µL L^{-1}) in hermitically sealed plastic containers (67 L) for 2 h at 20°C and 54.03 ± 8.17% RH following data recorded as mentioned in Chapter 3, Section 3.3. Earlier, Soegiarto et al. (2003) reported that NO is sufficiently stable at the low concentrations and short times required for produce to be treated in normal air. Similarly, Singh et al. (2009) reported that fruit fumigated with NO in containers exhibited normal air without depletion of O_2. Untreated fruit (controls) were kept in the same sealed plastic containers without NO. A compressed cylinder of NO purchased from BOC Gases Ltd., Sydney, NSW, Australia, contained 4, 810 ± 100 µL L^{-1} NO. After fumigation for 1.5 h, the average concentrations of CO_2 in the headspace of treatment containers injected with 0 or 20 µL L^{-1} NO were 1.42 ± 0.16% and 1.25 ± 0.17% for fruit treated in experiment I (Section 7.2.3), and 2.10 ± 0.44% and 1.90 ± 0.18% for fruit treated in experiment II (Section 7.2.4), respectively.

7.2.3 Experiment I: Effects of postharvest NO fumigation on ethylene biosynthesis and the activities of fruit softening enzymes during fruit ripening at ambient temperature

NO-fumigated and control fruit were packed in soft-board trays and allowed to ripen at ambient temperature (21 ± 1°C, 53.2 ± 9.2% RH as explained earlier in Chapter 3, Section 3.3) until the fruit attained the eating soft stage. Ethylene production, respiration rate, and fruit softness were determined daily during fruit ripening. The ACC content, and the activities of ethylene biosynthesis enzymes (ACS and ACO) and fruit softening enzymes, including *exo*-, *endo*-PG, PE and EGase, were determined from the pulp tissue at 2 days intervals during ripening. Pulp tissues samples from dorsal and ventral mesocarp part were taken and immediately immersed in liquid N_2 and kept at -80°C freezer prior to determining the activities of ethylene biosynthesis and fruit softening enzymes. The experiment used a completely randomised two-factorial design, including treatments and ripening period, with three replications of ten fruit were treated as an experimental unit.

7.2.4 Experiment II: Effects of postharvest NO fumigation on ethylene biosynthesis and the activities of fruit softening enzymes during cool storage (13 ± 1°C)

NO-fumigated and control fruit were stored for 21 days at 13 ± 1°C and 95 ± 0.6% RH as recorded using a method explained in Chapter 3, Section 3.3. The ethylene production and respiration rates were recorded daily. The fruit were removed from cold storage and equilibrated to ambient temperature prior to determining ethylene and respiration rate. The ACC content, activities of ACS, ACO, *exo*-, *endo*-PG, PE, and EGase were determined from pulp tissues at 7 days intervals during cold storage. The experiment used a completely randomised two-factorial design, including treatments and cold storage period with three as replications of ten fruit constituted as an experimental unit.

7.2.5 Determination of ethylene production

Ethylene was estimated using a gas chromatograph (6890N Network GC system; Agilent Technology, Palo Alto, CA^2, USA) fitted with a 2 m-long stainless steel column (Porapaq-Q, 3.18 mm, 80/100 mesh size; Supelco, Bellefonte, PA, USA) and

a flame ionisation detector (FID) as explained in detail in Chapter 3, Section 3.4.2. The rate of ethylene production was expressed as nmol C_2H_4 kg^{-1} h^{-1}.

7.2.6 Determination of respiration rate

The respiration rate was determined as carbon dioxide production using an infrared gas analyzer [Servomex Gas Analyzer, Analyzer series 1450 Food Package Analyzer, Servomex (UK) Ltd., East Sussex, UK] and expressed as mmol CO_2 kg^{-1} h^{-1} following a detail method in Chapter 3, Section 3.5.

7.2.7 Determination of ACC content, and activity of ACS and ACO in pulp tissue

The ACC content from pulp tissue was determined as described by Khan and Singh (2007) as detailed in Chapter 3, Section 3.7.1. The ACC content was expressed as pmol g^{-1} FW. The activity of ACS was determined from pulp tissue following the method of Nair et al. (2004b) and Khan and Singh (2007) with some modifications as outlined in Chapter 3, Section 3.7.2. The activity of ACS enzyme was expressed as pmol ACC mg $protein^{-1}$ h^{-1}. The activity of ACO was determined following a method described earlier by Gorny and Kader (1996), and with some modifications as explained in Chapter 3, Section 3.7.3. The activity of ACO enzyme was expressed as nmol C_2H_4 mg $protein^{-1}$ h^{-1}.

7.2.8 Rheological properties of fruit pulp

Rheological properties of pulp (hardness, springiness, cohesiveness, chewiness, adhesiveness, and stiffness) of ripe fruit was also determined using a texture analyser (TPA Plus, AMETEK Lloyd Instruments Ltd, Hampshire, UK) fitted with a 7/16 inch Magness-Taylor probe as detailed in Chapter 3, Section 3.6.2.2. The rheological properties of mango pulp for fruit hardness, springiness, chewiness and adhesiveness, and stiffness were expressed in Newtons (N), millimetre (mm), Newton millimetres (Nmm), and kilogram force per millimetre (kg f mm^{-1}). No specific units were used for measurements of cohesiveness.

7.2.9 Determination of fruit softening enzymes activities in pulp tissue

Activities of *exo*-, *endo*-PG, PE and EGase were determined in pulp following the method as described by Khan and Singh (2007) with some modification as detailed in Chapter 3, Section 3.8. The *exo*-, *endo*-PG, PE, and EGase enzymes activities

were expressed as μg galacturonic acid mg protein^{-1} h^{-1}, Δ viscosity mg protein^{-1} h^{-1}, mM NaOH mg protein^{-1} h^{-1} and Δ viscosity mg protein^{-1} h^{-1}, respectively.

7.2.10 Protein determination

Protein content in fruit pulp was determined following by Bradford (1976) method explained in Chapter 3, Section 3.9, using bovine serum albumin (Sigma-Aldrich Pty. Ltd., NSW, Australia) as a standard and expressed as mg mL^{-1} of enzyme extract.

7.2.11 Statistical analysis

The experimental data were subjected to two-way (i.e. treatment × ripening or storage period) analysis of variance (ANOVA) using SAS (release 9.1.3, SAS Institute Inc., Cary, NC, USA). Fisher's Least Significant Differences (LSD) were calculated following a significant ($P \leq 0.05$) F-test. All the assumptions of ANOVA were checked to ensure validity of statistical analysis.

7.3 Results

7.3.1 Ethylene production during fruit ripening and storage

NO fumigation (20 μL L^{-1}) suppressed (2-fold) and delayed the climacteric ethylene peak (1 day) as compared to control fruit during ripening (Figure 7.1A). Similarly, NO-fumigated fruit exhibited suppressed ethylene production compared with the control during 21 days cool storage (13°C), and suppression was more pronounced between 14 to 21 days storage (Figure 7.1B). Averaged over storage period, mean ethylene production was 3.02-fold lower in the NO-fumigated fruit than in the control (Figure 7.1B). The interaction between NO fumigation treatment and ripening period as well as storage period were significant ($P \leq 0.05$) for ethylene production.

7.3.2 Respiration rate during fruit ripening and storage

NO fumigation suppressed (1.24-fold) and delayed the climacteric respiration peak (2 days) compared with control fruit during ripening period (Figure 7.1C). Averaged over the ripening period, mean respiration rate was lower (2.19 mmol CO_2 kg^{-1} h^{-1}) in NO-fumigated fruit than control (2.87 mmol CO_2 kg^{-1} h^{-1}) (Figure 7.1C).

Similarly, NO-fumigated fruit exhibited suppressed respiration rate compared with the control fruit during 21 days cold storage, and the suppression was more pronounced between 7 to 14 days storage (Figure 7.1D). Averaged over the storage period, mean respiration rate was lower (2.08 mmol CO_2 kg^{-1} h^{-1}) in NO-fumigated fruit than in the control (2.54 mmol CO_2 kg^{-1} h^{-1}) (Figure 7.1D). The interaction between NO fumigation treatment and ripening period as well as storage period was found to be significant for respiration rate.

7.3.3 ACC content and activities of ACO, ACS during fruit ripening and cool storage

NO fumigation reduced the ACC content, and activities of ACS and ACO in pulp during 2 to 6 days of fruit ripening period compared with untreated fruit (Figure 7.2A, 7.2C and 7.2E). NO treatment completely suppressed the activity of ACO in the fruit pulp during the fruit ripening when compared to the control (Figure 7.2E). Averaged over the ripening period, mean ACC content, activities of ACS, and ACO enzymes in the pulp tissues were significantly ($P \leq 0.05$) 1.51-, 1.49-, and 3.30-fold lower in NO-fumigated fruit than in the control, respectively.

NO-fumigated fruit exhibited lower ACC content as compared to the control during 7 and 14 days of cool storage (Figure 7.2B). Activities of ACS and ACO enzymes suppressed, but did not significantly differ in NO-treated fruit as compared to the control during 7 days of cool storage. The suppression of ACC content and the activities of ACS and ACO were more pronounced and significantly different ($P \leq 0.05$) by 21 days of storage (Figure 7.2B, 7.2D, and 7.2F). Averaged over the storage period, mean ACC content, the activities of ACS, and ACO enzymes in the pulp were lower (0.03 pmol g^{-1} FW, 0.63 pmol ACC mg $protein^{-1}$ h^{-1}, 0.72 nmol C_2H_4 mg $protein^{-1}$ h^{-1}) in NO-fumigated fruit compared with the control treatment (0.04 pmol g^{-1} FW, 1.72 pmol ACC mg $protein^{-1}$ h^{-1}, 1.40 nmol C_2H_4 mg $protein^{-1}$ h^{-1}) respectively. The interaction between NO fumigation treatment and ripening period as well as storage period were found to be significant for ACC content and activities of ACS and ACO enzymes in pulp tissue.

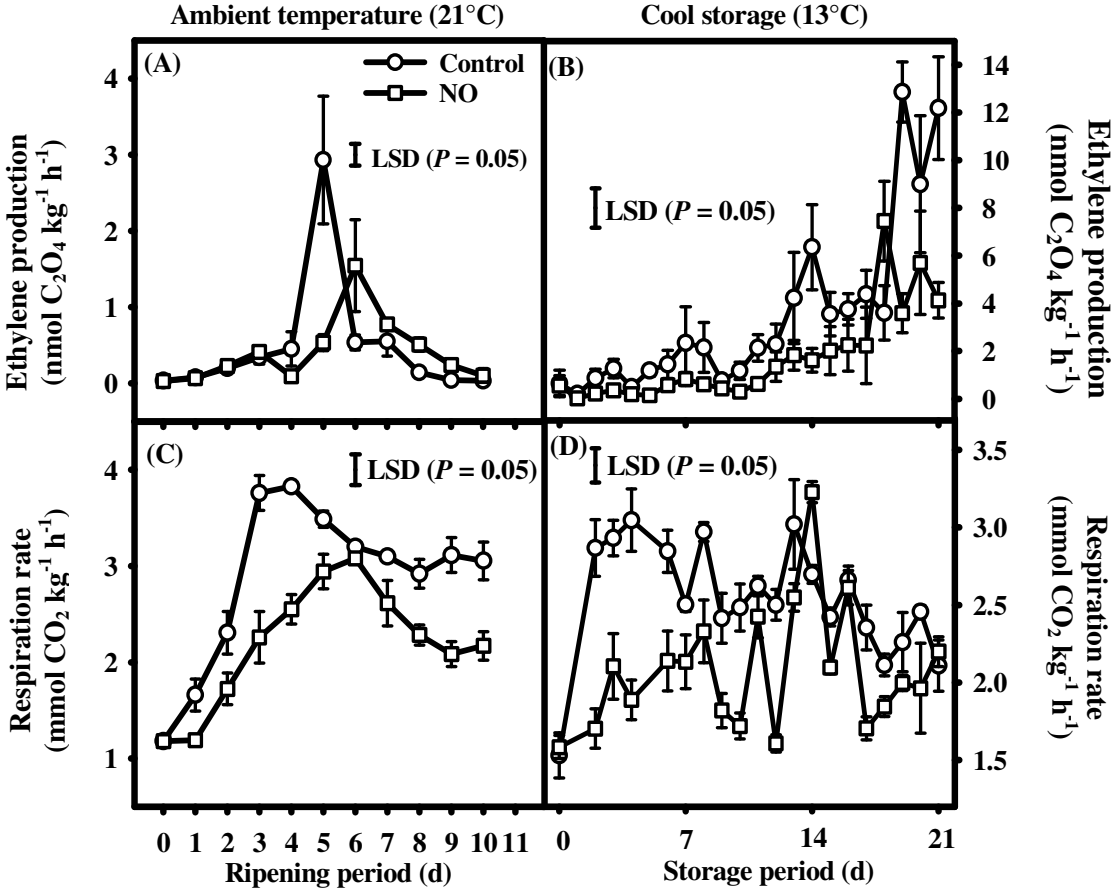

Figure 7.1. Ethylene production (A and B) and respiration rate (C and D) as influenced by NO fumigation (T) and ripening period (RP) at ambient temperature and during cool storage (SP).

Vertical bars represent S.E. of means and are invisible when the values are smaller than the symbol. n = 6 (2 fruit × 3 replications), LSD (***, **, * represents significantly different at $P \leq 0.001$, 0.01, 0.05 and NS = not significant. Ethylene production during ripening: T = NS, RP = 0.47^{***}, T × RP = 0.67^{***} and cold storage: T = 0.61^{***}, SP = 2.02^{***}, T × SP = 2.86^{***}; Respiration rate during ripening: T = 0.17^{***}, RP = 0.39^{***}, T × RP = 0.56^{*} and cold storage: T = 0.09^{***}, SP = 0.27^{***}, T × SP = 0.39^{***}.

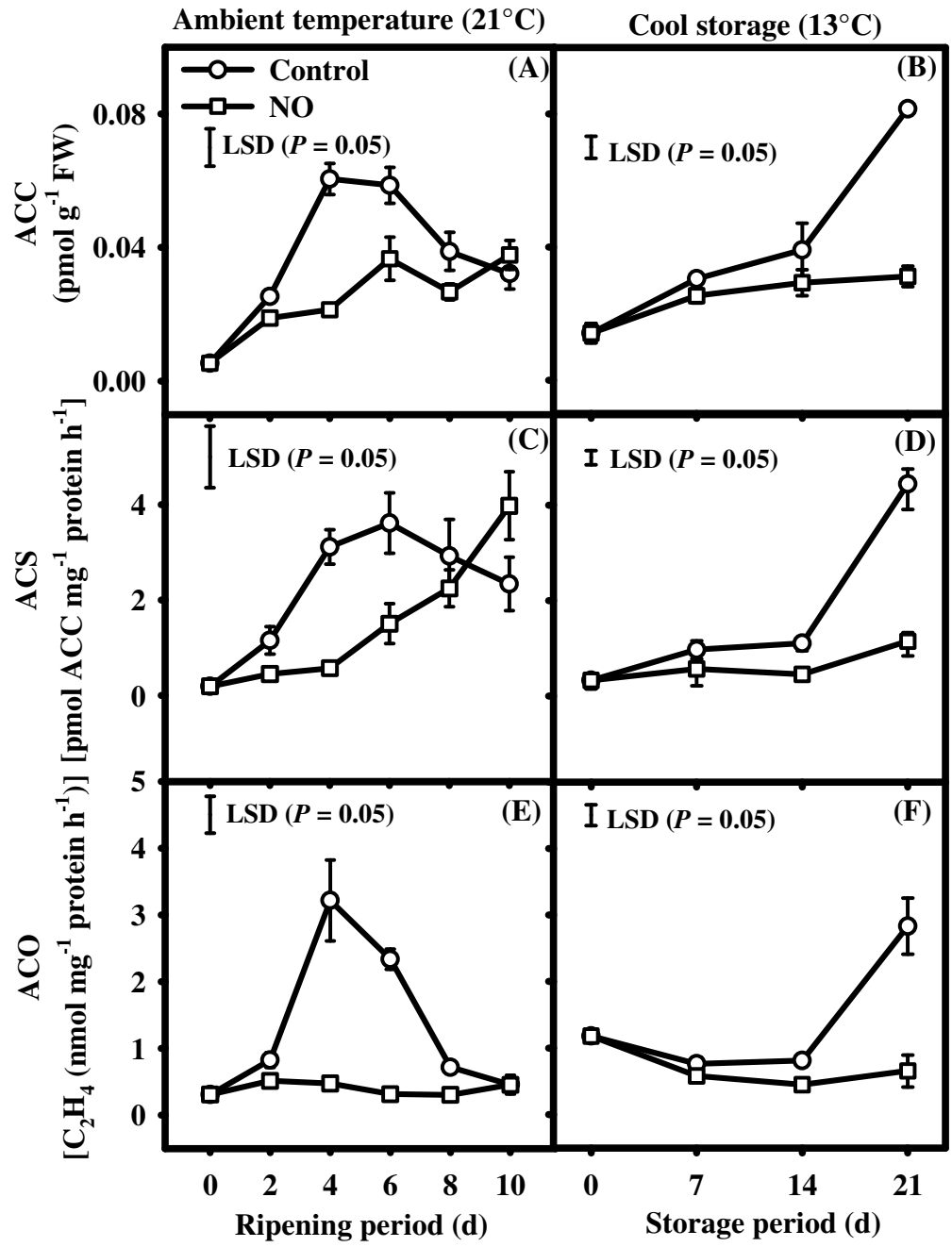

Figure 7.2. Ethylene biosynthesis enzymes, ACC content (A and B), ACS (C and D) and ACO enzymes (E and F) as influenced by NO fumigation (T) and ripening period (RP) at ambient temperature and during cool storage (SP).
Vertical bars represent S.E. of means and are invisible when the values are smaller than the symbol. n = 3, LSD (***, **, * represents significantly different at $P \leq 0.001$, 0.01, 0.05 and NS = not significant. ACC content during ripening: T = 0.005***, RP = 0.008***, T × RP = 0.01***; and cold storage: T = 0.006***, SP = 0.008***, T × SP = 0.01***; ACS activity during ripening: T = 0.53**, RP = 0.91*** , T × RP = 1.29**; and cold storage: T = 0.26***, SP = 0.36***, T × SP = 0.52*** and ACO activity during ripening: T = 0.23***, RP = 0.39***, T × RP = 0.55*** and cold storage: T = 0.27***, SP = 0.36***, T × SP = 0.55***.

