Ripening behaviour of capsicum (*Capsicum annuum* L.) fruit

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Abstract

Fruit of *Capsicum annuum L.* (capsicum or pepper) are one of the major sources of red food colourant and pungency for spice production. In the spice production industry, fruit are mechanically harvested at different ripeness stages and fruit colour needs to be synchronised before being processed. However, even though capsicum ripens normally on the plant it often fails to ripen fully and turn red once harvested at the green stage. Attempts to promote ripening of harvested fruits have had limited success and the reason for this has been unclear. This project, therefore, investigated ripening behaviour on and off the plant of capsicum fruit grown in Australia and examined effects of pre- and postharvest applications on ripening of green harvested fruit.

To examine ripening behaviour on and off the plant, capsicum fruit from three different cultivars (a mild paprika type *cv.* "Papri Queen", a cayenne chilli *cv.* "Caysan", and a sweet type bell pepper *cv.* "Aries") were either allowed to ripen naturally on the plant or harvested at three different maturity stages: light green, deep green and breaker. Harvested fruit were stored individually at room temperature and several ripening characteristics including internal ethylene (C_2H_4) and carbon dioxide (CO2) concentration, extractable colour, 1-aminocyclopropane-1-carboxylic acid (ACC) synthase and oxidase activity, and total soluble solid content (TSSC) were studied during storage.

There was very limited involvement of C_2H_4 during ripening of capsicum and the change in ACC synthase and ACC oxidase (two enzymes in C_2H_4 biosynthesis pathway) activity was not closely related to that of C_2H_4. However, it appeared that colour development in *cv.* "Papri Queen" was closely associated with what C_2H_4 production did occur while a climacteric-like peak of C_2H_4 could be observed in all fruit from *cv.* "Caysan".

For all three cultivars, the level of internal CO_2 concentration, extractable colour and TSSC were greater in fruit ripened on the plant followed by fruit harvested at the breaker, deep green and light green stage, respectively. Fruit harvested at the light

green stage failed to change colour properly and had very low levels of internal CO_2 concentration and TSSC while fruit harvested from the breaker stage onwards ripened normally and developed sufficient colour for spice processing. This may suggest a role of external carbon-supply during ripening.

To study the effect of the external-carbon supply during ripening, the stem of fruit were cinctured when fruit reached the light green stage and fruit were left to ripen on the plant. Cincturing delayed colour development of fruit by approximately five days but cinctured fruit were still able to turn red and develop extractable colour higher than the acceptable level of 140 ASTA units. Cincturing did not significantly alter other ripening behaviour such as CO_2 concentration or TSSC. The lack of external carbon-supply is, therefore, unlikely to play a major role in the failure of green harvested fruit to ripen.

To study the effect of application of plant growth regulators (both pre- and postharvest), an effective method of solution application utilising cincturing was firstly developed. Different plant growth regulator solutions including ethephon, naphthalene acetic acid, abscisic acid, jasmonic acid, sucrose, and different combinations of these were applied to fruit at the light green stage to study preharvest effects on ripening parameters during storage. Only treatment with high concentrations of ethephon increased the extractable colour higher than the acceptable level of 140 ASTA units and induced the complete degradation of chlorophyll. To study effects of postharvest application, 10 µL of various plant growth regulators was dropped into the hole created on the stem of harvested fruit for ten consecutive days. Treatment with ethephon significantly increased extractable colour and degraded chlorophyll content of fruit. Pre- and postharvest ethephon treatment strongly up-regulated *Capsanthin-capsorubin synthase* (*Ccs)* gene expression in a manner similar to the up-regulation of *Ccs* observed in fruit ripened on the plant. This explains the effect of C_2H_4 on colour development and also indicates the possible reason for the failure of green harvested fruit to ripen. However, the *Ccs* gene expression and chlorophyll degradation induced by ethephon was not visible until 14 days after harvest which indicated it may not be a direct effect and other signal transduction factors may be involved. When fruit are ripened on the plant, colour development may, therefore, be induced by ripening-related

factors (other than C_2H_4) which is possibly inhibited or inactivated when fruit are harvested at the green stage. C_2H_4 application to fruit at this stage may help to reactivate or recover these factors which in turn induce colour development. Thus, although capsicum fruit show typical non-climacteric behaviour, C_2H_4 appears to be involved in some aspects of the ripening process.

Statement

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university.

To the best of my knowledge and belief, this thesis contains no material previously published or written by any other person except where due reference is made in the text.

I consent to this thesis being made available for photocopying and loan.

Pham Thi Ngoc Thang
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List of Abbreviations

A	absorbance
ABA	abscisic acid
ACC	1-aminocyclopropane-1-carboxylic acid
AOAC	Association of Official Analytical Chemist
ASTA	American Spice Trade Association
B	breaker
BR	breaker red
^{o}C	Degree Celsius
Ccs	capsanthin-capsorubin synthase
CH_3COONa	sodium acetate
C_2H_4	ethylene
CO_2	carbon dioxide
cm	centimetre
DAA	days after anthesis
DAH	days after harvest
DG	deep green
DNA	deoxyribonucleic acid
DR	deep red
DR & D	deep red and partially dried
DW	dry weight
EDTA	ethylenediaminetetraacetic acid
ethephon	2-chloroethylphosphonic acid
FW	fresh weight
g	gram
Hepes	4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid
h	hour
$HgCl_2$	mercury chloride
i.d.	internal diameter

JA	jasmonic acid
KOH	potassium hydroxide
Lcy	lycopene-β-cyclase
LG	light green
LR	light red
LSD	least significant difference
mg	milligram
mL	millilitre
mm	millimetre
mM	millimole
MOPS	4-morpholinopropanesulfonic acid
NaCl	sodium chloride
NaOCl	sodium hypo chloride
NaOH	sodium hydroxide
Na_2HPO_4	sodium phosphate dibasic
nL	nanolitre
nm	nanometre
NAA	naphthalene acetic acid
PVPP	poly(vinylpolypirrolidone)
RNA	ribonucleic acid
s	second
SAM	S-5’- adenosyl methionine
sds	sodium dodecyl sulphate
SE	standard error of the mean
SUC	sucrose
Tris	tris (hydroxymethyl) aminomethane
TSSC	total soluble solid content
UC	University of California (potting mix)
v/v	volume by volume
w/v	weight by volume
µL	microlitre
µmol	micromole
%	percent

Chapter One

Chapter One - General introduction

1.1 Introduction

Capsicum fruits, also called peppers, are one of the major sources of red food colourant and of pungency for spice production. In addition, fruits can be used as fresh vegetables or ornamentals due to their pleasant taste, attractive appearance, medicinal properties, and vitamin content. The world demand for capsicums has been continuously increasing recently, and production increased by 40 % between 1990 and 2000 with about 1.4 million hectares cultivated (Food and Agricultural Organisation) (FAO, 2000).

Capsicums are ranked in the middle range of vegetables in terms of popularity, with a world annual output of 18 million metric tons and 1.4 million hectares of cultivated land used for their production (FAO, 2000). Asian production of fresh capsicums accounts for 56.4 % of world production, followed by the southern European region with about 15 % of world production in 2000. Africa and North and Central America do not produce as many fresh capsicums as other regions, and use most of the total production for dried chilli powder, which is one of the important industries in these regions (Wien, 1997).