7.3.4 Rheological properties of fruit pulp during fruit ripening and cold storage

Fruit firmness substantially declined in both NO-treated and control fruit with advancement of ripening (Table 7.1). NO-fumigated fruit showed significantly higher firmness, cohesiveness, springiness, chewiness, adhesiveness, and stiffness of the pulp during the ripening period when compared to the control (Table 7.1). Averaged over the ripening period, mean firmness, springiness, cohesiveness, chewiness, adhesiveness, and stiffness of pulp were also significantly ($P \leq 0.05$) higher (70.87 N, 0.09, 2.65 mm, 22.16 Nmm, 0.26 Nmm, and 2.04 kg f mm^{-1}) in NO-fumigated fruit than in the controls (52.17 N, 0.07, 2.26 mm, 15.09 Nmm, -0.01 Nmm, and 1.38 kg f mm^{-1}) respectively (Table 7.1).

As expected, pulp firmness declined in both NO-treated and control fruits with extension of the cool storage (Table 7.2). The NO-fumigated fruit exhibited 1.49- and 1.37-fold higher firmness than control fruit at the end of 2- and 3-week in cool storage, respectively (Table 7.2). NO-fumigated fruit maintained higher ($P \leq 0.05$) cohesiveness, chewiness, adhesiveness and stiffness of fruit pulp up to 7, 7 - 14, 7 – 21, and 7 - 21 days of storage, respectively, than the controls (Table 7.2). NO treatment did not significant ($P \leq 0.05$) affect pulp springiness after 21 days of storage (Table 7.2). Averaged over the storage period, mean cohesiveness, chewiness, adhesiveness and stiffness of fruit pulp were 1.11-, 1.16-, 7.84-, and 1.25-fold higher respectively in NO-fumigated fruit than in control fruit (Table 7.2). The interaction between NO fumigation treatment and storage period were found to be significant for pulp cohesiveness, chewiness, adhesiveness, and stiffness (Table 7.2).

Table 7.1. Changes in rheological properties of fruit pulp as influenced by NO fumigation during ripening at 21°C.

Ripening period (d)	HD (N)		CH (-)		SP (mm)		CHE (Nmm)		AD (Nmm)		ST (kg f mm^{-1})	
	Control	NO	Control	NO	Control	NO	Control	NO	Control	NO	Control	NO
0	186.8a	186.8a	0.097a	0.097b	3.8a	3.8a	62.2a	62.2a	0.08a	0.08c	4.8a	4.8a
2	85.6bB	149.5bA	0.082bB	0.107aA	2.9bB	3.5bA	23.5bB	54.8bA	-0.09cB	0.19bcA	2.3bB	4.4bA
4	15.2cB	47.7cA	0.058cdB	0.088bcA	1.9cB	2.6cA	2.1cB	9.9cA	-0.08cB	0.30abA	0.5cB	1.5cA
6	10.0cB	19.0dA	0.051dB	0.072dA	1.6dB	2.0dA	1.0cB	2.7dA	0.02abB	0.34abA	0.3cdB	0.7dA
8	9.0cB	13.0dA	0.057cdB	0.082cA	1.7dB	2.0dA	1.0cB	1.9dA	0.03abB	0.31abA	0.3cdB	0.5dA
10	6.5cB	9.2dA	0.059cB	0.083cA	1.6dB	2.0dA	0.7cB	1.4dA	-0.03bcB	0.36aA	0.2dB	0.4dA
Mean	52.17B	70.87A	0.07B	0.09A	2.26B	2.65A	15.09B	22.16A	-0.01B	0.26A	1.38B	2.04A
LSD of means at $P \leq 0.05$ level and levels of significance for a two-factor ANOVA												
T	4.13***		0.003***		0.07***		2.59***		0.05***		0.13***	
RP	7.15***		0.006***		0.11***		4.48***		0.08***		0.22***	
T × RP	13.06***		0.01***		0.21***		8.18***		0.15***		0.41***	

Means followed by the same lowercase letters within a column and means followed by the same uppercase letters within a row are not significantly different at $P \leq 0.05$ by the Least Significant Difference (LSD) with n = 30 (10 fruit × 3 replications). *** significant different at $P \leq 0.001$. RP = Ripening Period, T = Treatments and RP × T = Interaction between RP and T. HD = hardness, CH = cohesiveness, SP = springiness, CHE = chewiness, AD = adhesiveness, ST = stiffness and (-) = without unit.

Table 7.2. Changes in rheological properties of fruit pulp as influenced by NO fumigation during storage at 13°C.

Storage periods (d)	HD (N)		CH (-)		SP (mm)		CHE (Nmm)		AD (Nmm)		ST (kg f mm^{-1})	
	Control	NO	Control	NO	Control	NO	Control	NO	Control	NO	Control	NO
0	154.39a	154.39a	0.10a	0.10b	3.55a	3.55a	53.95a	53.95a	0.28a	0.28b	4.08a	4.08a
7	30.36b	39.47b	0.1aB	0.13aA	3.22b	3.26b	5.68bB	13.32bA	0.01bB	0.69aA	0.77bB	1.73bA
14	7.97cB	11.9cA	0.09a	0.09b	2.5c	2.74c	2.19cB	4.4cA	-0.02bcB	0.35bA	0.25cB	0.42cA
21	6.7cB	9.15cA	0.06b	0.07c	2.07d	1.99d	1.37c	1.47c	-0.07cB	0.24bA	0.2cB	0.39cA
Mean	49.86	53.73	0.09B	0.10A	2.84	2.89	15.80 B	18.28A	0.07B	0.39A	1.33B	1.66A
LSD of means at $P \leq 0.05$ level and levels of significance for a two-factor ANOVA												
T	NS		0.01^{***}		NS		2.90^{***}		0.09^{***}		1.98^{***}	
SP	5.74^{***}		0.01^{***}		0.18^{***}		2.05^{*}		0.13^{***}		0.20^{***}	
T × SP	NS		0.007^{***}		NS		2.05^{*}		0.09^{***}		0.14^{***}	

Means followed by the same lowercase letters within a column and means followed by the same uppercase letters within a row are not significantly different at $P \leq 0.05$ by the Least Significant Difference (LSD) with n = 30 (10 fruit × 3 replications). *, **, *** significant different at $P \leq 0.05$, 0.01, 0.001, respectively and NS = not significant. SP = Storage Period, T = Treatments and SP × T = Interaction between SP and T. HD = hardness, CH = cohesiveness, SP = springiness, CHE = chewiness, AD = adhesiveness, ST = stiffness and (-) = without unit.

7.3.5 Activity of exo-PG during fruit ripening and cool storage

NO fumigation reduced the activity of *exo*-PG in the pulp up to 6 days ripening period than control (Figure 7.3A). On 8 and 10 days of ripening period, the activity of *exo*-PG in the pulp of NO-fumigated was higher than in the control. The peak of *exo*-PG activity in the pulp of NO-fumigated fruit occurred 2 days after the control during ripening.

NO fumigation was effective in reducing the activity of *exo*-PG in the fruit pulp during 7 days of cool storage. During 14 days of cool storage, activity of *exo*-PG did not differ significantly (Figure 7.3B), but the activity of *exo*-PG continued to increase in the pulp of NO-fumigated fruit. The activity of *exo*-PG peaked 14 days later in NO-fumigated fruit than in control fruit during cool storage. Averaged over the storage period, mean *exo*-PG activity in the fruit pulp was 1.15-fold higher in NO-fumigated fruit than in the control fruit. There was a significant interaction between NO fumigation treatment and ripening period as well as storage period for the activity of *exo*-PG in the pulp.

7.3.6 Activity of endo-PG during fruit ripening and cool storage

NO fumigation was effective in reducing the activity of *endo*-PG in the pulp for up to 4 days of ripening period (Figure 7.3C). The activity of *endo*-PG in the pulp substantially declined from 6 to 10 days during ripening, but the differences were not significant between NO-fumigated and control fruit (Figure 7.3C). The peak activity of *endo*-PG in the pulp of NO-fumigated fruit was significant ($P \leq 0.05$) 1.79-fold lower and appeared 2 days later than in the control treatment (Figure 7.3C). The activities of *endo*-PG in the pulp of both NO-fumigated and control fruit peaked 7 days after cool storage, and then substantially declined to similar level during 14 and 21 days of cool storage (Figure 7.3D).

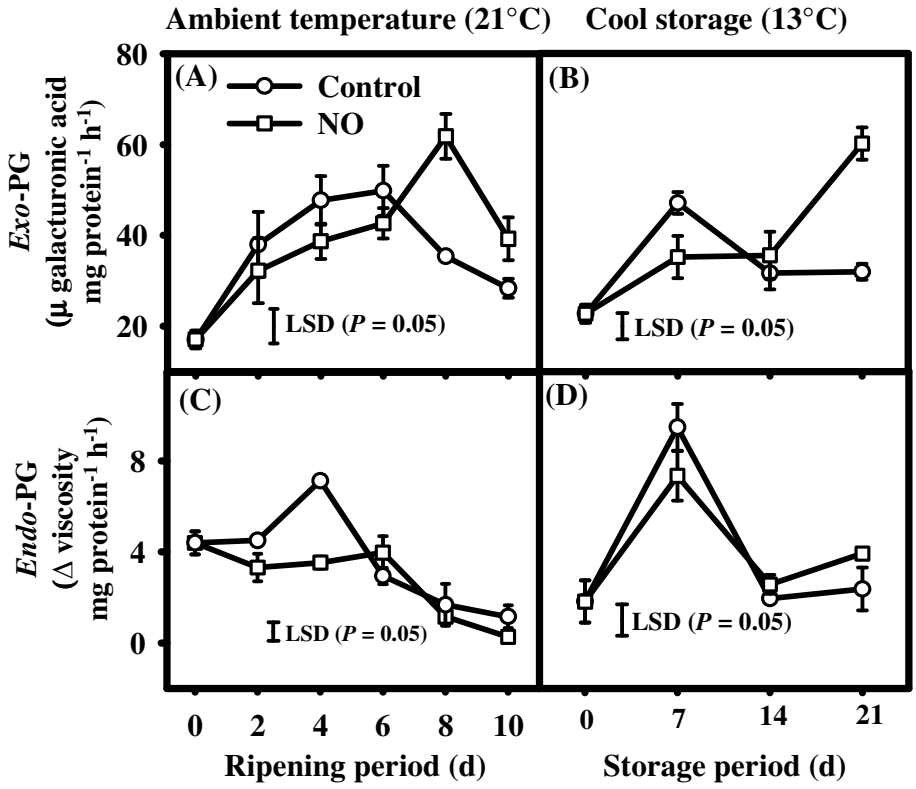

Figure 7.3. Fruit softening enzymes, *exo*-PG (A and B) and *endo*-PG, (C and D) as influenced by NO fumigation (T) and ripening period (RP) at ambient temperature and during cool storage (SP).

Vertical bars represent S.E. of means and are invisible when the values are smaller than the symbol. n = 3, LSD (***, **, * represents significantly different at $P \leq 0.001$, 0.01, 0.05 and NS = not significant. *Exo*-PG activity during ripening: T = NS, RP = 9.35^{***}, T × RP = 13.23^{***}; and cold storage: T = 5.02^{***}, SP = 7.10^{*}, T × SP = 10.04^{***}; *endo*-PG during ripening: T = 0.57^{**}, RP = 0.99^{***}, T × RP = 1.40^{**} and cold storage: T = 1.18^{***}, SP = NS, T × SP = NS.

7.3.7 Activity of PE during fruit ripening and cool storage

The activity of PE in the fruit pulp declined during ripening (2 - 10 days) and cool storage (21 days) in both NO-fumigated and control fruit (Figure 7.4A and 7.4B). NO-fumigated fruit exhibited significantly higher activity of PE in the pulp from the 2^{nd} to 10^{th} day of ripening when compared with the controls (Figure 7.4A). Averaged over the ripening period, mean PE enzyme activity was 1.33-fold higher in NO-fumigated fruit than in the control treatment.

NO-fumigated fruit exhibited significantly ($P \leq 0.05$) higher (1.46-, 2.06- and 1.82-fold) activities of PE in the pulp during 7, 14, and 21 days storage period, respectively, as compared with the controls (Figure 7.4B). Averaged over the 21 days storage period, mean PE activity was significantly ($P \leq 0.05$) higher (0.35 mM NaOH mg protein^{-1} h^{-1}) in NO-fumigated fruit than in the control treatment (0.26 mM NaOH mg protein^{-1} h^{-1}). The activity of PE in the pulp exhibited significant interaction between NO fumigation treatment and ripening period as well as storage period.

7.3.8 Activity of EGase during fruit ripening and cool storage

NO fumigation was effective in reducing the activity of EGase in the pulp for up to 4 days during the ripening period (Figure 7.4C). The activity of EGase in the pulp was significantly ($P \leq 0.05$) 2.97-fold lower on the 4^{th} day of the ripening period in NO-fumigated fruit than in the control fruit. The peak of EGase activity in the pulp of NO-fumigated fruit was 1.27-fold higher and appeared four days later than in the controls treatment (Figure 7.4C).

NO fumigation suppressed the activity of EGase in the pulp during 7 to 14 days of cool storage (Figure 7.4D). The peak of EGase activity in the pulp of NO-fumigated fruit appeared 14 days later and it was 1.06-fold lower than in the controls treatment (Figure 7.4D). The activity of EGase in the pulp exhibited significant interaction between NO fumigation treatment and ripening period as well as storage period.

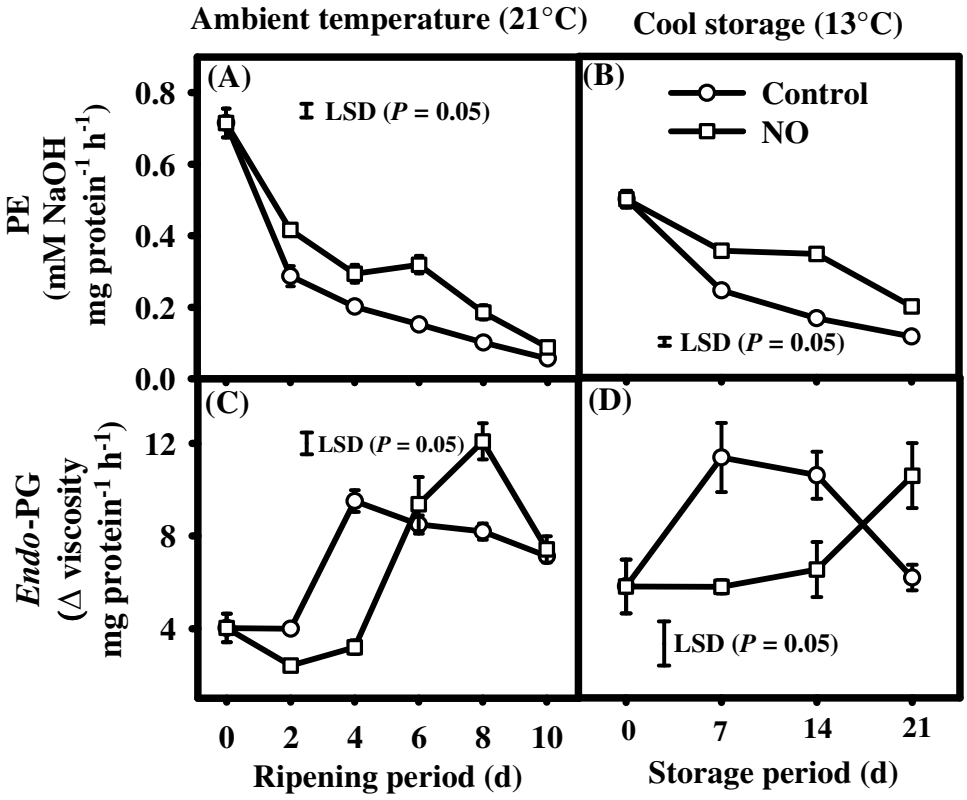

Figure 7.4. Activities of fruit softening enzymes, PE (A and B) and EGase (C and D) as influenced by NO fumigation (T) and ripening period (RP) at ambient temperature and during cool storage (SP).
Vertical bars represent S.E. of means and are invisible when the values are smaller than the symbol. n = 3. LSD (***, **, * represents significantly different at $P \leq 0.001$, 0.01, 0.05 and NS = not significant. PE activity during ripening: T = 0.03^{***}, RP = 0.05^{***}, T × RP = 0.07^{*} and cold storage: T = 0.02^{***}, SP = 0.03^{***}, T × SP = 0.04^{***}; EGase activity during ripening: T = NS, RP = 1.16^{***}, T × RP = 1.63^{***} and cold storage: T = NS, SP = NS, T × SP = 2.82^{**}.