Depending on use, fruits can be harvested at two stages: fully mature green for fresh use, and coloured for fresh and other uses. In the spice production industry, once-over harvest machines are used to reduce labour costs, so harvested fruits are usually at different ripeness stages and cannot be directly processed. In order to achieve high colour quality of spice powder, green fruits must turn fully red before being processed and induction of ripening of green harvested fruit is essential.

This chapter reviews the research that has been conducted to characterise the ripening pattern of fruit in general, and of capsicum fruits in particular, as well as factors that can affect that ripening process.

1.2 Literature review

1.2.1 Capsicum species

Cultivated *Capsicum* species are thought to have originated in tropical areas such as Mexico and South America and have spread to the rest of the world over several centuries. There are five main species in the Capsicum genus*: Capsicum annuum, C. baccatum*, *C. chinense*, *C. frutescens* and *C. pubescens*, but the most important species is *C. annuum* (Wien, 1997). There are many distinctive cultivars in *C. annuum* including most of the common commercial types such as sweet pepper (bell, large-fruited pepper), paprika (sweet, smaller-fruited pepper), and chilli (hot pepper) (Rajput and Paruleke, 1998).

1.2.2 Fruit maturation and ripening

The pattern of growth v”Aries” among different fruits, but it may be one of three common types: a single, double, or triple sigmoidal curve of growth (Coombe, 1976). Many fruits, such as apple (Dennis, 1986), banana (Israeli, 1986), and capsicum (Pretel *et al.*, 1995), are reported to have a single sigmoidal growth curve in which there is an initial phase of slow growth, followed by a rapid growth period, and finally a period of declining growth rate in which ripening is often initiated. Other fruits including stone fruits, figs, and grapes show a double sigmoid curve in which there are two rapid growth phases (respectively named Periods I and III) interrupted by one period of little or no growth (Period II). The growth pattern of the kiwifruit (*Actinidia chinensis)* can be described as triple sigmoidal (Bollard, 1970).

The growth rate of capsicum fruit is variable among fruits on the same plant, and depends on many different factors such as flowering order and growing conditions (Wien, 1997). Under most growing conditions, the capsicum plant terminates in a flower after producing eight to ten nodes on the main stem, and this is followed by the primary division of the main stem to form two or three main branches. These will again terminate in a flower after producing one node, and this pattern will repeat several times. In the field, the main stem usually produces five to eight nodes, and

beside the main stem there are typically three or four basal branches (side branches) rising from main-stem nodes that also produce more nodes. Yield of a capsicum plant growth in the field mainly comprises fruits at nodes three and four of the main branch and at nodes one and two of the side branches (Gaye *et al.*, 1992). The active development of these fruits will inhibit the fruit-set at later-formed nodes, so this group of fruit is often at a similar development stage, and the harvest season is quite short. When the capsicum plant is grown commercially in the glasshouse, the main stem can produce many more nodes because basal branches are pruned, and therefore the harvest season will last longer than that of fruit grown in the field.

The carbohydrate level in capsicum fruit during growth and maturation is closely related to the growth rate. During the early rapid growth period, mainly glucose and fructose are accumulated while the accumulation of starch and sucrose dominates the next period, the slow growth phase. The level of starch and sucrose keeps increasing until fruit commence ripening when the level of glucose and sucrose rapidly increases by the degradation of starch and sucrose (Nielsen *et al.*, 1991; Hubbard and Pharr, 1992).

Fruit can be divided into two groups: climacteric and non-climacteric, based on their respiratory behaviour during ripening (Biale and Young, 1981). Climacteric fruit show a sharp increase in respiration associated with significant changes in composition, colour and texture of fruit, whereas non-climacteric fruit demonstrate no dramatic change in either respiration or composition. Another feature distinguishing climacteric fruit from others is the ability to produce C_2H_4 in response to a low internal C_2H_4 concentration, termed autocatalytic production. This capacity is reported to be absent in non-climacteric fruits (McMurchie *et al.*, 1972). Although these criteria seem to be quite clear, there are several variations within the two groups that will be discussed in more detail in the next section.

1.2.3 Respiration and ethylene production during fruit ripening

1.2.3.1 Respiration and ethylene production in climacteric fruits

In typical climacteric fruits, such as banana or apple, the respiratory course has quite a common pattern, in which there is a decline to a lowest value called “pre-climacteric” minimum, followed by a sharp rise to a “climacteric peak” at the onset of ripening, and a gradual decline during a “post-climacteric” period (Biale and Young, 1981). Although the classification of fruits as climacteric or not is mostly based on the respiratory behaviour of harvested fruits (Abeles *et al.*, 1992), many authors report that some climacteric fruits also show the climacteric pattern of respiration when they normally ripen on the mother plant (Rhodes, 1980). However, there are some exceptions with climacteric fruits that have different ripening behaviour when they are either on or off the mother plant.

One of the most outstanding exceptions to the ripening classification is avocado. The detached fruit show a climacteric increase and ripen normally, while they do not ripen when they are on the plant, especially on a branch with functional leaves. This might be explained by the hypothesis that the leaves of the tree supply a ripening inhibitor, and fruits can ripen only when this inhibitor disappears or becomes inactive (Biale and Young, 1970).

Another example of different ripening behaviour of attached and detached fruits is observed in tomato and muskmelon. Saltveit Jr. (1993) investigated and compared internal CO_2 and C_2H_4 levels in ripening tomatoes on and off the mother plant and found that detached tomatoes exhibited a two-fold increase in respiration coinciding with a ten-fold rise in C_2H_4 concentration as in other typical climacteric fruits. However, tomato fruits which ripened on the plant showed a climacteric increase (about 20-fold) in C_2H_4 levels only, while carbon dioxide slightly increased throughout ripening. The same observations were reported by Shellie and Saltveit (1993) for ripening of muskmelon. Further work to determine the factors responsible for the differences in ripening characteristics of attached and detached fruits is, therefore, essential to find factors responsible for the difference in ripening behaviour.

The involvement of C_2H_4 in fruit ripening has been studied for several decades, especially since the development of advanced techniques to measure C_2H_4. The role of C_2H_4 in ripening of climacteric fruit has attracted many researchers because of its capacity to be produced autocatalytically (Biale and Young, 1981). Climacteric fruit are reported to produce C_2H_4 during their whole life (Burg and Burg, 1965). C_2H_4 production, which is at high levels during flowering, remains at low levels while fruits grow and mature. Around the onset of ripening and the climacteric rise of respiration, C_2H_4 production increases dramatically to reach a peak, and is maintained at relatively high rates throughout the remaining period (Rhodes, 1980). Depending on species, the increase in C_2H_4 production can precede (avocado, banana), coincide with (apple, pear) or follow (tomato, papaya) the peak in respiration (Biale and Young, 1981).