7.4 Discussion

7.4.1 Ethylene production and activities of ethylene biosynthesis enzymes

Postharvest NO fumigation (20 μL L^{-1}) reduced ethylene biosynthesis in 'Kensington Pride' mango during ripening at ambient temperature (21 ± 1°C) and cool storage (13 ± 1°C) (Figure 7.1A and 7.1B). Reduction in ethylene biosynthesis in NO-fumigated fruit during ripening may be ascribed to suppression of ACO activity (Figure 7.2E) as well as reduced activity of ACS and lowered ACC content in fruit pulp (Figure 7.2A and 7.2C). The activity of ACO in NO-treated fruit was substantial lower than control fruit but may be adequate for converting ACC to ethylene (Figure 7.2E) as also reported earlier in broccoli following high temperature treatment (Suzuki et al., 2005). The reduction in ethylene biosynthesis in NO-fumigated mango during cool storage may be attributed to the suppression of ACS and ACO activities as well as reduced ACC content (Figure 7.2B, 7.2D, and 7.2F). NO fumigation may have blocked the activity of ACO through down regulation of the expression of the ACO gene in the fruit pulp during ripening. The suppression in ACS and ACO activities in the fruit pulp during cool storage may be ascribed to the down-regulation of expression of genes coding for both ACS and ACO enzymes with NO fumigation treatment, and low temperature, during storage. Similarly, the inhibition in ethylene production in NO-treated banana slices has been suggested to be due to down-regulation of *MA-ACS1*, *MA-ACO1* (Cheng et al., 2009) as well as *MaERS2* and *MaExp1* gene (Yang et al., 2010), leading to delayed ripening. The delayed accumulation of ACO in NO-fumigated tomato fruit has been ascribed to the delayed expression of *LeACO1*, *LeACOH2*, and *LeACO4* genes in both mature green and colour break stage fruit (Eum et al., 2009). The reduction in ethylene production in NO-fumigated mango fruit during ripening may be due to binding of NO with ACO and ACC to form a stable ternary ACC-ACO-NO complex, thus limiting ethylene production as has been proposed by Manjunatha et al. (2010). Reduction in ethylene production during ripening or cool storage has been reported in various fruits treated with NO, including mango (Zaharah and Singh, 2011b; c), plum (Singh et al., 2009), banana (Cheng et al., 2009), peach (Flores et al., 2008; Liu et al., 2007; Zhu et al., 2006), pear (Sozzi et al., 2003), tomato (Eum et al., 2009), kiwifruit (Zhu et al., 2010b), and strawberry (Zhu and Zhou, 2007).

NO fumigation reduced ACC content in the fruit pulp during ripening and storage period when compared with the control, leading to a suppressed, and delayed the climacteric peak of ethylene production (Figure 7.1A and 7.1B). The reduction in ACC content in pulp tissues of NO-fumigated fruit seems to be associated with reduced activity of ACS in the pulp (Figure 7.2C and 7.2D). The reduction in ACC content due to reduced activity of ACS in NO-fumigated strawberry and peach fruit has also been reported by Zhu and Zhou (2007), and Liu et al. (2007).

7.4.2 Respiration rate

NO fumigation reduced the rate of respiration in mango fruit during ripening and storage, but the reduction was more pronounced during ripening. The differential reduction in fruit respiration rates during ripening and storage with NO fumigation (Figure 7.1C and 7.1D) may be ascribed to the corresponding reduction in ethylene biosynthesis (Figure 7.1A and 7.1B). It may also be argued that NO fumigation reduced respiration rate in mango fruit by inhibiting the activity of cytochrome oxidase. Millar and Day (1996) reported that NO inhibited cytochrome oxidase but not alternate oxidase in plant mitochondria. The suppression of respiration rate in NO–fumigated mango fruit during the first 4 days of ripening and 7 days cold storage suggests that NO fumigation is most effective in reducing cytochrome chain components of respiration rate during early ripening particularly before the climacteric burst of respiration rate rather than later in the post-climacteric phase. Cytochrome chain components have been reported to play an important role in facilitating the climacteric burst of respiration before the alternative oxidase and uncoupling proteins, which play a role in post-climacteric senescence in mango fruit (Considine et al., 2001). The suppression of respiration rate in NO-treated fruit during ripening has also been previously reported in mango (Zaharah and Singh, 2011c), plum (Singh et al., 2009), peach (Flores et al., 2008), strawberry (Zhu and Zhou, 2007), and tomato (Zhang et al., 2005).

7.4.3 Fruit softening and activities of fruit softening enzymes

Ironically, fruit firmness dictates storage and shelf life of a fruit, and it changes substantially during storage and ripening. Cell wall polymers such as pectin, cellulose and hemicellulose undergo substantial transformation and solubilisation during ripening which result in cell wall disintegration and softening of fruit. NO-

fumigated fruit exhibited higher firmness, cohesiveness, springiness, chewiness, adhesiveness, and stiffness of pulp during ripening and cool storage (Table 7.1 and 7.2). The reduced loss of pulp firmness, along with other rheological properties in NO-fumigated fruit, may be attributed to the suppressed and delayed the ethylene production (Figure 7.1A and 7.1B), reduced ACC content, activities of ACS and ACO (Figure 7.2A – 7.2F), as well as reduced activities of softening-related enzymes such as *exo*-PG, and EGase enzymes (Figure 7.3A -7.3B and 7.4C - 7.4D) in mango fruit during ripening and cold storage. Similarly, the application of 1-MCP significantly delayed and suppressed the climacteric ethylene production with reduction in the activities of ethylene biosynthesis enzymes (ACS, ACO) and ACC content, and the activities of fruit softening enzymes (EGase, *exo*- and *endo*-PG) in the skin as well as in pulp tissues of Japanese plum fruit (Khan and Singh 2007). Reduced fruit softening in NO-fumigated mango during ripening or storage at chilling temperature, has previously been reported in mango (Zaharah and Singh, 2011c), kiwifruit (Zhu et al., 2010b), plums (Singh et al., 2009; Zhang et al., 2008), banana slice (Cheng et al., 2009) and peaches (Flores et al., 2008; Zhu et al., 2006).

PG is responsible for degrading the (1-4)-linked galacturonic acid residues, and has been reported in mango fruit during ripening (Abu-Sarra and Abu-Goukh, 1992). PG can exist in two forms either as *exo*- or *endo*-PG. In mango, *exo*-PG seems to dominate and the activity of *exo*-PG increases as the fruit ripen (Ali et al., 2004). NO-fumigated fruit showed suppressed activity of *exo*- and *endo*-PG up to 6 and 4 days during ripening, respectively (Figure 7.3A and 7.3C) as well as 7 days of cool storage (Figure 7.3B and 7.3D), and that delayed fruit softening. The suppressed activities of *exo*- and *endo*-PG during mango fruit ripening and cool storage with NO treatment coincides with decreased loss of fruit firmness and changes in rheological properties of pulp (Table 7.1 and 7.2), which inturn, may be attributed to suppressed and delayed ethylene production (Figure 7.1A and 7.1B). The higher activity of *exo*- than *endo*-PG in mango fruit pulp during ripening and cool storage (Figure 7.1A and 7.1B) suggest that the former enzymes play a key role in mango fruit softening as also reported by Ali et al. (2004). There were direct significant ($P \leq 0.001$ and 0.05) negative linear correlations (r = -071 and r = -0.68) between fruit firmness and the activity of *exo*-PG during fruit ripening and cold storage, respectively. NO-treated

peach fruit have also been reported to exhibit reduced activity of PG and higher firmness during 20 days of cold storage (Zhu et al., 2010a).

PE activity in the pulp of 'Kensington Pride' mango fruit irrespective of NO treatment declined with advancement of fruit ripening and extension of storage period (Figure 7.4A and 7.4B). Similarly, the activities of PE have been reported to decrease with progression of fruit ripening and extension of the cool storage in various cultivars of mango such as 'Kitcher', 'Dr Knight' (Abu-Sarra and Abu-Goukh, 1992), 'Nam Dok Mai' (Ketsa and Daengkanit, 1999), 'Chiin Hwang No. 1' (Ueda et al., 2001) and 'Keitt' (Roe and Bruemmer, 1981). NO fumigation delayed the decline in the activity of PE in fruit pulp during ripening and 21 days cold storage period (Figure 7.4A and 7.4B). It is well known that PE catalyses the de-esterification of pectin into pectate and methanol. Higher PE activity in NO-fumigated fruit could probably be due to interaction between pectate with free calcium to form stronger cell wall in the pulp of mango fruit. NO-fumigated fruit with higher PE activity also had higher level of pulp firmness regardless of ripening or cool storage. Similarly, higher activity of PE was found in apple fruit on harvest day and it decreased during ripening and softening (Goulao et al., 2007). In pears, after 5 months of cold storage the expression of encoding PE gene (*PcPME3* and *PcPME4*) reduced with ripening and softening (Sekinea et al., 2006). Recently, low oxygen stress or 1-MCP which delays ethylene and softening in apple caused increase in PE expression after 4 months of cold storage (Pesis et al., 2010). Previously, NO treatment had been reported to suppress PE activity in banana slices during ripening (Cheng et al., 2009). There were also significant ($P \leq 0.001$) positive linear correlations ($r = 0.90$ and $r = 0.86$) between PE activity and fruit firmness during ripening and cool storage, respectively. Roe and Bruemmer (1981) also reported a significant positive correlation between loss of fruit softening and activity of PE during fruit ripening.

Endo-1,4-β-D-glucanase (EGase) was one of the major cell wall hydrolyses and contributed to mango fruit softening during the later stages of ripening (Chourasia et al., 2008). The activity of EGase has been reported to increase during ripening or cool storage in various mango varieties (Abu-Sarra and Abu-Goukh, 1992; Ketsa and Daengkanit, 1999; Roe and Bruemmer, 1981). The activity of EGase in pulp of 'Kensington Pride' mango fruit increased with the advancement of

ripening and extended cool storage (Figure 7.4C and 7.4D). NO fumigation reduced the activity of EGase up to the 4th day during ripening, and the first 7 days of cool storage, respectively (Figure 7.4C and 7.4D). The reduction in EGase activity may be ascribed to delayed and suppressed ethylene production during fruit ripening at ambient and cool storage due to the NO treatment that down-regulated the *MiCel1* gene encoding for EGase. Recently, Chourasia et al. (2008) claimed that expression of the *MiCel1* gene was ripening related, and enhanced the activity of EGase during ripening and contributed towards softening in mango fruit during the later stages of ripening. NO treatment had also been reported to reduce the activity of EGase in banana slices during ripening at 24°C (Cheng et al., 2009).

In conclusion, NO fumigation suppressed ethylene biosynthesis through inhibition of ACS and ACO activities leading to reduced ACC content in the pulp of 'Kensington Pride' mango fruit that subsequently reduced the activities fruit softening enzymes such as *exo*-, *endo*-PG as well as EGase during ripening and cool storage.

CHAPTER 8

Postharvest nitric oxide fumigation alleviates chilling injury, delays fruit ripening and maintains quality in cold stored 'Kensington Pride' mango

Summary

The effects of postharvest nitric oxide (NO) fumigation (0, 5, 10, 20, and 40 μL L^{-1}) on chilling injury (CI), ripening and quality of mango (*Mangifera indica* cv. 'Kensington Pride') during fruit ripening at 21 ± 1°C was investigated. The fruit had been cold-stored at 5 ± 1°C for either 2- or 4-week. NO treatments (10, 20, and 40 μL L^{-1}) significantly alleviated CI index in cold stored fruit during ripening. All NO fumigation treatments significantly suppressed ethylene production and respiration rates irrespective of cold storage period. NO fumigation of higher than 5 μL L^{-1} significantly delayed fruit softening up to 2 days and retarded colour development, and fumigation after 2- and 4-week of cold storage significantly delayed the increase in total sugar and fructose concentrations at the full-ripe stage. All NO fumigation treatments significantly increased tartaric and shikimic acid during ripening period in 2- and 4-week-cold-stored fruit, except for the 5 μL L^{-1} treatment in 2-week fruit. NO fumigation treatments did not significantly influence the concentrations of sucrose, glucose, total acid, citric, malic, fumaric, ascorbic acid, carotenoid in pulp and total antioxidant (in pulp and skin), as compared to non-fumigated fruit in both storage periods. Averaged over NO concentrations, 2-week of cold storage resulted in significantly higher mean concentration of total sugar, sucrose, fructose, tartaric acid, malic acid, total carotenoid and total antioxidant (skin) than in 4-week stored fruit and the trend was reverse for total acids, citric, shikimic, and fumaric acid. In conclusion, the postharvest application of NO (10, 20, and 40 μL L^{-1}) alleviated CI

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index, delayed fruit colour development, softening and ripening as well as maintained quality through reduction of ethylene production and respiration during ripening in 2- and 4-week cold-stored fruit.

8.1 Introduction

Mango fruit are highly susceptible to chilling injury (CI) when stored at the temperatures below 13°C (Nair and Singh, 2003). CI limits the exploitation of cold storage in extending storage life of mango. CI symptoms on mango fruit are expressed as skin discoloration particularly blackening, sunken lesions on the peel, lenticels spotting, uneven ripening, poor colour, reduced aroma, flavour, and carotenoid, and increase susceptibility to decay (Nair and Singh, 2009; Nair et al., 2003; Phakawatmongkol et al., 2004). CI impairs the cell structure including membrane and other organelles as well as modulating ethylene production, enhancing respiration, ethanol and acetaldehyde production, peroxidase and cellulase activities, increases the level of glutathione and phenolic compounds, and consequently hinders fruit ripening and in mango fruit (Nair and Singh, 2009; Nair et al., 2004a; Zauberman et al., 1988; Zhou et al., 2006).

Various approaches have been tested to ameliorate CI in mango fruit such as modified and controlled atmosphere storage (Pesis et al., 2000), heat treatment (Nair et al., 2001), intermittent warming (Zhang et al., 1997), cold-shock treatment (Zhao et al., 2006), application of ethrel (Nair and Singh, 2003), polyamines (Nair et al., 2004a), methyl jasmonte (Gonzalez-Aguilar et al., 2000), oxalic or salicylic acid (Ding et al., 2007), and 2,4-D (Wang et al., 2008a) prior to storage.

Chill injured mango fruit exhibited reduced ethylene production due to lower activities of 1-aminocyclopropane-1-carboxylic acid (ACC) synthase (ACS) and ACC oxidase (ACO) consequently failed to ripen (Nair et al., 2003). Exogenous application of ethrel prior to storage alleviated the CI development in 'Kensington Pride′ mangoes at 5°C for 28 days (Nair and Singh, 2003). This suggests the involvement of ethylene biosynthesis in development of CI. It has also been argued that chilling temperature during storage induces oxidative stress through generation of reactive oxygen species (ROS) which causes damage to the membrane and consequently CI in mango fruit (Ding et al., 2007; Zhou et al., 2006).

Nitric oxide (NO) acts as a multifunctional signalling molecule in plants, involving in many physiological processes including ripening of climacteric and non-climacteric fruit (Leshem and Pinchasov, 2000; Leshem et al., 1998). Postharvest exogenous application of NO has been reported to delay fruit ripening in a range of climacteric or non-climacteric fruit such as banana, plum, tomato, strawberry, and kiwifruit through different mechanisms such as suppressed respiration rate, reduced ethylene biosynthesis and CI, delayed development of browning disorder, disease incidence and skin colour changes, flesh softening and reduced activity of softening enzymes (Ku et al., 2000; Manjunatha et al., 2010). Recently postharvest NO fumigation has been reported to alleviate CI in cold stored Japanese plum and peach fruit (Singh et al., 2009; Zhu et al., 2010b). However, no research work has been reported on the effects of NO fumigation on development of CI during fruit ripening following cold storage in mango and other tropical fruits.