C_2H_4 induction of ripening may be better demonstrated by considering not the exact timing of the rise in C_2H_4 production, but rather the internal C_2H_4 concentration in the tissue of fruits (McGlasson *et al.*, 1978). Studying internal C_2H_4 concentration in fruits at the pre-climacteric stage and applying propylene (an analogue of C_2H_4) to induce ripening allowed researchers to introduce the concept of a threshold concentration of C_2H_4, which must accumulate in the tissue to trigger ripening (Biale and Young, 1970; Rhodes, 1980). This minimum sufficient level which can be obtained by both internally developed or externally applied C_2H_4, may not be exactly the same among different fruits, but many authors have reported that 0.1 $\mu L\ L^{-1}$ was sufficient to initiate the climacteric rise (Burg, 1962).

There is also a wide range of internal C_2H_4 concentrations reported in climacteric fruit during maturation and ripening (Rhodes, 1980). The lowest value is often found in fruits at the pre-climacteric stage, while the highest value, several times higher than the lowest, is usually found from fruits at the onset of ripening as a result of autocatalytic C_2H_4 activity. McMurchie *et al.* (1972) proposed two systems of C_2H_4 production: *System 1 and System 2*. In *System 1*, C_2H_4 presents at low levels in pre-climacteric fruits and its accumulation is initiated by a factor involved in the process of aging in the tissue, resulting in a sufficient threshold level to induce the ripening

process and to produce C_2H_4 autocatalytically in *System 2.* More details of the two systems of C_2H_4 as well as the biosynthesis of C_2H_4 will be discussed in the section on genetic control (section 1.2.5.3).

1.2.3.2 Respiration and ethylene production in non-climacteric fruits

Non-climacteric fruit are characterised by a low rate of respiration and C_2H_4 production (Biale and Young, 1981). As a typical example of non-climacteric fruits, citrus fruits show a downward trend in respiration accompanying slow changes in fruit composition. In addition, internal C_2H_4 concentration in these fruits remains at low levels with some slight fluctuation after harvest (Rhodes, 1980). Non-climacteric fruits are considered to lack the autocatalytic capacity to produce endogenous C_2H_4 in response to exogenous C_2H_4 application, and to produce C_2H_4 under the control of *System 1* (Oetiker and Yang, 1995). However, non-climacteric fruit can also demonstrate an increase in respiration under various conditions and at different stages of maturity.

Grapes display one of the variations in non-climacteric fruits. They show a respiratory increase in the onset of veraison (period III of the double-sigmoidal growth curve) accompanying both changes in composition and increase in fruit size due to renewed cell expansion (Rhodes, 1980). However, Biale and Young (1981) suggested that the increase in respiration in veraison could not be considered as a "climacteric" pattern, since veraison occurred during the growth phase of the fruit and fully mature fruits showed only the downward trend of respiration as for other non-climacteric fruits. Moreover, there is no increase in C_2H_4 production corresponding to the rise in respiration at veraison, and the internal C_2H_4 concentration even falls before veraison and slowly declines throughout period III (Rhodes, 1980). Finally, the lack of autocatalytic ability to produce endogenous C_2H_4 in response to exogenous C_2H_4 application in grapes is a characteristic of non-climacteric fruits.

Capsicum fruits are difficult to classify as climacteric or non-climacteric, because ripening behaviour is often quite different among species and cultivars, as well as

between attached and detached fruits. Gross *et al.* (1986) found that the mature green harvested chilli from *cv*. Chooraehong showed a climacteric increase in respiration when fruits were about 50 % red, although C_2H_4 production was at low levels. However the chilli from *cv.* Changjiao was reported to be non-climacteric since there was no increase in respiration and this fruit responded to C_2H_4 application as other non-climacteric types (Lu *et al.*, 1990). Sweet capsicums or bell peppers also behave differently depending on cultivar and on whether they are on or off the mother plant. Bell peppers from *cv.* Maor, which were harvested at green and red stages, showed a non-climacteric pattern of both external production and internal concentration of CO_2, but external production and internal concentration of C_2H_4 increased significantly during storage (Lurie *et al.*, 1986). Another cultivar of bell pepper, Yolo Wonder, when harvested green mature, was found to exhibit a non-climacteric pattern of both CO_2 and C_2H_4 production during colour development (Villavicencio *et al.*, 1999).

By measuring CO_2 and C_2H_4 production of just-harvested bell peppers at different stages from mature green to red, Villavicencio *et al.* (2001) observed wide variations of respiration and C_2H_4 pattern among different cultivars. He found that in some cultivars respiration activity of fruits at the mature green stage was higher than that at other stages, while in others there was an increase in CO_2 production when fruit turned red. In most cultivars, C_2H_4 production showed a climacteric-like pattern in which there was a peak observed in fruits at either breaking or light red stage. Therefore he suggested that it might be very difficult to give an exact definition to this genus. Chile peppers from *cv.* New Mexican also exhibited a decline in respiration along the course of senescence, but C_2H_4 production reached two peaks: the lower when fruits were at the rapid growth phase and the higher when fruits were totally red (Biles *et al.*, 1993). Differences in behaviour on and off the plant were observed for fruit from paprika *cv.*PS72285 and "Caysan" chilli *cv.*SPS705, which behaved as non-climacteric during storage, but showed climacteric-like pattern of respiration and C_2H_4 production if measured immediately after harvest (Krajayklang *et al.*, 2000). An increase in CO_2 production was observed in fruits changing colour from deep green to breaker, and C_2H_4 evolution reached a peak in fruits at the light red stage. Experimenting with attached pepper fruits, Villavicencio *et al.* (2001) detected a climacteric-like pattern of CO_2 and C_2H_4 concentration in almost all

cultivars. The ripening behaviour of capsicum fruit, therefore, seems to be dependent upon their attachment to the mother plant.

1.2.4 Major changes during fruit ripening

1.2.4.1 Colour change

Colour change is one of the visible signs of ripening, and plays a key role in determining eating and processing quality. Most fruits undergo colour change during ripening, except some kinds of fruit that retain their green colour when fully ripe such as certain varieties of green apple and pear. Colour change during ripening is caused by the transition of chloroplasts containing green pigments into chromoplasts rich in red or yellow pigments due to the degradation of chlorophyll. This, in turn, uncovers other previously existing pigments in chloroplasts, especially β-carotene. The biosynthesis of new pigments during ripening also results in changing fruit colour (Tucker, 1987).

The mechanism of chlorophyll degradation during fruit ripening is not completely understood, but it has been recently reported that chlorophyll may undergo three main steps catalysed by enzymes to form colourless catabolites (Figure 1.1).

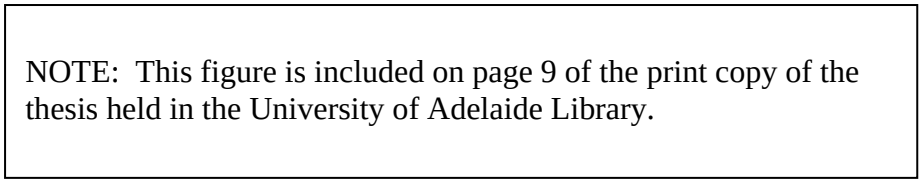

Figure 1.1 The chlorophyll degradation pathway in higher plants adapted from Hortensteiner (1999). The enzymes involved in each step are in italics.

There are several studies on chlorophyll degradation and related enzyme activities during ripening of capsicums (Mosquera and Mendez, 1994; Mendez *et al.*, 2000; Deli *et al.*, 2001). In the common types of capsicums, chlorophyll content decreases during the course of ripening so that there is no chlorophyll present when fruits turn fully red. In other cultivars, for instance paprika cv. Negral, fully ripe fruits have a brownish colour and retain a significant level of chlorophyll, about 0.15 to 0.2 mg g^{-1}DW (Biles *et al.*, 1993; Mendez *et al.*, 2000). However, there is not much information on the change in chlorophyll and the activities of the enzymes that catalysed the degradation of chlorophyll in fruits after harvest, especially in green harvested fruits, which were reported not to turn red post-harvest. Further research on this may help to determine possible factors responsible for abnormal colour change in ripening of green harvested fruits.

Carotenoids, which are located within chloroplasts and chromoplasts, are pigments contributing to the yellow to red colour in many fruits. There are various types of carotenoids, of which the most common ones are lycopene and β-carotene, and their

amounts in fruit vary depending on species (Goodwin and Goad, 1970). Carotenoids can be synthesised in green tissue and these carotenoids often change from chloroplast-types to chromoplast-types during ripening. Carotenoid levels in the peel of banana remain almost constant during ripening, but fruits turn from green to yellow due to the disappearance of chlorophyll (Palmer, 1970). However, in many fruits, levels of carotenoids often increase dramatically due to *de novo* biosynthesis during fruit ripening (Seymour *et al.*, 1993). The primary carotenoid biosynthesised from acetyl-CoA is phytoene; this compound in turn is converted into further acyclic carotenoids such as lycopene, which in turn becomes the precursor for β-carotene biosynthesis (Goodwin and Goad, 1970).

Figure 1.2 The carotenoid biosynthesis pathway in capsicum fruit adapted from Camara *et al.* (1995). The enzymes for each step are in italics.

The biosynthesis of carotenoids during ripening of capsicums shown in Figure 1.2 have been investigated by many authors (Camara *et al.*, 1995; Mendez *et al.*, 2000; Deli *et al.*, 2001). All carotenoids in capsicums can be divided into two isochromatic families: red containing the pigments specific to the capsicum genus such as capsanthin, capsanthin-5, 6-epoxide, and capsorubin; and yellow comprising of the pigments zeaxanthin, violaxanthin, antheraxanthin, β-cryptoxanthin, β-carotene, and curcubitaxanthin A (Mendez *et al.*, 2000). The total amount of carotenoids in unripe capsicum fruit is 0.18 to 0.3 g kg^{-1}DW, and it increases to 7 to 13 g kg^{-1}DW at the full ripe stage (Deli *et al.*, 2001). There are several studies conducted on the change and the biosynthesis of carotenoid compounds during pepper fruit ripening. The disappearance of lutein and neoxanthin that exist in the chloroplast, the rise of

concentrations of β-carotene, antheraxanthin, violaxanthin and the *de novo* biosynthesis of zeaxanthin, capsanthin, β-cryptoxanthin, curcubitaxanthin A, capsanthin-5,6-epoxide, and capsorubin are three main factors contributing to the red colour of ripe capsicums (Mendez *et al*., 2000). In the cultivar Negral, the brownish colour of ripe fruits results from both remaining chlorophyll and newly biosynthesised carotenoids (Amela *et al.*, 1996). However, there is not much research on the biosynthesis of carotenoids in harvested fruits. Further study on chlorophyll degradation and the carotenoid biosynthesis pathway in harvested fruits will help to find which steps in the pathways or which enzyme activities are inhibited leading to the lack of red colour development in ripe fruits.

In the spice industry, the minimum acceptable level of extractable colour v"Aries" among manufacturers and processors and the price for the spice product increases with the level of extractable colour. According to the US Government standard, the minimum acceptable level for paprika and chilli powder was 110 and 70 ASTA units respectively (Purseglove *et al*., 1981). However, the level between 140 and 300 ASTA units was usually preferred by the processor (Larkcom, 1995) and the acceptable level of 140 for paprika and of 110 ASTA units for chillies were used in this study.

1.2.4.2 Fruit softening

The main factor contributing to fruit softening is modification of the fruit cell walls (Seymour *et al.*, 1993). The major change in fruit cell walls during ripening is thought to be the loss of galactose and arabinose of the neutral pectins, and increase of soluble pectins (Seymour *et al.*, 1993). These changes are considered to result from the action of cell-wall hydrolase enzymes such as polygalacturonase (PG), cellulase, pectinesterase (PE), and β-galactosidase. PG and cellulase are reported to be absent or inactive in unripe fruit, but have high activities during fruit ripening, especially PG (Harpster *et al*., 1997; Rao and Paran, 2003), whereas the highest activity of PE and β-galactosidase can be found during both development and ripening of fruit. Thus, the modification of cell wall may not be regulated by a single enzyme through a single mechanism and probably involves an interaction of several

enzymes via different mechanisms (Harpster *et al.*, 2002). Further research, therefore, is needed to determine how exactly the cell walls are modified, the precise relationship between these changes and actual softening of fruit as well as the collaboration between different physiological and biochemical changes during the ripening process of capsicum fruit.

1.2.5 Ripening regulation

1.2.5.1 Growing conditions

Growing conditions usually play a key role in fruit set and development, and thus influence the ripening process. It is obvious that growing conditions vary with species, variety, and cultivar. Even for capsicum fruits, the optimal conditions of environment and cultivation are not the same among different cultivars. Capsicum plants can be grown in tropical climates all year round as well as sub-tropical climates during the warmer months (Wien, 1997). Paprika and sweet pepper may demand lower temperatures than hot pepper, because the higher temperature results in higher levels of the pungent compound, capsaicin, in capsicums (Rajput and Paruleke, 1998). Although growing conditions, especially in the post-anthesis period, have a great impact on fruit set and development, there is little available information on the influence of environmental factors and agricultural practice on ripening of capsicum. Some studies reported the importance of potassium and phosphorous fertilisers because low levels of these nutrients leads to poor colour (Boronat *et al.*, 2002). To hasten the ripening process, spraying the plant with the C_2H_4-generating compound (ethephon) before harvest is considered highly effective (Wien, 1997). However, its effectiveness v”Aries” with cultivar, concentration of ethephon, number of sprays, and many other environmental and physiological factors (Cantliffe and Goodwin, 1975). Some studies on pepper cv. Tabasco reported an increase in immature fruit abscission accompanying improvement of fruit colour with applied ethephon, with the negative impact minimised by the addition of $Ca(OH)_2$ (Conrad and Sundstrom, 1987). In contrast, when ethephon was applied to hasten ripening of paprika, there was no effect on either the proportion of red fruits or the extractable colour of paprika powder (Cooksey *et al.*, 1994).

1.2.5.2 Plant growth regulators

Plant growth regulators (PGR) such as auxins, gibberellins, cytokinins, C_2H_4 and abscisic acid, salicylic acid and jasmonic acid are considered to play an important role in the regulation of several different processes in plants including the ripening process (McGlasson *et al.*, 1978). C_2H_4 acts as a ripening hormone by initiating the ripening process of many fruits, especially climacteric fruits. However, the involvement of other PGRs cannot be ignored, and the complex interaction between C_2H_4 and one or more other PGRs is the most likely control mechanism during fruit ripening (Seymour *et al.*, 1993).