I hypothesized that NO fumigation may alleviate CI symptoms in cold-stored mango fruit and delay fruit ripening. These observations prompted investigating the effects of different concentrations of NO fumigation on CI, ethylene production, respiration rate, and fruit quality in 'Kensington Pride' mangoes during fruit ripening at ambient temperature following 2- and 4-week of cold storage at chilling temperature of 5 ± 1°C. To my best knowledge, this is the first report on the NO fumigation on alleviation of CI and extending storage life in mango.

8.2 Materials and methods

8.2.1 Plant materials

Hard mature (green skin and light cream pulp) mango fruit (*Mangifera indica* L. cv. 'Kensington Pride′) were obtained from a commercial orchard located at Carnarvon (lat. 24°52´S; long. 113°38´E), Western Australia (WA). The fruit were firm (139 ± 6.08 N), produced ethylene at 0.06 ± 0.01 nmol C_2H_4 kg^{-1} h^{-1} and had a respiration rate of 1.6 ± 0.04 mmol CO_2 kg^{-1} h^{-1}. They were desapped, fungicide-treated (Sportak 0.55 mL L^{-1} with Prochloraz as an a.i.), air dried, packed in soft-board trays, and transported to Perth, WA, by a refrigerated truck (13°C). Fruit of uniform size, free from visual blemishes and diseases were used for the experiments.

8.2.2 NO fumigation treatments and experimental design

Fruit were fumigated with different concentration of NO (0, 5, 10, 20, and 40 μL L^{-1}) in a sealed plastic container (67 L). Different concentrations of NO were obtained from a cylinder containing 4, 810 ± 100 μL L^{-1} NO in N_2 (BOC Gases Ltd., Sydney, NSW, Australia) and injected into the container using 50 mL syringe through an injection port of the container lid. Fruit were fumigated in an atmosphere containing NO for 2 h at ambient temperature (21 ± 1°C). The O_2 gas in the container was not depleted because NO has been reported to be sufficiently stable at the low concentrations and short treatment times was required for the fruit to be treated in normal air (Soegiarto et al., 2003). Control fruit were placed in the plastic container with the same duration of incubation without any NO treatment.

The fruit fumigated with different concentrations of NO were stored at 5 ± 1°C, 93.81 ± 2.02% RH. After 2- and 4-week, 30 fruit of each treatment (3 replications and 10 fruit per replication) were transferred from the cold storage to ambient temperature (21 ± 1°C, 61.3 ± 6.2% RH) and allowed to ripen. The changes in temperature and RH during cold storage and ripening at ambient were recorded as detail mentioned in Chapter 3, Section 3.3. CI, ethylene production, respiration rate, fruit firmness and visual colour were determined daily during ripening period. Fruit quality such as concentrations of sugars and organic acids were also determined at eating soft ripe fruit. The experiment was completely randomised with two factors, NO fumigation and storage period.

8.2.3 Chilling injury (CI) index

The incidence of CI was assessed everyday during fruit ripening period following 2- and 4-week of cold storage. CI index was determined using a rating scale ranging from 0 to 4 based on its common visual symptoms of dark coloration and prominence of lenticels on the skin area of fruit affected. The scale used was: 0 = no damage; 1 = very light damage; 2 = light damage; 3 = moderate damage and 4 = severe damage, as previously described by Nair et al. (2004b). CI index was calculated by following the formula: [Σ (A×B)/5C], in which A represents the injury score of individual fruit, B is the number of fruit affected, 5 is the total number of score (0 - 4) used and C is the total number of fruit recorded.

8.2.4 Ethylene production

Mango fruit were sealed in an airtight jar (1, 000 mL) fitted with a rubber septum for one h at ambient temperature (21 ± 1°C). One mL of headspace gas sample was injected into a gas chromatograph (6890N Network GC system; Agilent Technology, Palo Alto, CA^2, USA) fitted with a 2 m-long stainless steel column (Porapaq-Q, 3.18 mm, 80/100 mesh size; Supelco, Bellefonte, PA, USA) and a flame ionisation detector (FID) as explained in detail in Chapter 3, Section 3.4.2. Ethylene production rate was expressed as nmol C_2H_4 kg^{-1} h^{-1}. All the estimations were performed twice.

8.2.5 Respiration rate

One mL of headspace gas sample was taken using a syringe through rubber septum from the same jar used for measurement of ethylene production as explained in Section 8.2.4, prior injection into an infrared gas analyser [Servomex Gas Analyzer, Analyzer series 1450 Food Package Analyzer, Servomex (UK) Ltd., East Sussex, UK] as explained in Chapter 3, Section 3.5 and expressed as mmol CO_2 kg^{-1} h^{-1}.

8.2.6 Fruit quality

8.2.6.1 Visual skin colour

The visual assessment of skin colour was estimated daily during ripening period by following a rating scale ranging from 1 to 5 as previously described by Dang et al. (2008a) and detail explained in Chapter 3, Section 3.6.1.1.

8.2.6.2 HunterLab ColourFlex

Fruit colour was also recorded from opposite positions of each fruit in Commission Internationale de L´ Eclairage (CIE) units using a ColorFlex 45°/0° Spectrophotometer (HunterLab ColorFlex, Hunter Associates Inc, Reston, VA, USA) as L*, a*, and b* colour coordinates on the fruit surface and chroma value (C*) and hue angel (hº) were determined as explained in Chapter 3, Section 3.6.1.2.

8.2.6.3 Determination of carotenoid

Total carotenoid from the pulp of ripe mango fruit were determined according to the methods previously described by Lalel (2002) as outlined in Chapter 3, Section 3.6.7.

8.2.6.4 Fruit softness

Daily softness of individual fruit from each replication was subjectively assessed using a rating scale of 1 to 5 as described in Chapter 3, Section 3.6.2.1.

8.2.6.5 Determination of sugars and organic acids

Mango pulp (~1 g) was extracted for determination of soluble sugars and organic acids. Following centrifugation at 10, 000 × *g* for 15 min at 15°C the supernatant was diluted with Milli Q water to 50 mL subsequently filtered through 0.2 µm nylon syringe filter [Alltech Associates, Ltd., NSW, Australia] and loaded into the 1.0 mL glass vial. The individual sugars and organic acids were quantified using reversed-phase high performance liquid chromatography (RP-HPLC system, Waters, Milford, MA, USA) as outlined in Chapter 3, Section 3.6.3. The concentrations of sucrose, glucose, fructose and citric acid were expressed as g 100 g^{-1} FW, whilst tartaric, malic, shikimic, fumaric, shikimic were expressed as mg 100 g^{-1} FW basis.

8.2.6.6 Total antioxidant

The level of total antioxidant in mango skin or pulp tissue of ripe fruit was determined as detail procedure outlined in Chapter 3, Section 3.6.8. Total antioxidant was expressed as mM Trolox Equivalent Antioxidant Activity (TEAC) 100 g^{-1} FW basis.

8.2.6.7 Ascorbic acid

Ascorbic acid was determined according to the detail method explained in Chapter 3, Section 3.6.9. The sample was read at 760 nm wavelength using a UV-VIS Spectrophotometer (Jenway Spectrophotometer Model 6405, Dunmow, Essex, UK). Ascorbic acid concentration was quantified using a standard curve of L-ascorbic acid and was expressed as mg 100 g^{-1} FW basis.

8.2.7 Statistical analysis

The experimental data were subjected to two-way (NO fumigation × ripening period) analysis of variance (ANOVA) using SAS (release 9.1.3, SAS Institute Inc., Cary, NC, USA). The effects of various treatments and ripening period or treatments and storage period were assessed using two-way ANOVA. Fisher's Least Significant

Differences (LSD) were calculated following a significant ($P \leq 0.05$) F-test. All the assumptions of ANOVA were checked to ensure validity of statistical analysis.

8.3 Results

8.3.1 CI index

The severity of CI increased during fruit ripening at ambient temperature following cold storage irrespective of NO treatments (Figure 8.1A and 8.1B). All NO fumigation treatments except 5 μL L^{-1} reduced CI index during fruit ripening in 2-week cold-stored fruit than control (Figure 8.1A). Fruit treated with 10 μL L^{-1} NO exhibited lowest CI at fully ripe following 2-week cold storage as compared to all other treatments. In 4-week cold-stored fruit, all NO fumigation treatments reduced CI index on 5 and 6 days of ripening as compared to control. CI index at fully ripe stage of 4-week stored-fruit was lowest with 40 μL L^{-1} NO treatment as compared to all others (Figure 8.1B). The interaction between NO fumigation treatments and ripening period was significantly affected the CI index during fruit ripening irrespective of storage.

8.3.2 Ethylene production

NO fumigation had significantly ($P \leq 0.001$) suppressed climacteric ethylene peak during fruit ripening irrespective of 2- or 4-week cold storage period (Figure 8.2A and 8.2B). Climacteric ethylene peak was suppressed by 1.26-, 1.66-, 2.13-, and 3.18-fold with 5, 10, 20, and 40 μL L^{-1} NO fumigation respectively than control during fruit ripening following 2-week of cold storage (Figure 8.2A).

In 4-week cold-stored fruit, the climacteric ethylene peak during fruit ripening was suppressed by 1.27-, 1.49-, 2.61-, and 2.31-fold with 5, 10, 20, and 40 μL L^{-1} NO respectively, than the control fruit (Figure 8.2B). The interaction between NO fumigation treatments and ripening period significantly ($P \leq 0.001$) affected the ethylene production in fruit stored for 2-week only.

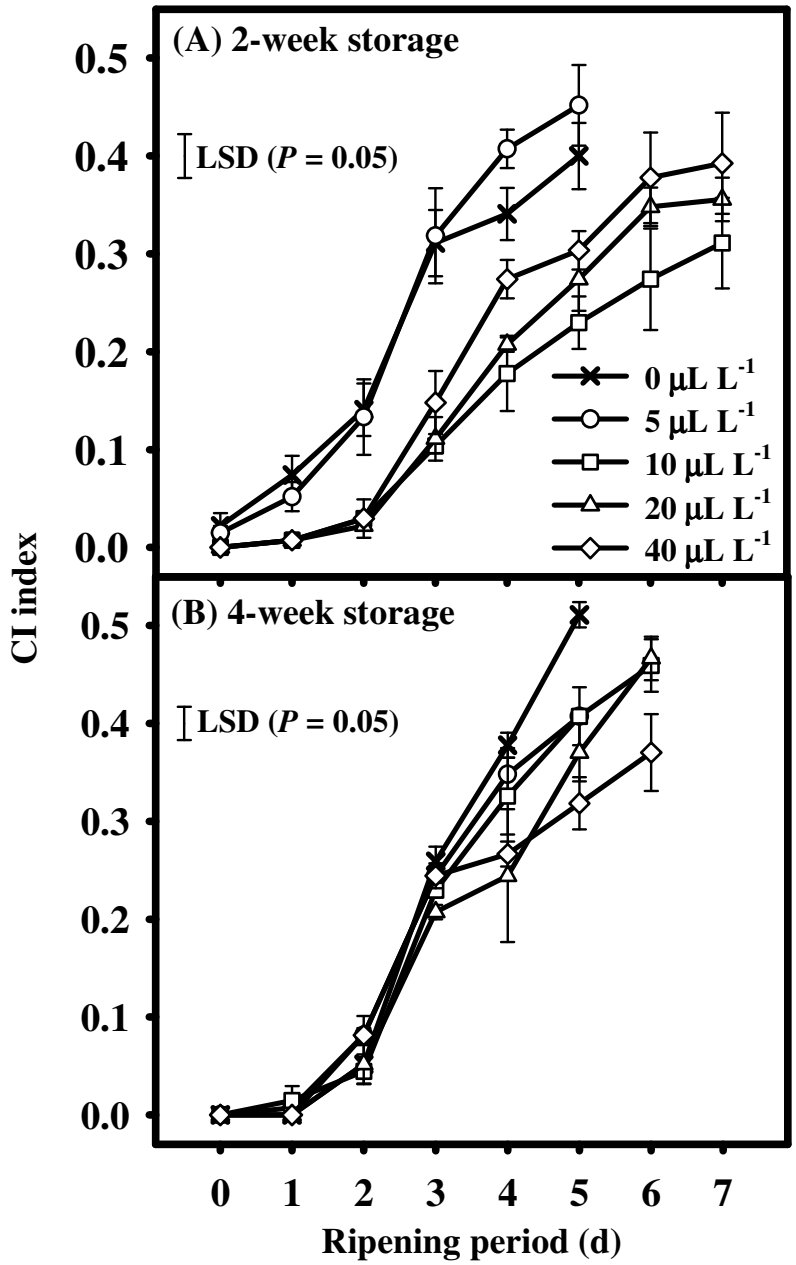

Figure 8.1. CI index as influenced by different concentrations of NO fumigation (T) and ripening period (RP) at ambient temperature following 2- (A) and 4-week of cold storage (B) of fruit at 5 ± 1°C.

n = 30 (10 fruit × 3 replications). Vertical bars represent S.E. of means and are invisible when the values are smaller than the symbol. LSD (*, **, *** significant at $P \leq 0.05$, 0.01, 0.001, respectively and NS = not significant): 2-week storage, T = 0.03^{***}, RP = 0.03^{***}, T × RP = 0.08^{**}; 4-week storage, T = 0.02^{***}, RP = 0.03^{***}, T × RP = 0.06^{**}.

8.3.3 Respiration rate

All NO fumigation treatments (10, 20, and 40 μL L^{-1}), except for the 5 μL L^{-1} treatment, significantly ($P \leq 0.01$) suppressed the respiratory climacteric peak by 16.1%, 18.1%, and 20.8% than control during fruit ripening following 2-week of cold storage (Figure 8.2C). However, the differences among these treatments in reducing the respiratory climacteric peak during ripening were not significant. In 4-week cold-stored fruit, NO treatments did not show any significant effect on the suppression of climacteric respiration peak during fruit ripening than control (Figure 8.2D). Fruit fumigated with 40 μL L^{-1} NO resulted in sharp declined after climacteric respiration peak as compared all other treatments. The interaction between NO fumigation and ripening period for respiration rate was found to be significant ($P \leq 0.01$) in 2-week cold-stored fruit only.

8.3.4 Fruit softness

NO fumigation treatments (10, 20, and 40 μL L^{-1}) resulted in lower rate of fruit softening as compared to 5 μL L^{-1} and control during ripening of 2-week cold-stored fruit (Figure 8.3A). At ripe eating stage, all NO-fumigated fruit following 2-week cold-stored did not show any significant difference in fruit softening as compared to control.

All NO fumigation treatments (except 5 μL L^{-1}) resulted in lower rate of fruit softening as compared to control during ripening (2 to 6 days) of 4-week cold-stored fruit (Figure 8.3B). Following 4-week cold storage, control and 5 μL L^{-1} NO treated fruit attained ripe eating soft stage at ambient temperature one day earlier as compared to all other treatments. The interaction between NO fumigation and ripening period was found to be significant ($P \leq 0.01$) for fruit softening in 2- and 4-week cold-stored fruit.