Climacteric fruits respond to C_2H_4 only if applications are carried out at the pre-climacteric stage prior to the onset of ripening, in other words, prior to the burst of autocatalytic C_2H_4 production of fruits. Treatments with C_2H_4 shorten the time to the onset of ripening, but do not change the respiratory pattern, and the height of a peak in respiration is independent of the concentration of C_2H_4. Once ripening is initiated by a sufficient concentration over a long enough period, generally 0.1 to 1 $\mu L\ L^{-1}$ of C_2H_4 for one day, removal of this gas will have no effect on fruits and they can ripen normally due to their autocatalytic C_2H_4 production capacity (Biale and Young, 1981). Recent work on molecular aspects of the mechanism of C_2H_4 action has demonstrated that the activity of ACC oxidase in the pre-harvest period was greatly induced by exogenous C_2H_4, and this would accelerate the stimulation of endogenous C_2H_4 to reach a threshold level (Oetiker and Yang, 1995).

Treatments of non-climacteric fruits with exogenous C_2H_4 at any time of the ripening process will elicit a rise in respiration which does not exist in normal ripening. The height of a respiratory peak is dependent on the concentration of C_2H_4 applied through a logarithmic relationship, but the time when a peak appears is C_2H_4-independent (McGlasson *et al.*, 1978). In addition, exogenous C_2H_4 applications do not change the low level of endogenous C_2H_4 produced by fruits, and removal of the gas will lead the respiration rate to drop to the basal level (Biale and Young, 1981).

Besides the factors mentioned, the effectiveness of applying C_2H_4 to induce ripening also depends on the sensitivity or the resistance of fruit tissue to C_2H_4 (McGlasson, 1985). In some kinds of fruit such as tomato, apple or pear, young fruits have high resistance to externally applied C_2H_4 and often require higher concentrations of C_2H_4 and a longer duration of exposure (McGlasson *et al.*, 1978). In other fruits such as avocado or mango, some unknown ripening "inhibitors" supplied by the mother plant are considered to retard the effect of C_2H_4, and it might take time for harvested fruits to increase sensitivity to C_2H_4 due to the disappearance of ripening inhibitors (McGlasson, 1985).

The response of capsicums to applied exogenous C_2H_4 is variable, and under some conditions, negligible. The respiratory rate of green harvested hot peppers from *cv.* Changjiao was found to increase significantly in proportion with concentrations of applied C_2H_4, and it dropped to the basal level if the gas was removed (Lu *et al.*, 1990). However, Saltveit Jr. (1997) found that there was no increase in either respiration or C_2H_4 evolution of mature green bell pepper fruits exposed to 500 ppm propylene for 48 h. Similar results were found with paprika from *cv.* PS 72285 and chilli from *cv.* SPS 705 stored in an atmosphere with 100 μL L^{-1} C_2H_4 (Krajayklang *et al.*, 2000). Application of C_2H_4 and C_2H_4-generating compounds to enhance red colour development in harvested peppers has long been reported, but its effectiveness is not as high as expected. Although C_2H_4 is accepted as a degreening agent for many fruits such as citrus, banana and muskmelon, treatments with C_2H_4 and C_2H_4-generating compounds were not able to enhance the red colour of green harvested capsicums. No promotion of colour change was found for harvested fruits placed in a closed atmosphere containing 100 μL L^{-1} C_2H_4 (Krajayklang *et al.*, 2000). Ethephon (2-chloroethyl phosphonic acid) is a common C_2H_4-generating agent used to accelerate the colour development of harvested fruits. However, as with C_2H_4, treatments with ethephon had little effects on green harvested fruits and it was highly effective only if treatments are carried out with fruits that have some red spots on their surface (Lockwood and Vines, 1971). Generally, applying exogenous C_2H_4 did not promote the colour development of green harvested fruits and the reason for this is far from clear.

Beside C_2H_4, abscisic acid (ABA) is another PGR that has long been known to be involved in fruit ripening. It has been reported that ABA played an important role in regulating the abscission of young and pre-harvest fruits (McGlasson *et al.*, 1978) but the role of ABA in regulating fruit ripening is quite unclear and most of the suggestions come from the results of applying exogenous ABA to fruit during ripening (Jiang and Joyce, 2003). It was found that ABA promoted ripening in climacteric fruits such as apple and banana mainly by enhancing C_2H_4 biosynthesis, whereas in non-climacteric fruits such as orange, ABA was shown to induce earlier degreening and to stimulate carotenoid biosynthesis (Buesa and Vendrell, 1989; Riov *et al.*, 1990; Jiang *et al.*, 2000). However, there were several other reports suggesting that increasing C_2H_4 levels in fruit tissue brought about a rise in ABA levels rather than the converse, and in order to elucidate the effects of exogenous ABA and its role during fruit ripening, further work is essential. The endogenous level of ABA and its change during fruit ripening v”Aries” depending on the species of fruit, and there is relatively little information on ABA levels, its change and its role during capsicum ripening.

Another PGR that has been shown to be involved in the ripening process is auxin. However, the role of auxins in the control of fruit ripening is not clear, and the effect of applying exogenous auxins is quite variable depending on the species of fruit and the method of application (McGlasson *et al.*, 1978). Davies (Davies *et al.*, 1997) found that treatment of grape berries with synthetic auxin delayed the onset of ripening by affecting the expression of genes regulating the ripening process, while auxin was shown to promote the ripening process in peach (Ohmiya, 2000). There have been several reports on the interaction between auxins and C_2H_4 that have suggested an auxin-induced increase in C_2H_4 production (Hansen and Grossmann, 2000). However, there is limited information on auxin levels and its role during ripening of capsicum fruits, or on the effect of applying auxins to induce ripening of harvested capsicum fruits.

1.2.5.3 Genetic control

The ripening process appears to be genetically controlled with many genes being upregulated during ripening resulting in physiological and biochemical changes in ripening fruit (Seymour *et al.*, 1993; Picton *et al.*, 1995). C2H4 has been considered to induce ripening in climacteric fruit by up-regulating several genes including C2H4 biosynthesis genes leading to the autocatalytic response in the presence of C2H4 (Biale and Young, 1981; McGlasson, 1985; Jones *et al.*, 2003; De Paepe *et al.*, 2004).

It has long been known that C2H4 was biosynthesised from methionine (Figure 1.3). Methionine, a biological precursor of C2H4, is activated by ATP to form S-adenosyl-L-methionine (SAM). The next step is the conversion of SAM to 1-aminocyclopropane-1-cacboxylic acid (ACC) catalysed by ACC synthase, and this step is considered as rate limiting in C2H4. The metabolism of ACC to C2H4 is catalysed by ACC oxidase.

NOTE: This figure is included on page 17 of the print copy of the thesis held in the University of Adelaide Library.

Figure 1.3 The ethylene biosynthesis pathway (Adams and Yang, 1979). The enzymes for each step are in italic.