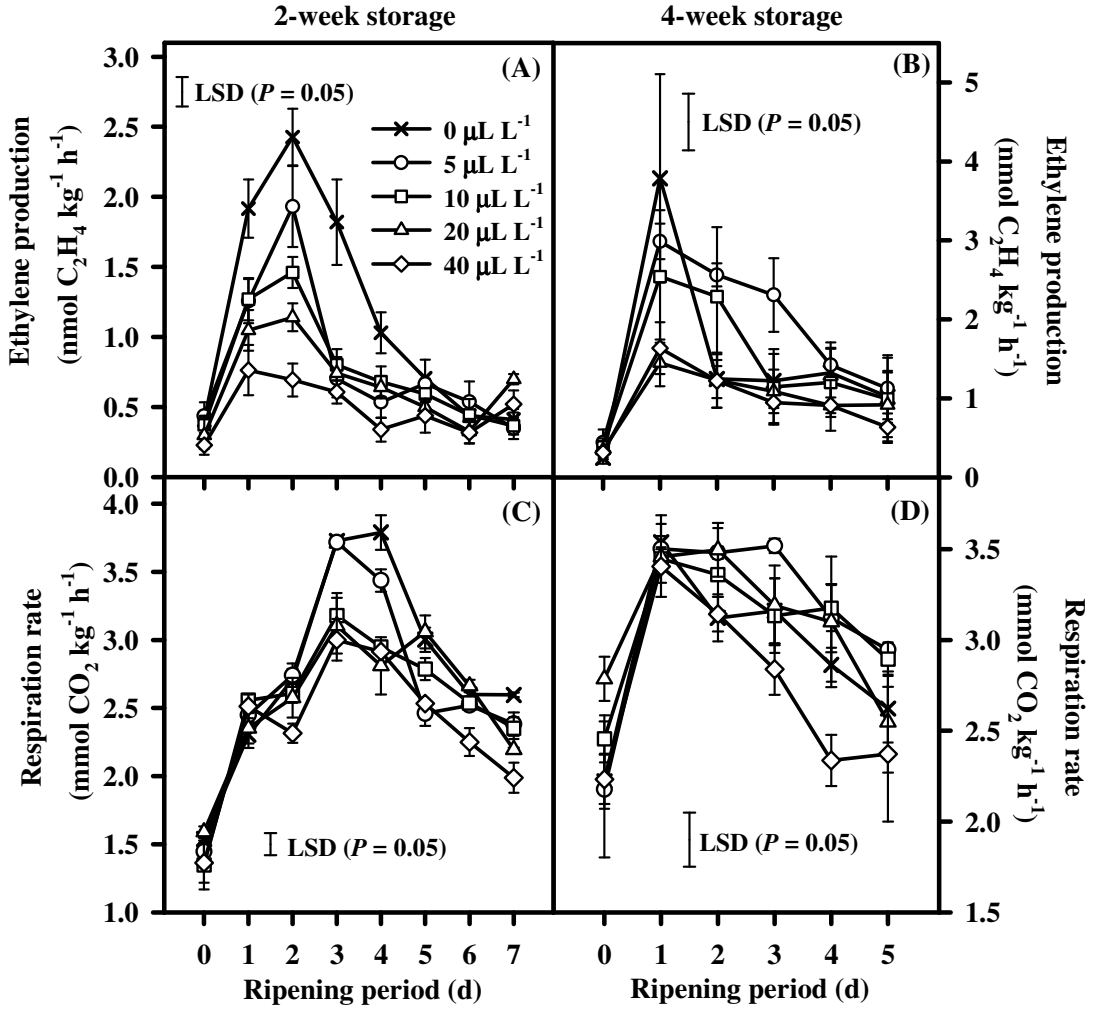

Figure 8.2. Ethylene production and respiration rates as influenced by different concentrations of NO fumigation (T) and ripening period (RP) at ambient temperature following 2- (A and C) and 4-week of cold storage (B and D) of fruit at 5 ± 1°C.

n = 6 (2 fruit × 3 replications). Vertical bars represent S.E. of means and are invisible when the values are smaller than the symbol. LSD (*, **, *** significant at $P \leq 0.05$, 0.01, 0.001, respectively) and NS = not significant): Ethylene (2-week storage), T = 0.13^{***}, RP = 0.16^{***}, T × RP = 0.36^{***}; (4-week storage) T = 0.51^{**}, RP = 0.56^{***}, T × RP = NS, Respiration (2-week storage), T = 0.10^{***}, RP = 0.12^{***}, T × RP = 0.14^{***}; (4-week storage), T = 0.21^{**}, RP = 0.23^{***}, T × RP = NS.

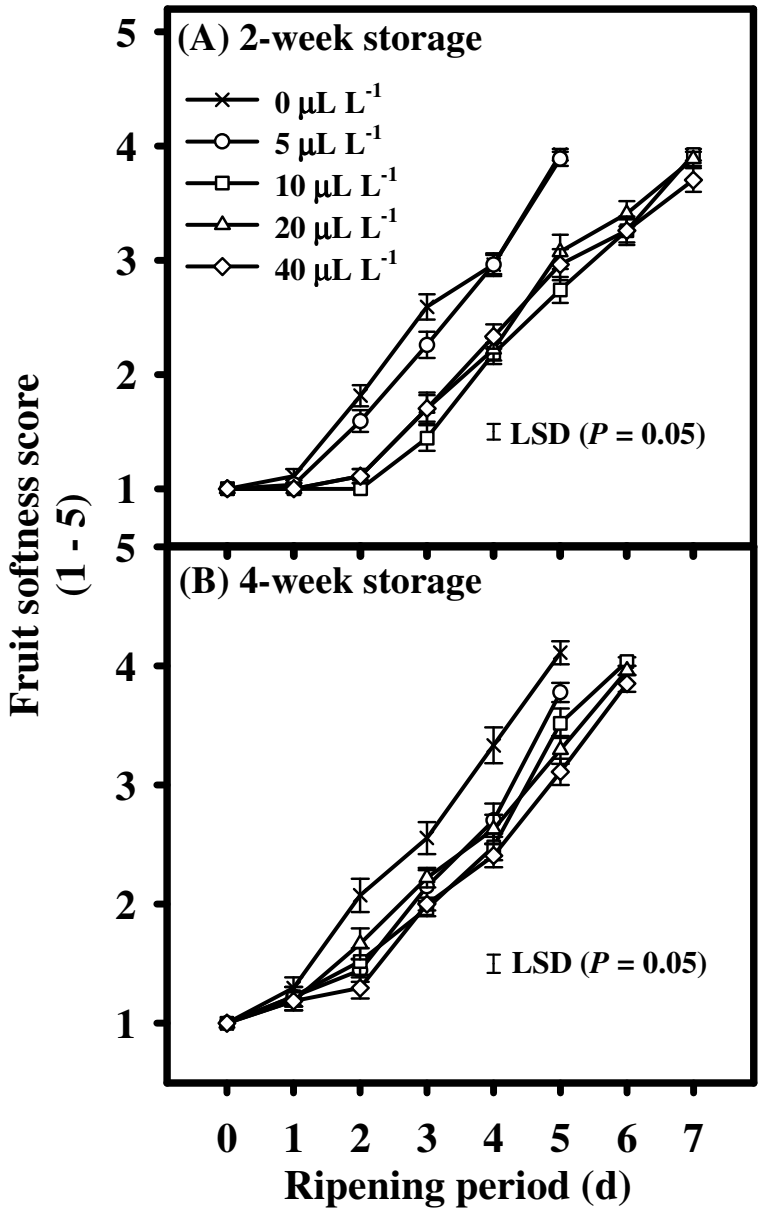

Figure 8.3. Fruit softness as influenced by different concentrations of NO fumigation (T) and ripening period (RP) at ambient temperature following 2- (A) and 4-week of cold storage (B) of fruit at 5 ± 1°C.
n = 30 (10 fruit × 3 replications). Vertical bars represent S.E. of means and are invisible when the values are smaller than the symbol. LSD (*, **, *** significant at $P \leq 0.05$, 0.01, 0.001, respectively and NS = not significant): 2-week storage, T = 0.08^{***}, RP = 0.11^{***}, T × RP = 0.12^{***}; 4-week storage, T = 0.10^{***}, RP = 0.12^{***}, T × RP = 0.13^{***}.

8.3.5 Skin colour

NO-fumigated fruit exhibited reduced chromaticity L*, a*, and b* values during fruit ripening following 2- and 4-week cold storage (Figure 8.4A – 8.4F). At fully ripe stage, the chromaticity L*, a* and b* values were remained lower in NO-fumigated fruit (10, 20, and 40 μL L^{-1}) irrespective of storage period (Figure 8.4A – 8.4F). The interaction between NO fumigation and ripening period was found to be significant ($P \leq 0.05$) only for the chromaticity a* and b* values in both 2- and 4-week cold-stored fruit. All NO fumigation treatments (10, 20, and 40 μL L^{-1}) except 5 μL L^{-1} significantly ($P \leq 0.05$) reduced chroma (C*) values at fully ripe stage following 2- and 4-week of cold storage. But the response was more pronounced in 2-week than 4-week cold-stored fruit (Figure 8.5A and 8.5B). The interaction between NO fumigation and ripening period was found to be significant ($P \leq 0.001$) for C* value in 2- and 4-week cold-stored fruit. NO fumigation treatments (10, 20, and 40 μL L^{-1}) delayed the reduction of hue angle (h°) during ripening period of both 2- and 4-week cold-stored fruit (Figure 8.5C and 8.5D). The interaction between NO fumigation and ripening period was found to be non-significant in h° values irrespective of cold storage period.

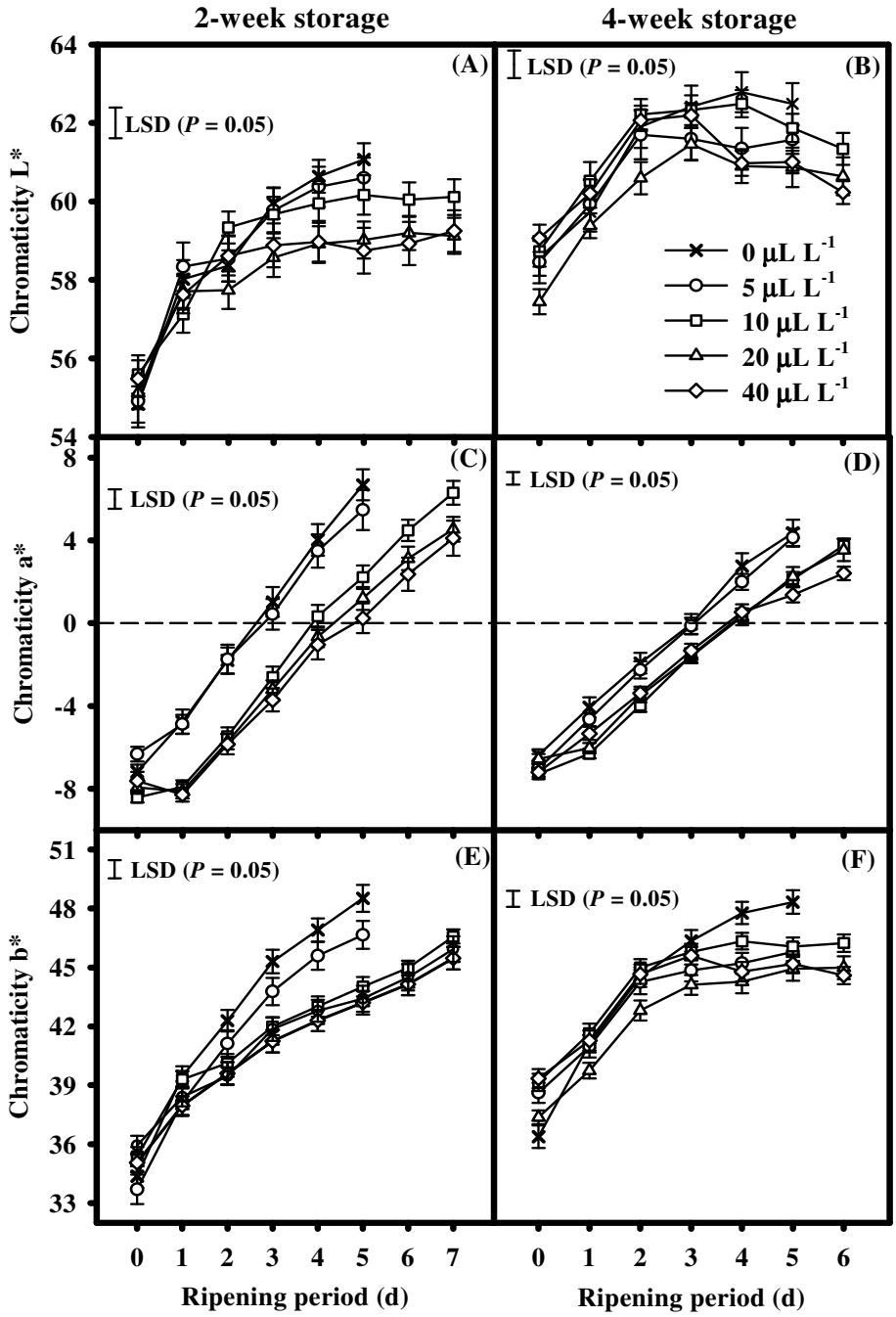

Figure 8.4. Skin colour development of chromaticity L*, a* and b* as influenced by different concentrations of NO-fumigation (T) and ripening period (RP) at ambient temperature following 2- (A, C, and E) and 4-week of cold storage (B, D, and F) of fruit at 5 ± 1°C.

n = 30 (10 fruit × 3 replications). Vertical bars represent S.E. of means and are invisible when the values are smaller than the symbol. LSD (*, **, *** significant at $P \leq 0.05$, 0.01, 0.001, respectively, and NS = not significant): 2-week storage, L*: T = 0.48^{**}, RP = 0.61^{***}, T × RP = NS; a*: T = 0.57^{***}, RP = 0.73^{***}, T × RP = 1.62^{*}; b*: T = 0.56^{***}, RP = 0.71^{***}, T × RP = 1.59^{***}; 4-week storage, L*: T = 0.46^{***}, RP = 0.54^{***}, T × RP = NS; a*: T = 0.40^{***}, RP = 0.47^{***}, T × RP = 1.05^{*}; b*: T = 0.53^{***}, RP = 0.62^{***}, T × RP = 1.39^{***}.

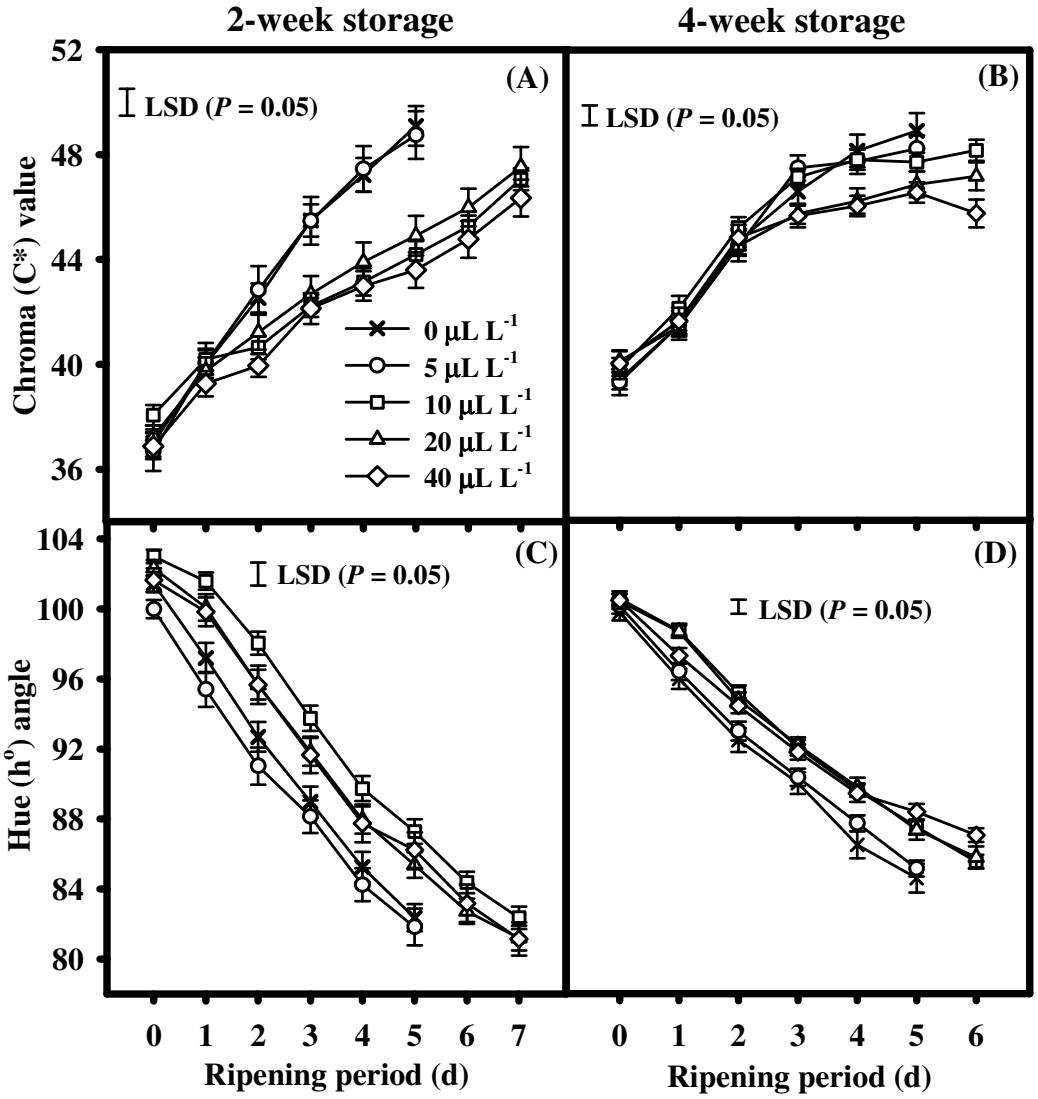

Figure 8.5. Skin colour development of C* and h° as influenced by different concentrations of NO fumigation (T) and ripening period (RP) at ambient temperature following 2- (A and B) and 4-week of cold storage (C and D) of fruit at 5 ± 1°C.

n = 30 (10 fruit × 3 replications). Vertical bars represent S.E. of means and are invisible when the values are smaller than the symbol. LSD (*, **, *** significant at $P \leq 0.05$, 0.01, 0.001, respectively and NS = not significant): 2-week storage, C^{*}: T = 0.62^{***}, RP = 0.79^{***}, T x RP = 1.76^{***}; h°: T = 0.80^{***}, RP = 1.02^{***}, T × RP = NS; 4-week storage, C^{*}: T = 0.49^{***}, RP = 0.58^{***}, T × RP = 1.29^{*}; h°: T = 0.52^{***}, RP = 0.62^{***}, T × RP = NS.

8.3.6 Changes in the concentrations of total and individual sugars

All NO fumigation treatments significantly ($P \leq 0.05$) reduced concentration of total sugar in the pulp of fully ripe fruit (1.09-, 1.14-, 1.12-, and 1.14-fold) and (1.19-, 1.20-, 1.13-, and 1.45-fold) as compared to control, in 2- and 4-week cold-stored fruit, respectively (Table 8.1). NO fumigation treatments did not significantly affect the concentrations of sucrose and glucose in pulp of ripe fruit following cold storage. The concentration of fructose in the pulp of ripe fruit was reduced with all the NO treatments but the reduction was highest with 20 and 40 μL L^{-1} NO. Whilst, in 4-week cold-stored fruit fumigated with 40 μL L^{-1} NO resulted in significantly highest reduction in fructose concentration (1.52-fold) than control.

The concentrations of total sugars, sucrose and fructose in the pulp of ripe fruit stored for 2-week were significantly ($P \leq 0.05$) higher (12.3%, 17.8%, and 9.5%, respectively) as compared to 4-week cold-stored fruit (Table 8.1). The interaction between NO fumigation and storage period was found to be significant only for total sugars in ripe fruit following 2- and 4-week cold storage (Table 8.1).

8.3.7 Changes in the concentrations of total and individual organic acids

Amongst all the organic acid determined from the pulp of ripe fruit, the NO fumigation treatments significantly affected the concentrations of tartaric and shikimic acid only irrespective of cold storage period (Table 8.2). The concentrations of both tartaric and shikimic acid increased with NO application in 2-week cold stored fruit. Similarly, at full-ripe stage following 4-week cold storage, NO-fumigation treatments (10 and 20 μL L^{-1}) exhibited 1.68- and 1.97-fold higher concentration of tartaric acid as compared to control and other treatments, respectively. All NO fumigation treatments (5, 10, 20, and 40 μL L^{-1}) significantly ($P \leq 0.05$) increased the concentration of shikimic acid (1.51-, 1.59-, 1.52-, and 2.14-fold, respectively) in the pulp of full-ripe fruit than control following 4-week cold storage. The concentrations of total acids, citric, shikimic and fumaric acid in the pulp of full-ripe fruit increased with extended cold storage period but tartaric and malic acid showed reverse trends (Table 8.2). The interaction between NO fumigation treatments and storage period was found to be significant for the malic and shikimic acid in pulp of full-ripe fruit.

8.3.8 Total antioxidant, ascorbic acid and total carotenoid

The levels of total antioxidant in the peel and pulp as well as total carotenoids in the pulp of full-ripe fruit were not significantly affected by NO treatments (Table 8.3). The levels of total antioxidants in fruit skin and carotenoids in pulp at full-ripe stage were significantly reduced with the extension of cold storage period from 2- to 4-week (Table 8.3). The interaction between NO fumigation and storage period was found to be significant ($P \leq 0.05$) only for the total antioxidant in the pulp of full-ripe fruit.

Table 8.1. The concentrations of total and individual sugars (sucrose, glucose and fructose) at eating soft ripe stage of fruit as influenced by different concentrations of NO fumigation and cold storage period.

NO ($\mu L\ L^{-1}$)	Storage period (Weeks)	Total sugars (g 100 g^{-1} FW)	Sucrose (g 100 g^{-1} FW)	Glucose (g 100 g^{-1} FW)	Fructose (g 100 g^{-1} FW)
0		10.75a	4.46	1.50	4.80a
5		9.85b	4.60	0.99	4.26ab
10	2	9.43b	3.56	1.36	4.51ab
20		9.54b	4.57	0.95	4.02b
40		9.46b	4.17	1.19	4.10b
	Mean	9.81A	4.27A	1.20	4.34A
0		10.11a	3.93	1.39	4.79a
5		8.47b	3.50	1.19	3.78bc
10	4	8.45b	3.50	1.19	3.76bc
20		8.96b	3.54	1.24	4.18ab
40		6.99c	3.06	0.78	3.16c
	Mean	8.60B	3.51B	1.16	3.93B
LSD of means at $P \leq 0.05$ level and levels of significance for a two-factor ANOVA					
NO-fumigation (T)		0.58^{***}	NS	NS	0.46^{***}
Storage period (SP)		0.37^{***}	0.52^{**}	NS	0.29^{**}
T × SP		0.82^{*}	NS	NS	NS

Means followed by the same small letters within a column and means followed by the same capital letters within a row are not significantly different at $P \leq 0.05$ by the Least Significant Difference (LSD) with n = 3. *, **, *** significantly different at $P \leq 0.05$, 0.01, 0.001, respectively, and NS = not significant.

Table 8.2. The concentrations of total and individual organic acids (citric, tartaric, malic, shikimic, and fumaric) at eating soft ripe stage of fruit as influenced by different concentrations of NO fumigation and cold storage period.

NO ($\mu L\ L^{-1}$)	Storage period (Week)	Total acids (g 100 g^{-1} FW)	Citric (g 100 g^{-1} FW)	Tartaric (mg 100 g^{-1} FW)	Malic (mg 100 g^{-1} FW)	Shikimic (mg 100 g^{-1} FW)	Fumaric (mg 100 g^{-1} FW)
0		0.63	0.46	9.41b	128.78	35.38b	0.14
5		0.68	0.51	9.15b	122.16	40.44ab	0.15
10	2	0.60	0.38	19.80a	153.30	49.88ab	0.19
20		0.64	0.43	12.43ab	153.09	50.36ab	0.21
40		0.64	0.42	13.98ab	147.28	56.99a	0.23
	Mean	0.64B	0.44B	12.95A	140.92A	46.61B	0.18B
0		0.90	0.74	5.09c	98.81	55.83c	0.39
5		1.01	0.83	7.10abc	88.11	84.40b	0.55
10	4	0.85	0.67	8.56ab	82.36	88.99b	0.49
20		0.82	0.66	10.06a	72.44	85.03b	0.51
40		1.15	0.95	6.33bc	75.61	117.25a	0.60
	Mean	0.95A	0.77A	7.43B	83.47B	86.30A	0.51A
LSD of means at $P \leq 0.05$ level and levels of significance for a two-factor ANOVA							
NO-fumigation (T)		NS	NS	3.02^{*}	NS	13.15^{***}	NS
Storage period (SP)		0.10^{***}	0.09^{***}	2.90^{***}	9.89^{***}	8.32^{***}	0.05^{***}
T × SP		NS	NS	NS	22.16^{**}	18.64^{*}	NS

Means followed by the same small letters within a column and means followed by the same capital letters within a row are not significantly different at $P \leq 0.05$ by the Least Significant Difference (LSD) with n = 3. *, **, *** significantly different at $P \leq 0.05$, 0.01, 0.001, respectively, and NS = not significant.

Table 8.3. Total antioxidant in pulp and skin, ascorbic acid and total carotenoid in the pulp at eating soft ripe stage of fruit as influenced by different concentrations of NO fumigation and cold storage period.

NO ($\mu L\ L^{-1}$)	Storage period (Week)	Total antioxidant (mM TEAC g^{-1} FW)		Ascorbic acid (mg 100 g^{-1} FW)	Total carotenoid (mg kg^{-1} FW)
		Pulp	Skin		
0		3.38	222.69	20.56	45.99
5		3.39	226.84	19.71	47.82
10	2	3.05	203.55	19.04	46.24
20		3.10	205.59	19.81	45.54
40		2.62	203.58	20.59	39.95
	Mean	3.11	212.45A	19.94	45.11A
0		2.77	218.04	21.09	29.03
5		2.80	173.42	18.63	26.08
10	4	2.69	188.06	20.46	27.59
20		2.94	177.69	19.67	28.74
40		3.33	197.12	19.44	24.53
	Mean	2.91	190.87B	19.86	27.19B
LSD of means at $P \leq 0.05$ level and levels of significance for a two-factor ANOVA					
NO-fumigation (T)		NS	NS	NS	NS
Storage period (SP)		NS	13.10^{**}	NS	2.91^{***}
T × SP		0.59^{*}	NS	NS	NS

Means followed by the same small letters within a column and means followed by the same capital letters within a row are not significantly different at $P \leq 0.05$ by the Least Significant Difference (LSD) with n = 3. *, **, *** significantly different at $P \leq 0.05$, 0.01, 0.001, respectively, and NS = not significant.

8.4 Discussion

8.4.1 Chilling injury (CI)

CI index increased during ripening period at ambient temperature following 2- and 4-week cold storage and was more pronounced in 4-week stored fruit. Similarly, Nair et al. (2003) and Nair et al. (2004a) reported that CI index increased during ripening period depending upon chilling temperature and storage period in 'Kensington Pride' mango fruit. All NO treatments reduced CI index in ripe fruit following 2- and 4-week cold storage (Figure 8.1A and 8.1B). Fruit treated with 10 to 40 μL L^{-1} NO and stored for 2- and 4-week exhibited lowest CI index at ripe stage as compared to other treatments (Figure 8.1A and 8.1B). The reduction of CI in mango fruit with NO treatment did not seem to be directly associated with reduced ethylene production. Previously, the suppression of ethylene production with increased CI and its reduction with exogenous application of ethrel has been reported to be associated with CI development in 'Kensington Pride' mango (Nair and Singh, 2003). Whilst, exogenous application of NO has reduced ethylene production consequently reduced CI in 'Amber Jewel′ plum (Singh et al., 2009) and 'Feicheng′ peach (Zhu et al., 2010a). Contrarily, the reduction in CI index in mango fruit with NO treatments may not be ascribed to the suppression of ethylene production. The relationships between ethylene and development of CI are complex in various climacteric and non-climacteric fruits, with no consistent relationship between the two factors (Watkins and Miller, 2005). Additionally, similar to the earlier findings of Ku et al. (2000), NO fumigation has also reduced water loss during mango fruit ripening period (data not shown). The reduction in water loss with NO fumigation may have contributed in reducing CI. Bower et al. (2003) also reported a link between postharvest water loss and CI in mango and avocado fruit. It may also be argued that chilling temperatures induce generation of ROS which induces oxidative stress and causes CI in mango fruit (Ding et al., 2007; Zhou et al., 2006). Possibly, the exogenous application of NO down regulates the generation of ROS consequently reducing oxidative stress in mango fruit stored at chilling temperature which alleviates CI. NO has antioxidant properties and also been reported to play important role in ROS metabolism and signalling network during normal and stress conditions (Hayat et al., 2010). Leshem and Kuiper (1996) claimed that NO is a natural stress-alleviating agent and may have component of the “general adaptation syndrome” reaction in which plants evoke identical stress-alleviating mechanism to combat different types of environmental

stress conditions. Such a mode of action of NO in reducing CI in mango fruit warrants to be investigated. Additionally, my results suggest that higher concentrations of NO are required to reduce CI in mango fruit stored at CI temperature for a longer period (4-week) than short period (2-week).

8.4.2 Ethylene production

NO fumigation significantly reduced ethylene production during fruit ripening at 21 ± 1°C after 2- and 4-week of cold storage and the reduction was more pronounced in 4-week stored fruit (Figure 8.2A and 8.2B). Possibly, the suppression of ethylene production with NO treatment may be attributed to the reduced activities of ACS and/or ACO. Similarly, the inhibition of ethylene biosynthesis with NO treatment has been reported in different fruits such as banana (Cheng et al., 2009), peach (Flores et al., 2008), strawberry (Zhu and Zhou, 2007), and tomato (Eum et al., 2009). NO has been reported to reduce ethylene production in strawberry and banana by inhibiting the activity of ACS (Cheng et al., 2009; Zhu and Zhou, 2007) and ACO activity as well as transcription of *MA-ACO1* gene, respectively (Cheng et al., 2009). Likewise, the inhibition of ethylene biosynthesis in NO-fumigated tomatoes has been ascribed to decrease and delay in the expression of ACO genes (*LeACO1*, *LeACOH2* and *LeACO4*) during fruit ripening, but treated fruit expressed extensively ACS genes in both mature green and breaker stage during ripening period (Eum et al., 2009). Recently, Manjunatha et al. (2010) claimed that NO binds to ACO to form a binary ACO-NO complex, which is chelated by 1-amino-cyclopropane carboxylic acid content (ACC) to produce a stable ternary ACC-ACO-NO complex, consequently decreasing ethylene production. Possibly, either one of above mentioned or any other mechanism of action of NO in inhibiting ethylene biosynthesis may exist in mango fruit and warrants to be investigated.

8.4.3 Respiration rate

NO fumigation reduced respiration rate during fruit ripening at 21 ± 1°C in 2- and 4-week cold-stored fruit (Figure 8.2C and 8.2D). Similarly, suppression of respiration rate during ripening in NO-fumigated fruit have been reported earlier, such as plum (Singh et al., 2009) and apple (Wang et al., 2008b).

8.4.4 Fruit softening

Most of the NO treatments delayed fruit softening during ripening following 2- and 4 week of cold storage (Figure 8.3A and 8.3B). NO fumigation was more effective in delaying fruit softening in 2-week stored fruit during ripening period than 4-week stored. The reduction in fruit softening may be ascribed to the reduced activities of fruit softening enzymes induced with the suppression of ethylene production in NO-treated fruit. Similarly, Khan and Singh (2008) reported that increased activities of fruit softening enzymes such as PE, EGase, *endo*- and *exo*-PG were associated with increased climacteric ethylene production during plum fruit ripening. Similarly, NO fumigation of plums and bananas slices with 1 and 5 mM NO, respectively, has been reported to retard fruit softening during storage and ripening (Cheng et al., 2009; Zhang et al., 2008). NO treatments (except 5 μL L^{-1}) also delayed fruit ripening by 2 and 1 day(s) in 2- and 4-week cold-stored fruit respectively (Figure 8.3A and 8.3B). Similarly, delays in fruit ripening by 3 and 4 days at 21 ± 1°C were also noticed in 'Amber Jewel' plums after fruit were fumigated with 10 μL L^{-1} NO gas after 6-week in cold storage at 0°C (Singh et al., 2009). The exact mode of action of NO in reducing fruit softening and modulating activities of various fruit softening enzymes during mango fruit ripening is yet to be investigated.

8.4.5 Chromaticity L, a*, b*, C*, and h°*

NO fumigation retards colour development including chromaticity L*, a*, b*, and croma (C*) and maintains higher hue angle (h°) of the skin during fruit ripening at 21 ± 1°C (Figure 8.4A – 8.4F and 8.5A – 8.5D). Restriction of L*, a*, b*, and C* value changes and maintaining high h° value in NO-fumigated fruit may due to suppression of ethylene production and consequently reducing degeneration of chlorophyll and reduced biosynthesis of carotenoids particularly in the skin as also reported in banana (Cheng et al., 2009).

8.4.6 Total and individual sugars

NO fumigation reduced the concentration of total sugars and fructose in ripe fruit stored following 2- and 4-week of cold storage. Ripe fruit stored for 4-week showed more pronounced reductions in the concentrations of total sugars and fructose than 2-week cold-stored ones possibly their utilisation in respiration during extended cold storage period (Table 8.1). These results suggest that NO fumigation influences

metabolism of sugars in mango fruit and warrants to be investigated. Similarly, Singh et al. (2009) reported ealier that the individual sugar (fructose, glucose, sucrose, and sorbitol) profiles of NO-fumigated fruit were significantly different from those of non-fumigated fruit after cold storage and ripening at 21 ± 1°C.

8.4.7 Total and individual organic acids

Citric and malic acids are major organic acids in mango fruit. The total organic acids, citric acid, malic acid and fumaric acid concentrations were not significantly affected with NO fumigation treatment irrespective of cold storage period (Table 8.2). The increased concentrations of citric, tartaric and shikimic acid and decreased tartaric and malic acid in 4-week stored fruit than 2-week one may be ascribed to the higher utilisation of tartaric and malic acid in respiration as compared to other organic acids. NO fumigation seems to be influencing the organic acid metabolism in mango fruit during ripening depending upon cold storage period.

8.4.8 Total antioxidant, ascorbic acids and total carotenoid

NO treatments did not affect the concentrations of total antioxidants (in fruit skin and pulp), as well as ascorbic acid and carotenoids in the pulp of ripe fruit (Table 8.3). In contrast, NO treatment (1 mM and 2 μmol L^{-1}) maintained high level of ascorbic acid (Duan et al., 2007) and decreased the activity of antioxidant enzyme in longan and kiwifruit, respectively (Duan et al., 2007; Zhu et al., 2008).

In conclusion, the postharvest application of NO (10, 20, and 40 μL L^{-1}) reduced CI index, retarded colour development, softening and delayed fruit ripening as well as maintained quality through reduction of ethylene production and respiration rate during fruit ripening period in 2- and 4-week cold-stored (5°C) fruit.