Research on molecular aspects of the biosynthesis of C2H4 ripening, reviewed by Oetiker (Oetiker and Yang, 1995), has contributed interpretation to the concept of

System 1 and *2* of C_2H_4 given by McMurchie (McMurchie *et al.*, 1972). During the pre-climacteric period, fruits produce a low level of C_2H_4 (*System 1* of C_2H_4 production), which induces the activity of ACC oxidase accompanied by an increase of the internal C_2H_4 concentration (IEC) to the autocatalytic threshold. In *System 1*, the activity of ACC synthase is low and the ACC content increases only slightly, but when a threshold of IEC is achieved, the activity of ACC synthase is induced by the up-regulation of ACC synthase expression leading to the burst of ACC, and of autocatalytic C_2H_4 production of *System 2* (Kim, 2006; Owino *et al.*, 2006; Park *et al.*, 2006).

Apart from C_2H_4 biosynthesis genes, the C_2H_4 receptors and the signal transduction pathway have been established (Bleecker and Kende, 2000; Chen *et al.*, 2005; Etheridge *et al.*, 2006). Many members of the C_2H_4 receptor family and different elements of the transduction pathway have been characterised and the C_2H_4 responsive-element in the promoter region of several genes has also been isolated (Montgomery *et al.*, 1993). These findings have contributed to the establishment of the C_2H_4 mechanism of action in many processes in plants. However, such a study in the perception and signal transduction of C_2H_4 during ripening of capsicum fruit has not been conducted yet.

In capsicum fruit, many ripening-related genes have been characterised including the C_2H_4 biosynthesis genes ACC synthase and ACC oxidase (Harpster *et al.*, 1996); the cell wall degradation genes polygalacturonase, cellulase (Harpster *et al.*, 2002; Rao and Paran, 2003); the chlorophyll degradation genes chlorophyllase, Mg-dechelatase and pheophorbide α oxigenase (Moser and Matile, 1997) and especially the carotenoid biosynthesis pathway genes including phytoene desaturase *Pds* (Hugueney *et al.*, 1992), ζ-carotene desaturase *Zds* (Breitenbach *et al.*, 1999), lycopene β cyclase *Lcy* (Hugueney *et al.*, 1995), zeaxanthin epoxidase *Zepd* (Bouvier *et al.*, 1996) and capsanthin-capsorubin synthase *Ccs* (Bouvier *et al.*, 1994). Although the expression of these genes during capsicum fruit ripening on the plant has been investigated, there is little information available on their expression in harvested fruit and whether or not these genes are C_2H_4-controlled.

1.3 Research aims

Several aspects of the ripening behaviour of capsicum fruits have been established, but a number of questions still remain. Firstly, many previous studies on ripening characteristics of capsicum fruit have led to different conclusions, and whether capsicum fruit may be described as climacteric is still controversial. Different conclusions may have been arrived at because of different pre-harvest factors, different cultivars used for experiments, and different sampling techniques and measuring methods. Secondly, most of the previously reported studies were conducted on either attached or detached fruit without comparing many characteristics of ripening between the two kinds. Thirdly, there has been limited research conducted on the degradation of chlorophyll and the biosynthesis of carotenoids during the post-harvest period of detached fruit, and which factors are responsible for the failure of red colour development in these fruits is not fully understood. Finally, there have been a limited number of studies on ripening on and off the plant of capsicum cultivars that are grown under Australian climatic conditions and agricultural practices. The objectives of this research, therefore are:

- To compare several ripening characteristics on and off the plant of fruit from three different cultivars.
- To examine the effect of pre- and postharvest factors including cincturing and application of several plant growth regulators on colour development of green harvested fruit.

Chapter Two

Chapter Two - General materials and methods

2.1 Introduction

This chapter describes the general common materials and methods that were used for the majority of the experiments. Specific materials and methods for particular experiments will be described in the relevant chapters.

2.2 Plant materials

2.2.1 Cultivar selection

Three cultivars of pepper (C*apsicum annuum L.)* were selected to reflect three main types of pepper (Figure 2.1): Cayenne "Caysan" hot chilli SPS705, mild type "Papri Queen" paprika SPS2591 and sweet type "Aries" bell pepper SPS608. Their characteristics are further described below.

"Papri Queen" paprika SPS2591 ("Papri Queen") is a mild-pungent type with long, slightly curved fruit that are around 100 to 150 mm long and 25 to 40 mm wide at the shoulder. The fruit are generally used for the production of ground and powdered paprika powder based on its high intensity of red colour.

Cayenne "Caysan" hot chilli SPS705 ("Caysan") is a hot type with linear, oblong and usually pointed fruit that are 80 to 120 mm long and 15 to 25 mm wide. "Caysan" can be used for both powder processing and for cooking due to its high level of pungency and flavour.

"Aries" bell pepper SPS608 ("Aries") is a non-pungent type with bell shaped fruit. These fruit are relatively large, being 500 to 700 mm long and 400 to 600 mm wide, with thick and juicy flesh. "Aries" is mostly consumed fresh.

In addition, “Caysan” and “Papri Queen” are the most appropriate type for spice production in Australia.

Figure 2.1 Three cultivars used for experimentation described in this thesis: “Papri Queen” (left), “Caysan” (middle), and “Aries” (right).

2.2.2 Plant growth

Hybrid seeds of the three selected cultivars (see section 2.2.1) were obtained from Peto Seed Company (New York, USA) through the distributor South Pacific Seeds (Adelaide, Australia). Seeds were sown in 4-inch diameter round pots containing “UC mix” with the rate two or three seeds per pot depending on the desired germination percentage. UC mix (the University of California mix, adapted version by Waite Plant Research Centre) consisted of two thirds of washed Waikerie sand, one third of peat moss with the addition of fertiliser (2.25kg/m^3 Osmocote® plus 15N:4.8P:10.8K:1.2Mg), hydrated lime (0.7kg/m^3) and Ag-lime (1kg/m^3).

Seedlings were germinated and grown in a temperature controlled glasshouse (25±5^{o}C for both day and night without humidity control) at the University of Adelaide, Waite Campus. During the period between June and October of each year, supplemental lighting was provided to ensure the 12h light/12h dark cycle. Daily

irrigation was provided throughout growing season by hand-watering to maintain soil moisture (pots watered until flow-through).

Seeds germinated within 1 to 2 weeks and seedlings reached the four leaf stage by 6 to 7 weeks (Figure 2.2). At this time, young seedlings were transplanted into larger 5-inch square pots containing the UC mix previously described and the plants were then randomly allocated on a bench such that the distance between pots was never less than 20 cm.

When the plant produced the first flower, a slow-release fertiliser (Osmocote® plus 15N:4.8P:10.8K:1.2Mg) was applied to the top of the soil at a rate of 2 to 3 g per plant and re-applied 6 weeks later. Pest and disease control were implemented when necessary (Dichlorvos fumigation for aphids and Omite or Gremlin spraying for mites).

Figure 2.2 Seedlings of “Papri Queen” (after germination) in 4-inch round pots (left) and transplanted seedlings in 5-inch square pots (right).

2.2.3 Pollination and fruit set

Generally, self-pollination was the common means of reproduction of all three cultivars. However, in “Aries” they were manually pollinated with a small brush to ensure proper fruit set. To maintain the homogenous population for experimentation, only one fruit from the first flower of each plant was used for experiments and all flowers used were labelled with the anthesis date (Figure 2.3). Fruit with abnormal development or deformation were discarded during pre-harvest assessment.