CHAPTER 9

General discussion and conclusions

9.1 Introduction

Plant hormone(s) play a key role in induction and regulating fruit ripening including softening during storage and ripening period. Mango is highly perishable fruit. It ripens in 7 to 9 days depending upon cultivar and harvest maturity. Mango is also susceptible to chilling injury (CI) when the fruit are stored at the cold storage below 13°C (Nair and Singh, 2009). Mango fruit ripening and softening mostly influenced by endogenous ethylene biosynthesis. Other than ethylene, brassinosteroids (BRs), abscisic acid (ABA), and auxin (indole-3-acetic acid, IAA) also been reported to play a role in regulating the ripening processes of other climacteric or non-climacteric fruits (Burg and Burg, 1962; Lara and Vendrell, 2000; Nilgun and Nihat, 2005; Ruan et al., 2005; Sheng et al., 2000; Symons et al., 2006). The exogenous application of S-(+)-*cis, trans*-abscisic acid (ABA) and inhibitor of its biosynthesis - nordihydroguaiaretic acid (NDGA), epibrassinolide [Epi-BL; (22R, 23R)-2α,3α,22,23-tetrahydroxy-7-oxa-B-homo-5α-ergostan-6-one] and nitric oxide (NO) has been reported to regulate ethylene biosynthesis and fruit softening in climacteric or non-climacteric fruits (Parikh et al., 1990; Symons et al., 2006; Zaharah and Singh, 2011a; Zhang et al., 2009a; Zhang et al., 2009b), however no research work has been reported on their role in regulating mango fruit ripening. It was hyphothesized that the changes in endogenous levels of BRs, ethylene, ABA, and/or IAA may play a role in modulating the ripening processes of 'Kensington Pride' mango fruit. The endogenous levels of these regulators were regulated using inhibitors of their biosynthesis and/or action to unfold their mechanism in delaying/hastening mango fruit ripening, extending storage life and improving fruit quality. The general aims of my research were to investigate the changes in the endogenous level of ethylene, ABA, IAA and BRs as well as regulating the endogenous levels of ABA and BRs with their exogenous application and/or inhibitors of their biosynthesis to underpin their role in mango fruit ripening. The role of NO fumigation in regulating ethylene biosynthesis, fruit softening, ameliorating CI and the ripening processes at ambient and cool-stored in 'Kensington Pride' mango fruit was also investigated.

9.2 Roles of brassinosteroids (BRs), ethylene, abscisic acid (ABA) and indole-3-acetic acid (IAA) in regulating mango fruit ripening

9.2.1 Roles of endogenous level of BRs, ethylene, ABA and IAA in regulating mango fruit ripening

Ethylene plays an important role in regulating mango fruit ripening (Brecht and Yahia, 2009). Its exogenous application has been reported to trigger ethylene production, consequently promote mango fruit ripening, but the application of its biosynthesis inhibitor - aminoethoxyvinylglycine (AVG) and 1-methylecyclopropene (1-MCP), suppressed and delayed the ripening process (Lalel et al., 2003e). Other than ethylene, the involvement of a steroidal plant hormones (BRs) in promoting the ripening of grape berry (Symons et al., 2006), caught my attention to uncover this role in mango fruit ripening. The endogenous level of IAA has been reported to decline during ripening period in kiwifruit (Chen et al., 1999) and tomato (Sheng et al., 2000). Whilst, the exogenous application of 2,4-Dichlorophenoxyacetic acid (2,4-D) has been reported to elevate the endogenous levels of ABA in 'Tainong' mango during ripening in cold-stored fruit (4°C) (Wang et al., 2008a), suggesting that both endogenous auxin and ABA may play a role in mango fruit ripening. To address the first objective of my research, the changes in endogenous levels of BRs, ethylene, ABA, and IAA along with the changes in the rate of respiration, pulp firmness and skin colour were determined in 'Kensington Pride' mango during 8 days ripening period at ambient temperature (21 ± 1°C) (Chapter 4, Section 4.2.2). The climacteric ethylene production and the respiration peak occurred on the 4th day of ripening period (Figure 4.1A and 4.2A). The endogenous level of ABA started to increase during the climacteric-rise (CR) stage on the 2nd day of ripening and peaked on the 4th day of ripening (Figure 4.1B). Similarly like other climacteric fruit, the endogenous level of ABA has been reported to increase in the pericarp of peach and it was associated with the climacteric ethylene and respiration peak during ripening period (Wu et al., 2003). On the other hand, the endogenous level of IAA was higher in the pulp of 'Kensington Pride' mango during the pre-climacteric (PC) minimum stage and declined during the climacteric and post-climacteric stages (Figure 4.1C). Similar results have been reported in kiwifruit (Chen et al., 1999) and tomato (Sheng et al., 2000). My experimental results also indicated that the changes in the endogenous level of BRs (castasterone and brassinolide) are unlikely to modulate

mango fruit ripening as it is present in a trace amounts in mango pulp tissues throughout the ripening period (Table 4.1).

During ripening, increased endogenous levels of ABA (Figure 4.1B) may probably triggered ethylene production by up-regulating the activities of 1-aminocyclopropane-1-carboxylic acid (ACC) synthase (ACS) and ACC oxidase (ACO) and consequently promoted fruit ripening. As reported earlier, the exogenous application of ethephon in combination with modified atmosphere packaging stored at 13.5 ± 0.5°C increased ethylene production and reduced fruit firmness in 'Kensington Pride' mango (Singh and Janes, 2001). Whilst, the exogenous application of 1-MCP reduced the ethylene production (Lalel et al., 2003e) and inhibited the loss of fruit firmness (Osuna et al., 2005) in 'Kensington Pride' and 'Kent' mango fruit, respectively, leading to delayed ripening processes. Increased pulp firmness losses also associated with the promotion of yellow skin colour development as the ripening period proceeds (Figure 4.2B and 4.2C). In conclusion, higher levels of endogenous IAA in fruit pulp during the PC stage and the accumulation of ABA prior to the climacteric stage might switch on ethylene production, which subsequently regulates fruit ripening.

9.2.2 Role of exogenous application of BRs in regulating mango fruit ripening

Exogenous application of BRs have been reported to promote ripening processes in a climacteric fruit such as tomato by inducing ethylene production in pericarp disc (Vardhini and Rao, 2002) as well as in non-climacteric fruit such as grape berry (Symons et al., 2006). The exogenous applications of BRs have not previously been implicated in regulating mango fruit ripening. Thus, role of different doses of Epi-BL on ethylene production, respiration rate, fruit softening and skin colour development during ripening at ambient temperature was investigated (Chapter 4, Section 4.2.3). The exogenous application of Epi-BL (45 and 60 ng g^{-1} FW) accelerated and advanced the onset of the climacteric peak of ethylene production and respiration rate by 2 and 1 day(s), respectively, accelerated fruit colour development and softening during fruit ripening period (Table 4.2, Figure 4.3A and 4.3B). Earlier, the exogenous application of BRs has been suggested to promote ethylene biosynthesis (Schlagnhaufer et al., 1984), but these results unlikely to support a direct role of BRs

in mango fruit ripening because fruit softening seems did not differ between treated and untreated fruit in full-ripe stage.

9.2.3 Roles of exogenous application of ABA in modulating mango fruit ripening

The exogenous application of ABA (10^{-6} M) has been reported earlier to hasten fruit ripening process in 'Alphonso' and 'Langra' mango (Palejwala et al., 1988; Parikh et al., 1990). However, the role of different concentration of exogenous application of ABA in regulating 'Kensington Pride' mango fruit ripening have not yet been studied. Hard mature green 'Kensington Pride' mango fruit was dipped in three different concentrations of ABA and its inhibitor biosynthesis (NDGA) as mentioned in Chapter 4, Section 4.2.4, to underpin the first aim of my research work. The exogenous application of ABA (1.0 mM and 2.0 mM) promoted skin colour development and rate of fruit softening, respectively, during ripening compared with the control, whilst the application of NDGA-treated exhibited the reversed trend (Figure 4.3C and 4.3D). Increased fruit softening and skin colour development probably might be due to activation in polygalacturonase (PG) activity and carotenoid biosynthesis, respectively. Likewise, the exogenous application of ABA also has been reported to accelerate the ripening process such as in apricot (Zhang and Lu, 1983), tomato (Hong et al., 1999), strawberry (Jiang and Joyce, 2003), banana (Lohani et al., 2004), and kiwifruit (Chen et al., 2005). In conclusion, the exogenous application of ABA promotes mango fruit ripening. However, the exact mechanism of ABA in promoting mango fruit ripening warrants to be investigated.

9.3 ABA regulates ethylene biosynthesis, activities of fruit softening enzymes and quality

The increased endogenous level of ABA coupled with the climacteric peak of ethylene production were determined in Chapter 4 (Figure 4.1A and 4.1B) and it demonstrates its pivotal role in mango fruit ripening. Moreover, a positive exponential relationship between endogenous levels of ABA and ethylene production during fruit ripening (Chapter 5, Figure 5.2) confirmed my hypothesis. The effects of exogenous application of S-(+)-*cis, trans*-abscisic acid (1.0 mM ABA) and its inhibitor of biosynthesis (0.2 mM NDGA) were most effective in regulating fruit colour development and fruit softening (Figure 4.3C and 4.3D) included in Chapter 4, Section 4.2.3. To address the second aim of my research, both the treatments of

ABA and its inhibitor were used to investigate their role in regulating ethylene biosynthesis and fruit softening including fruit quality (Chapter 5, Section 5.2.3, Experiment II).

The exogenous application of 1.0 mM ABA accelerated the climacteric peak of ethylene production during mango fruit ripening at ambient temperature (Figure 5.3). It may be ascribed to the increased activities of ethylene biosynthesis enzymes such as ACS and ACO, and ACC content in pulp tissues, consequently hastened fruit ripening (Figure 5.4A – 5.4C). The exogenous application of inhibitor of ABA biosynthesis treatment (NDGA) in 'Kensington Pride' mango fruit suppressed the climacteric peak of ethylene production, lower level of ACC content through inhibition of the activity of ACS enzyme and fully suppressed the activity of ACO enzyme (Figure 5.3, 5.4A – 5.4C), leading to delayed ripening processes. Similarly, higher concentrations of ABA also preceded the climacteric increase of ethylene production in tomato fruit, and its exogenous application has been reported to induce ethylene biosynthesis *via* up-regulating the expression of ACS (*LeACS2*) and ACO (*LeACO1*) gene consequently promoted fruit ripening, and fluridone and NDGA treatment delayed the ripening process (Zhang et al., 2009b).

The exogenous application of ABA advanced fruit softening during ripening period as compared to control (Figure 5.5), which may be ascribed to ABA accelerated ethylene biosynthesis, and consequently increased fruit softening. The increased fruit softening during ripening may also be attributed to the induced activities of *endo*-PG and PE in the pulp tissues in ABA-treated fruit (Table 5.1). Similarly, Zhou et al. (1996) claimed that the activity of PG increased in ABA-treated 'Zihua' mango, leading to increased fruit softening. The application of inhibitor of its biosynthesis (NDGA treatment) delayed fruit softening, which may be ascribed to the inhibition of fruit softening enzymes activities (*endo*-PG and PE), subsequently delayed fruit softening. The reduction in fruit softening during ripening as well as activities of different fruit softening enzymes with postharvest application of 1-MCP (inhibitor of ethylene action) further support this hypothesis (Khan and Singh, 2007).

The exogenous application of ABA exhibited higher accumulation of total sugars and sucrose as well as degradation of total organic acids, citric and fumaric acid in 'Kensington Pride' mango fruit during ripening, and the trends were reversed in NDGA-treated fruit (Table 5.2 and 5.3). Increased accumulation of total sugars and sucrose as well as higher reduction of total organic acids also has been reported in ABA-treated (10^{-6} M) 'Alphonso' and 'Langra' mango fruit during the ripening period (Palejwala et al., 1988).

In conclusion, a significant positive relationship between the endogenous level of ABA and ethylene suggest that ABA play a key role in regulating mango fruit ripening. The exogenous application of ABA hastened the ripening process in 'Kensington Pride' mango with acceleration of ethylene biosynthesis due to increased activities of ACS, ACO enzymes, ACC content in the fruit pulp which consequently promoted fruit softening and the activities fruit softening enzymes, higher concentration of total sugars and sucrose as well as degradation of total organic acids, citric and fumaric acid, whilst, the trend was reversed in NDGA-treated fruit. However, the effect of exogenous application of ABA on the changes of the endogenous level of ABA during mango fruit ripening have not yet been investigated and warrants to be elucidated.

9.4 Role of NO in regulating mango fruit ripening

Autocatalytic ethylene production triggers the ripening process but reduces shelf life under normal ripening conditions (Burg and Burg, 1962). As a prelude, the overall effects of NO on fruit ripening and quality are presumed to be similar to those of 1-MCP. The effects of NO treatment in reducing the autocatalytic ethylene production and consequently in delaying 'Kensington Pride' mango fruit ripening at ambient or in cool/cold storage was investigated. Hence, different experiments have been carried out to address objective number 3, 4, and 5 of my research work which forms Chapter 6, 7, and 8.

9.4.1 Postharvest NO fumigation at the pre-climacteric or climacteric-rise stages influences ripening and quality in mango fruit

The postharvest NO treatment plays an important role in modulating fruit ripening in both climacteric and non-climacteric fruits (Manjunatha et al., 2010). The exogenous

application of NO has been reported to delay fruit ripening in plum (Singh et al., 2009; Zhang et al., 2008), banana (Cheng et al., 2009; Yang et al., 2010), peach (Flores et al., 2008; Liu et al., 2007; Zhu and Zhou, 2006), pear (Sozzi et al., 2003), longan (Duan et al., 2007), and strawberry (Zhu and Zhou, 2007). To address the third aim of my research work (Chapter 6), we investigated the effects of postharvest NO fumigation (0, 5, 10, 20, and 40 μL L^{-1}) applied at the PC or CR stage on ripening and quality of mango (*Mangifera indica* L. cv. 'Kensington Pride') during fruit ripening at 21 ± 1°C.

NO fumigation treatments (20 μL L^{-1} or 40 μL L^{-1}) applied at the PC or CR stages delayed and suppressed ethylene production (Figure 6.1A and 6.1B). Lower ethylene production in NO-fumigated fruit possibly reflects the reduced activities of ACS and/or ACO as also reported earlier in strawberry (Zhu and Zhou, 2007), banana (Cheng et al., 2009), and tomato (Eum et al., 2009). The reduction in the rate of respiration of NO-fumigated fruit at the PC stage was more pronounced than at the CR stage (Figure 6.2A and 6.2B). Effects of NO fumigation was more pronounced when applied at the PC stage is probably due to their effectiveness in reducing the activity of cytochrome oxidase components during early ripening period particularly before the climacteric burst of respiration rather than later in the post-climacteric phase.

Delayed rate of fruit softening in all NO-treated fruit above 5 μL L^{-1} applied at the PC or the CR stage up to 2 days, as well as retained slightly higher rheological properties (cohesiveness, springiness, chewiness, adhesiveness, and stiffness) of pulp of ripe fruit (Figure 6.3A, 6.3B and Table 6.1) may be attributed to the reduced ethylene production (Figure 6.1A and 6.1B). Lower skin colour development as well as lower chromaticity L*, a*, b*, C* and higher h° as well as in all NO fumigation at the PC and the CR stage (Figure 6.4 - 6.5 and Table 6.2) might be due to reduced chlorophyll degeneration.

Lower concentration of total sugars, sucrose, fructose and malic acid was observed in the fruit fumigated at both the stages (PC and CR) but lower concentration of SSC and glucose at the PC stage, and lower tartaric acid and reverse trend in shikimic acid at the CR stage were also observed (Table 6.3 and 6.4). The

differential responses to sugar and organic acid accumulation in fruit treated at different climacteric stages may also be partially attributed to the harvest maturity stage.

All NO fumigation treatments had significant effects but no clear trend was absenced on TA and SSC:TA ratio applied at both stages (Table 6.3). Earlier, higher amount of TA in NO-treated (20 μL L^{-1} or 40 μL L^{-1}) fruits has been reported, without significant effect on SSC during ripening in Japanese plum cv. 'Amber Jewel' (Singh et al., 2009). NO-treated fruit did not influence fruit firmness (hardness), SSC, sucrose, glucose, total acid, citric, tartaric, malic, fumaric, total carotenoid, and ascorbic acid content in the pulp of full-ripe fruit as compared to control in both climacteric stages applied (Table 6.1, 6.3 - 6.4). Averaged over all NO treatments applied at PC stage, the concentrations of springiness, chewiness, adhesiveness, chromaticity b*, C*, TA, total acid, citric, tartaric, malic, shikimic, and fumaric were higher than applied at CR stage and the trend was reversed for stiffness, chromaticity L*, SSC, SSC:TA ratio, total sugars, glucose, fructose, and total carotenoid (Table 6.1 - 6.4). Singh et al. (2009) also claimed that the changes in the ripening related parameters including skin colour, firmness, TA and individual sugars (fructose, glucose, sucrose, and sorbitol) in NO-treated plum fruit had significant effect during ripening or after cold storage followed by ripening at 21°C. The results of this experiment suggest that fruit maturation is one of the important factors that would influence the effectiveness of NO treatment in regulating mango fruit ripening. In conclusion, the postharvest application of NO (20 μL L^{-1} or 40 μL L^{-1}) has more potential to delay fruit ripening if applied to mango fruit at the PC stage by suppressing ethylene production and the rate of respiration, with minimum losses in fruit quality, than NO fumigation at the CR stage.