Figure 2.3 The first flower of plants was labelled with the anthesis date. A “Caysan” plant is shown as an example.

2.3 Fruit sampling and assessment

2.3.1 Fruit sampling

Fruit were hand-harvested and stored individually in aluminium foil trays loosely covered with polyethylene bags temperature-controlled room at 21 ± 1^{o}C (Figure 2.4). When samplings were required, the weight of an intact fruit was measured and then an internal gas sample for carbon dioxide (CO_2) and ethylene (C_2H_4) measurements was extracted. Based on the experimental design, fruit tissues were either used fresh for enzyme assay, dried for extractable colour measurement or frozen with liquid nitrogen and stored at -80^{o}C for further analysis.

Figure 2.4 Fruit were stored individually in an aluminium foil tray (18x10x5 cm) during postharvest storage. “Papri Queen” fruit are shown as an example.

2.3.2 Gas measurement

The CO_2 and C_2H_4 concentration of the internal atmosphere inside fruit were quantified using gas chromatography (Figure 2.5). Two 1 mL gas samples were withdrawn from the cavity of the fruit using a 1mL syringe with a 25-inch gauge needle.

C_2H_4 concentration was quantified using a Varian chromatograph model 3400 with a flame ionisation detector (Varian Australia, Mulgrave, Vic) and a Porapak Q stainless column (60 cm x 3.1 mm i.d.) of 80/100 mesh. Temperatures for the column, injector and detector were set at 50, 135 and 150 ^oC, respectively. The flow rate was set at 50 mL min^{-1} for the carrier gas (food grade nitrogen), 300 mL min^{-1} for compressed air and 40 mL min^{-1} for hydrogen. The results were expressed as µL L^{-1} for internal concentration of C_2H_4. A standard C_2H_4 sample (concentration of 2.05 µL L^{-1}; BOC gases, NSW) was used for calibration.

CO_2 concentration was measured using a Varian gas chromatograph model 3300 with a thermal conductivity detector (Varian, Mulgrave, Vic) and a silica column (35 cm x 3.1 nm i.d.) of 80/100 mesh. Temperature was set at 29 ^oC for the column and 90 ^oC for both detector and injector. Helium as a carrier gas was supplied at the flow rate of 5 mL min^{-1}. Calibration was set up with 1 mL injection of the standard gas sample containing 5.25 mL L^{-1} CO_2 in air (BOC gases, NSW) and the results were presented as mL L^{-1} g^{-1} for CO_2 concentration per gram of fresh weight.

Figure 2.5 Gas chromatography system (Varian Australia): GC 3400 for measuring CO_2 concentration (left) and GC 3300 for C_2H_4 concentration (right)

2.3.3 Extractable colour measurement

Extractable colour was measured based on the standard method of the American Spice Trade Association (ASTA) and presented as ASTA units (ASTA, 1986; AOAC, 1995). Fruit tissue was cut into small pieces and dried at 40 ^{o}C in an oven until the moisture of samples was approximately 10%. The dry sample was ground by an electric mill (Kika-werke GMBH, Germany; Figure 2.7) into such a fine grade powder that it passed through a No 18 sieve with circular openings of 1 mm diameter. The ground powder was kept in sealed plastic bags and stored in the dark and in dry conditions for no longer than 2 weeks before measurement.

Figure 2.6 Electric mill to grind dry samples (Kika-werke GMBH, Germany).

Ground sample (70 to 100 mg) was placed in a 100 mL volumetric flask and filled to the volume with acetone. The flask was tightly stoppered, shaken and placed in the dark at room temperature (20 to 24 ^{o}C) for 16 h. The supernatant extract was transferred to a 1 mL glass cuvette to measure the absorbance (A) at 460 nm using a spectrophotometer (SP8001, Metertech). Acetone was used as a blank and for each measurement day, an instrumental correction factor was determined by reading the standard glass reference (SRM 2030 glass filter 30%-T26; Figure 2.7) at 465 nm

where A_{465} of the standard glass reference was specified at 0.4962 by the National Institute of Standards and Technology (USA).

The final ASTA value for each measurement was calculated using the following formula:

$$\text{ASTA colour} = \frac{A_{460} \times 16.4 \times \text{instrumental correction factor } (I_f)}{\text{Sample dry weight (g)}}$$

$$\text{Where} \quad I_f = \frac{\text{Declared } A_{465} \text{ of standard glass (0.4962)}}{\text{Actual } A_{465} \text{ of standard glass}}$$

Figure 2.7 The standard glass reference for extractable colour measurement (NIST-USA)

2.3.4 Total soluble solid content (TSSC)

Fresh fruit tissues were quickly cut into small pieces with a scalpel and then ground with a mortar and pestle. The juice was extracted from ground sample by using cheese cloth as the filter. A tiny drop of fresh juice was dropped onto the glass

surface of a refractometer (Bellingham and Stanley, England) using a micropipette. The total soluble solid content was read and presented as ^{o}Brix.

2.3.5 Photography

All pictures in this thesis were taken using a Sony digital camera (DSC-P43, Sony Corp., Japan).

2.4 Statistical analysis

GenStat Release 6.1 for Window (GenStat software, Lawes Agricultural Trust, Rothamsted Experimental Station, England) was used to statistically analyse the data. One-way ANOVA and two-way ANOVA were used to compare the treatment means with the level of least significant difference (LSD) at $P<0.05$.

Chapter Three

Chapter Three - Ripening behaviour of capsicum fruit on and off the plant

3.1 Introduction

Ripening, an important phase of fruit growth and senescence includes many irreversible events that change fruit texture, composition, flavour and colour so that fruit become acceptable and desirable for consumption.

A dramatic change in respiration, ethylene (C_2H_4) production, colour, texture, and flavour is observed during ripening of climacteric fruit while a gradual change is characterised in non-climacteric fruit. Climacteric fruit are also capable of producing C_2H_4 auto-catalytically in response to C_2H_4 while non-climacteric fruit lack this capacity (Biale and Young, 1981). The classification of capsicum fruit as climacteric or non-climacteric has been difficult due to the diversity of ripening behaviour from different cultivars. Sweet capsicum has been widely reported as non-climacteric because of the absence of a climacteric peak in both CO_2 and C_2H_4 production (Biles *et al.*, 1993). In contrast, some hot pepper cultivars were considered as climacteric with the peak reached when fruit surface colour was 30 to 40 % red (Gross *et al.*, 1986).

Capsicum fruit harvested at different maturity stages also behave differently during ripening in comparison to fruit left to ripen on the plant. Interestingly, the climacteric-like pattern in CO_2 and C_2H_4 production has been observed in fruit ripening on the plant or in just-harvested fruit (Villavicencio *et al.*, 1999; Krajayklang *et al.*, 2000), but often not if harvested green.

Because the research thus far has been contradictory, *the aim of this study was to examine the ripening behaviour of fruit from three capsicum cultivars when left to ripen on the plant and when harvested at different ripeness stages.*

3.2 Materials and methods

3.2.1 Plant materials

The fruit used for experimentation described in this chapter were grown in a glasshouse at the University of Adelaide (as described in section 2.2.2). “Papri Queen” and “Caysan” were planted in March 2004 with fruit initially setting from June to July 2004. “Aries” and an additional “Papri Queen” crop were planted in October/November 2004 with fruit setting in January/February 2005. The “Papri Queen” crop planted in March 2004 was used to develop the growth curve only. Preparation and fruit setting assessment were carried out as described in section 2.3.