9.4.2 Mode of action of NO in inhibiting ethylene biosynthesis and fruit softening during ripening and cool storage of 'Kensington Pride' mango

The changes in endogenous level of ethylene production influence fruit texture during ripening through regulating the activities of fruit softening enzymes (Chourasia et al., 2006; Chourasia et al., 2008). The postharvest application of NO (20 μL L^{-1}) suppressed and delayed the climacteric peak of ethylene production during ripening at ambient temperature (21 ± 1°C) as well as during cool storage (13

± 1°C) (Figure 7.1A and 7.1B). The reduction in ethylene production during ripening or cool storage probably due to stoichiometric relationships between NO and endogenous level of ethylene production as well as NO bound to ACO to form ACO-NO complex and then chelated to ACC to produce a stable ACC-ACO-NO complex (Manjunatha et al., 2010). The suppressed activities of ethylene biosynthesis (ACS and ACO) and ACC content in pulp tissues was higher in NO-fumigated fruit during ripening or cool storage than control fruit (Figure 7.2A – 7.2F), could also be ascribed to reduced ethylene production (Figure 7.1A and 7.1B). Similarly, reduced ethylene production and/or ethylene biosynthesis enzymes with postharvest application of NO during ripening or cold storage has also been reported in banana (Cheng et al., 2009), plum (Singh et al., 2009), kiwifruit (Zhu et al., 2010b), peach (Flores et al., 2008; Liu et al., 2007; Zhu et al., 2006), pear (Sozzi et al., 2003), strawberry (Zhu and Zhou, 2007), and tomato (Zhang et al., 2005).

NO fumigation inhibited the rate of respiration in both ripening at ambient or at low temperature storage (Figure 7.1C and 7.1D). NO treatment significantly delayed (2 days) and suppressed (19.54%) the climacteric respiration peak during ripening (Figure 7.1C), whilst, the suppression of respiration rate was more pronounced during 7 to 14 days of cool storage (Figure 7.1D), indicating that NO treatment effectively reduced cytochrome chain components of respiration rate before the climacteric peak of respiration rate rather than at the post-climacteric stage.

NO-fumigated fruit maintained higher rheological properties of pulp (firmness, springiness, cohesiveness, chewiness, adhesiveness, and stiffness) during ripening or cool storage as compared to untreated fruit (Table 7.1 and 7.2). Likewise, NO treatment has been reported to retain higher fruit firmness during ripening or cold storage in banana (Cheng et al., 2009), plum (Singh et al., 2009), kiwifruit (Zhu et al., 2010b), peach (Flores et al., 2008; Liu et al., 2007; Sun et al., 2011; Zhu et al., 2006), and pear (Sozzi et al., 2003). The reduction in mango fruit softening with NO treatment might be due to decreased activities of *exo*-, *endo*-PG and EGase, but maintained higher PE activity in pulp tissues during ripening and cool storage (Figure 7.3A – 7.3D and 7.4A – 7.4D). Earlier, the decreased activities of fruit softening enzymes (PG, PE and EGase) has been reported in NO-treated banana

slices (Cheng et al., 2009; Yang et al., 2010) and peach (Zhu et al., 2010a) during ripening at ambient temperature or cold storage (5°C) respectively. In conclusion, the application of 20 μL L^{-1} NO fumigation to 'Kensington Pride' mango reduced fruit softening through inhibiting the activities of fruit softening and ethylene biosynthesis enzymes answering the fourth objective of my research work.

9.4.3 Postharvest NO fumigation alleviates chilling injury, delays fruit ripening and maintains quality in cold stored 'Kensington Pride' mango

To address the fifth objective of my research, the effects of NO fumigation in alleviating CI, ethylene production, regulating ripening as well as maintaining quality following 2- or 4-week of cold-stored fruit. The fruit fumigated with 10, 20, and 40 μL L^{-1} NO showed significantly reduced CI index following 2- or 4-week cold-stored at 5 ± 1°C (Figure 8.1A and 8.1B). This might be due to suppressed and reduced ethylene production irrespective of cold storage period (Figure 8.2A and 8.2B). Similarly, the alleviation of CI symptoms has been reported to associate with inhibition of ethylene production during cold storage in Japanese plum cvs. 'Amber Jewel' (Singh et al., 2009) and 'Feicheng' peach (Zhu et al., 2010a). The reduced CI index is possibly due to decreased water loss (data not shown) as well as low oxidative stress in NO-treated fruit during mango fruit ripening period.

NO fumigation reduced the rate of respiration during fruit ripening at 21 ± 1°C following 2- and 4-week of cold-stored fruit (Figure 8.2C and 8.2D). The suppression rate of respiration during ripening in NO-fumigated fruit has also been reported in plum (Singh et al., 2009) and apple (Wang et al., 2008b).

The exogenous application of NO treatment above 5 μL L^{-1} significantly delayed fruit softening up to 2 and 1 day(s) in 2- or 4-week of storage period (Figure 8.3A and 8.3B). The reduction in fruit softening may be ascribed to reduced suppression of ethylene production in NO-treated fruit in both storage periods (Figure 8.2A and 8.2B). This also maybe due to NO-treated fruit suppressed the activities of fruit softening enzymes as reported earlier in Chapter 7 and discussed in this chapter, Section 9.4.2.

NO fumigation retards skin colour development (chromaticity L*, a*, b*, and C*) and maintains higher h° value during fruit ripening at 21 ± 1°C, irrespective of cold-storage period (Figure 8.4A - 8.4F and Figure 8.5A - 8.5D). The reduction in skin colour development might be due to suppression of ethylene production (Figure 8.2A and 8.2B) and consequently reducing degeneration of chlorophyll and reduced biosynthesis of carotenoid in skin as reported earlier in NO-treated banana (Cheng et al., 2009).

NO fumigation delayed the accumulation of total sugars and fructose at full-ripe stage after 2- and 4-week of cold storage (Table 8.1). All NO fumigation treatments significantly increased the concentrations of tartaric and shikimic acid during ripening period in 2- and 4-week cold-stored fruit, except for the 5 μL L^{-1} treatment in 2-week cold-stored fruit (Table 8.2). However, NO treatments did not significantly influence the concentration of sucrose, glucose, total acid, citric acid, malic acid, and fumaric acid than control fruit in both storage periods (Table 8.1 and 8.2). These results suggest that NO fumigation influences metabolism of sugars in mango fruit and warrants further investigation.

Averaged over all NO concentrations, the 2-week cold-stored fruit resulted in significantly higher mean concentrations of total sugars, sucrose, fructose, tartaric acid, malic acid than 4-week cold-stored fruit, which could possibly be due to their utilisation in respiration during extended cold storage period, and the trend was reverse for total acids, citric, shikimic, and fumaric acid (Table 8.1 and 8.2).

In conclusion, the postharvest application of NO (10, 20, and 40 μL L^{-1}) alleviated CI index, reduced skin colour development, softening and delayed fruit ripening as well as maintained quality *via* inhibition in the ethylene production and rate of respiration during ripening of mango fruit at ambient temperature (21°C) following 2- or 4-week cold-storage.

9.5 General conclusions

1. The higher levels of endogenous IAA in fruit pulp during the PC stage and the accumulation of ABA prior to the climacteric stage might switch on ethylene production that triggers fruit ripening. There is a significant non-linear (quadratic) relationship between endogenous level of ABA in the pulp and ethylene production during fruit ripening period. Whilst exogenous Epi-BL promoted fruit ripening endogenous measurements suggest that changes in BRs levels are unlikely to modulate mango fruit ripening.

2. Additionally, the exogenous application of ABA (1.0 mM) accelerated ethylene biosynthesis with elevation in the activities of ethylene biosynthesis enzymes (ACS and ACO) and ACC content, promoted fruit softening enzyme activity (*endo*-PG) and retained higher PE activity in the pulp. Whilst, the activities of ethylene biosynthesis and softening enzymes was significantly delayed and/or suppressed in the pulp of NDGA-treated fruit. These results suggested that ABA is involved in regulating mango fruit ripening and its effects are, at least in part, mediated by changes in ethylene production.

3. Post-harvest fumigation with NO at 20 μL L^{-1} or 40 μL L^{-1}, applied to mango fruit at the PC stage, was more effective in suppressing ethylene production and the rate of respiration, and in retarding skin colour development and fruit softening during ripening at ambient temperatures as well as increasing the rheological properties (springiness) of pulp, TA, total and individual organic acids level in full-ripe fruit, than NO fumigation at the CR stage.

4. NO fumigation suppressed ethylene biosynthesis through inhibition of ACS and ACO activities leading to reduced ACC content in the pulp of 'Kensington Pride' mango fruit that subsequently reduced the activities of fruit softening enzymes such as *exo*-, *endo*-PG and EGase during ripening and cool storage.

5. The postharvest application of NO (10, 20, and 40 μL L^{-1}) alleviated CI index, delayed fruit colour development, softening and ripening as well as maintained quality through reduction of ethylene production and respiration rate during ripening period following 2- and 4-week cold-stored fruit.

CHAPTER 10

Future research

This study has provided some basic information about the changes in the endogenous level of plant hormone(s) and their regulation in mango fruit ripening. It also opens up further research into the following areas:

- The effects of exogenous application of ABA and its NDGA on the expression of genes encoding ACS, ACO, *exo*-, e*ndo*-PG, and EGase during ripening period warrants to be investigated.

- Postharvest NO fumigation suppressed and delayed the climacteric peak of ethylene and delay mango fruit softening. Changes in the endogenous levels of NO during mango fruit ripening yet to be investigated.

- The effects of NO fumigation on the expression of genes encoding ACS, ACO, *exo*-, e*ndo*-PG, and EGase during ripening period warrants to be studied.

- NO fumigation alleviated CI in 2- to 4-week cold stored (5 ± 1°C) mango fruit. The effect of NO fumigation on enzymatic and non-enzymatic antioxidants in order to reduce lipid peroxidation during cold storage period of mango is worth of investigations in the future.

- The complex interaction among various plant hormones and NO as a signaling molecule during fruit ripening need to be investigated.

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APPENDIX 1

Analysis/Chemical used	MW (g/mol)	Formula	Product name
Titratable acidity			
1.0 N Sodium hydroxide	NA	NA	EcoLab
1% Phenolphthalein solution	318.32	$C_{20}H_{14}O_4$	Fluka
Total Carotenoid			
Magnesium carbonate	84.31	$MgCO_3 \cdot xH_2O$	Sigma
Acetone	58.08	CH_3COCH_3	Sigma
n-hexane	86.18	$CH_3(CH_2)_4CH_3$	APS
Sodium chloride	54.44	NaCl	Sigma
Determination of ACC content			
Mercury (II) chloride	271.5	$HgCl_2$	Sigma
1-aminocyclopropane-1-carboxylic acid (ACC)	101.1	$C_4H_7NO_2$	Sigma
Sodium hypochlorite	74.44	NaClO	Fisher
Sodium hydroxide (Pellet)	40	NaOH	Sigma
Determination of ACS enzyme			
Potassium phosphate monobasic	136.09	KH_2PO_4	Sigma
Potassium phosphate di-basic	174.18	K_2HPO_4	Sigma
Ethylene diamine tetra acetic acid (EDTA)	292.24	$(HO_2CCH_2)_2NCH_2CH_2N(CH_2CO_2H)_2$	Sigma
2-Mercaptoethanol	78.13	$HSCH_2CH_2OH$	Sigma
Pyridoxal-5'-phosphate	265.16	$C_8H_{10}NO_6P \cdot H_2O$	Merck
Glycerol	92.09	$HOCH_2CH(OH)CH_2OH$	Bio-Rad
Dithiotheritol	154.25	$HSCH_2CH(OH)CH(OH)CH_2SH$	Roche
Polyvinylpyrrolidone (PVP)	Average 10, 000	$(C_6H_9NO)_n$	Sigma
SAM (S-adenosyl methionine)	399.4	$C_{15}H_{22}N_6O_5S \cdot xC_7H_8O_3S$	Sigma
Hepes	238.3	$C_8H_{18}N_2O_4S$	Sigma
Potassium hydroxide	56.11	KOH	Sigma
Triton X-100	Average 625	*t*-Oct-C_6H_4-$(OCH_2CH_2)_xOH$, x= 9-10	Sigma
Mercury (II) chloride	271.5	$HgCl_2$	Sigma
Sodium hydroxide	40	NaOH	Sigma
Sodium hypochlorite	74.44	NaClO	Fisher

Analysis/Chemical used	MW (g/mol)	Formula	Product name
Determination of ACO enzyme			
Tris (hydroxymethyl) methylamine (Tris-base)	121.4	$NH_2 \cdot C(CH_2OH)_3$	Merck
Trizma hydrochloride	157.6	$NH_2C(CH_2OH)_3 \cdot HCl$	Sigma
Sodium bicarbonate	84.01	$NaHCO_3$	Sigma
Glycerol	92.09	$C_3H_8O_3$	Sigma
(+)-Sodium L-ascorbate	198.11	$C_6H_7NaO_6$	Sigma
PVP phosphate	NA	NA	Sigma
ACC	101.1	$C_4H_7NO_2$	Sigma
Ferrous sulphate	278	$FeSO_4.7H_2O$	Sigma
Dithiotheritol	154.25	$HSCH_2CH(OH)CH(OH)CH_2SH$	Roche
Extraction PG, PE, and EGase			
Polyethylene-glycol	62.07	$C_2H_6O_2$	Sigma
Sodium bisulphate	NA	NA	Sigma
Acetic acid	60.05	CH_3CO_2H	Sigma
Sodium acetate	82.03	$C_2H_3O_2Na$	Sigma
Sodium chloride	54.44	NaCl	Sigma
Exo-PG			
Acetic acid	60.05	CH_3CO_2H	Sigma
Sodium acetate	82.03	$C_2H_3O_2Na$	Sigma
Polygalacturonic acid	25, 000-50, 000	$(C_6H_8O_6)n$	Fluka
Borax (Sodium tetraborate)	381.4	$Na_2B_4O_7 \cdot 10H_2O$	Sigma
Boric acid	61.83	BH_3O_3	Fluka
2-Cyanoacetamide	84.08	$C_3H_4N_2O$	Across
Endo-PG			
Polygalacturonic acid	25, 000-50, 000	$(C_6H_8O_6)n$	Fluka
Acetic acid	60.05	CH_3CO_2H	Sigma
Sodium acetate	82.03	$C_2H_3O_2Na$	Sigma
PE			
Sodium chloride	54.44	NaCl	Sigma
Ethylenediamine tetra-acetic acid (EDTA)	292.24	$C_{10}H_{16}N_2O_8$	Sigma
Citrus pectin	NA	NA	Sigma
EGase			
Phosphate-citrate buffer tablets	NA	NA	Sigma
Sodium phosphate di-Basic	141.96	Na_2HPO_4	Sigma
Sodium chloride	54.44	NaCl	Sigma
Citric acid	192.12	$C_6H_8O_7$	Sigma
Carboxymethyl cellulose			Sigma

Analysis/Chemical used	MW (g/mol)	Formula	Product name
Protein analysis			
Coomassie brilliant blue	825.97	$C_{45}H_{44}N_3NaO_7S_2$	Fluka
95% ethanol	46.07	CH_3CH_2OH	Fisher
85% phosphoric acid	98	H_3PO_4	Sigma
Sodium chloride	54.44	NaCl	Sigma
Sugar standard compound			
Sucrose	342.3	$C_{12}H_{22}O_{11}$	Sigma
D-Glucose anhydrous	180.16	$C_6H_{12}O_6$	APS
D-(-)-Fructose	180.16	$C_6H_{12}O_6$	Sigma
Organic acid standard compound			
Ammonium tartrate di-basic	184.15	$C_4H_6O_6 \cdot 2H_3N$	Sigma
Shikimic acid	174.15	$C_7H_{10}O_5$	Sigma
Fumaric acid	116.07	$C_4H_4O_4$	Sigma
Citric acid anhydrous	192.43	$C_6H_8O_7$	Fluka
DL-Malic acid	134.1	$C_4H_6O_5$	Sigma
Total Antioxidant			
Methanol	32.04	CH_3OH	Sigma
Sodium Fluoride	41.99	NaF	Fluka
DPPH (2,2-dipheynyl-1-picrylhydrazyl)	394.32	$C_{18}H_{12}N_5O_6$	Sigma
Ascorbic acid			
Metaphosphoric acid	NA	$(HPO_3)_n$	Merck
EDTA disodium salt	372.24	$C_{10}H_{14}N_2Na_2O_8 \cdot 2H_2O$	Fisher
Folins reagent	NA	NA	Merck

Note:

EcoLab - EcoLab Pty. Ltd., NSW, Australia

Sigma - Sigma-Aldrich Pty. Ltd., NSW, Australia

APS - Asia Pasific Specialty (APS), Chemicals, Ltd., NSW, Australia

Fisher - Fisher Scientific, Victoria, Australia

Bio-Rad - Bio-Rad Laboratories Pty. Ltd., NSW, Australia

Roche - Roche Dianostics GMBH, Mannheim, Germany

Fluka - Fluka, Buchs, Switzerland

Across - Across organics, New Jersey, USA

Merck - Merck Pty, Ltd., Victoria, Australia