3.2.2 Growth curve determination

From each population, 10 to 15 fruit were randomly selected to determine their growth curves. Fruit length and diameter were measured with a digital caliper (DigiMax, United Kingdom) from 21 to 28 days after anthesis to avoid any damage to young fruit. Measurements were carried out weekly during the early growth and late ripening periods and every second day when fruit were nearly mature and had started ripening. The onset of ripening was determined as when fruit growth eventually ceased and fruit at this stage were referred to as light green (LG) fruit.

3.2.3 Sampling time determination

Sampling times were determined based on the change of skin colour of fruit ripened on the plant (Table 3.1). The first stage, the LG stage (scored as 0) was determined from the growth curve. Six other stages were identified by scoring fruit visual colour every day from the LG stage based on a subjective scale developed by Krajayklang (Krajayklang, 2001). Sampling was at colour score 0, 1, 2, 5, 9, 10 and 11 (light green, deep green, breaker, breaker red, light red, deep red, and deep red and partially dried).

Table 3.1 Score for external colour of capsicum fruit ripened on the plant and seven sampling times (highlighted in yellow).

Colour score	Colour stage	Description
0	Light green (LG)	Totally green but with low intensity - ***Sampling time 1***
1	Deep green (DG)	Completely green and intense colour - ***Sampling time 2***
2	Breaker (B)	Chocolate colouration with no reddening - ***Sampling time 3***
3	Breaker red	20 to 30% red surface colouration
4	Breaker red	30 to 40% red surface colouration
5	Breaker red (BR)	40 to 50% red surface colouration- ***Sampling time 4***
6	Breaker red	50 to 60% red surface colouration
7	Breaker red	60 to 75% red surface colouration
8	Breaker red	<100% red surface colouration
9	Light red (LR)	100% red with low intensity – ***Sampling time 5***
10	Deep red (DR)	100% red, high intensity and succulent – ***Sampling time 6***
11	Deep red and partially dry (DR&D)	100 % red with intense colour, partially dried – ***Sampling time 7***

The duration from the anthesis date to each of these seven stages was recorded and used for the sampling dates, which are presented as days after anthesis (DAA).

3.2.4 Experimental design and methods

3.2.4.1 Experimental design

The experiments to study ripening behaviour of fruit on and off the plant were designed as completely randomised for each cultivar with harvest date as the treatment factor for each independent experiment. The experimental unit was a single fruit on a single plant and each treatment contained six fruits as six replicates. Fruit were either left to ripen on the plant or harvested at three different stages: light green (LG), deep green (DG), and breaker (B). These three harvest dates were determined as per section 3.2.3.

Harvested fruit were stored individually at room temperature in an aluminium foil tray covered with a polyethylene bag to maintain humidity. During ripening or storage, fruit were sampled several times as described in sections 2.3.1 and 3.2.3 (Table 3.1).

Several ripening parameters were examined including internal CO_2 and C_2H_4 concentrations, extractable colour, total soluble solid content (TSSC) and 1-aminocyclopropane-1-carboxylic acid (ACC) oxidase and synthase enzyme activities. Methods for determining CO_2 and C_2H_4 as well as extractable colour and TSSC were as described in section 2.3.

3.2.4.2 ACC synthase activity

To measure the capacity of the tissue to convert exogenous S-5'-adenosyl methionine (SAM) to ACC, approximately 2 g of fruit tissue were quickly cut into 1 mm thick-discs with a sharp scalpel and incubated in a 14 x 160 mm test tube containing 10 mL of extraction buffer consisting of 0.1 M Hepes-KOH pH 8.5, 5 µM Pyridoxal 5'-phosphate (PLP), and 100 µM SAM as substrate (Satoh *et al.*, 1997). The tube was closed and placed in a water bath at 30 ^{0}C for 2 h and 0.5 mL of the reaction mixture was used to determine ACC content according to the method described by Lizada (Lizada and Yang, 1979). The mixture containing ACC was added to 0.1 mL of 1mM $HgCl_2$ in a 12 x 75 mm test tube and water was added to a total volume of 0.9 mL. The reaction tube was sealed with a serum rubber stopper and kept in ice while a cold mixture of 5 % NaOCl and saturated NaOH (2:1; v/v) was prepared. 10 drops (approximately 0.1 mL) of this mixture were dropped into the reaction tube through the stopper using a 1 mL syringe fitted with a 25-gauge needle. The reaction mixture was shaken for 5 s before incubation in ice for a further 2.5 min. It was agitated for 5 s prior to gas sampling and C_2H_4 concentration of the internal atmosphere was measured using gas chromatography as described in section 2.3.2. The total amount of C_2H_4 produced by 1 g of fresh tissue in 1h was calculated.

The amount of ACC was determined using a standard curve presenting the relationship between ACC and C_2H_4. Different amounts of ACC were added to the 0.5 mL mixture obtained from the enzyme reaction and amounts of C_2H_4 produced

by each sample were recorded to determine this curve. To predict the amount of ACC from obtained C_2H_4 data, GenStat Release 6.1 for Window (GenStat software, Lawes Agricultural Trust, Rothamsted Experimental Station, England) was used. ACC synthase activity was determined as the amount of ACC converted by 1 g of fruit tissue in 1 h ($\mu mol\ g^{-1}\ h^{-1}$).

3.2.4.3 ACC oxidase activity

The capacity of the tissue to convert exogenous ACC to C_2H_4 was measured in a similar manner to ACC synthase activity. Approximately 1 g of fruit discs was incubated in a 14 x 160 mm test tube containing 10 mL of extraction buffer consisting of 0.025 M Hepes-Tris pH 7.5, 0.5 M sorbitol, and 1 mM ACC as substrate for 1 h (Pretel *et al.*, 1995). The reaction tube was then tightly closed and incubated at 30 ^{0}C with shaking at 50 rpm for 1 h. A gas sample of the internal atmosphere (1 mL) was withdrawn using a 1 mL syringe with a 25-gauge needle and C_2H_4 concentration was determined as described in section 2.3.2. The activity of ACC oxidase was determined as the volume of C_2H_4 converted by 1 g of fruit tissue in 1 h ($nL\ g^{-1}\ h^{-1}$).

3.3 Results

3.3.1 "Papri Queen" fruit

3.3.1.1 Growth curve

The growth rate of fruit from "Papri Queen" grown in winter time (June to August) was fastest during the first 30 days after anthesis but the rate slowed for the next 10 to 14 days and was almost unchanged afterwards (Figure 3.1A). The 44th days after anthesis (DAA) was determined as the day fruit ceased to develop and commenced the ripening phase. Full size fruit were on average 76.4 ± 3.7 mm long and 24.0 ± 0.4 mm wide and fruit at this stage were identified as light green (LG).

During summer, fruit developed more quickly and were significantly larger than fruit grown in winter (Figure 3.1B). Fruit reached the LG stage at 34 DAA and the

average length and diameter at this stage were 105.1 ± 4.1 and 31.4 ± 0.6 mm, respectively.

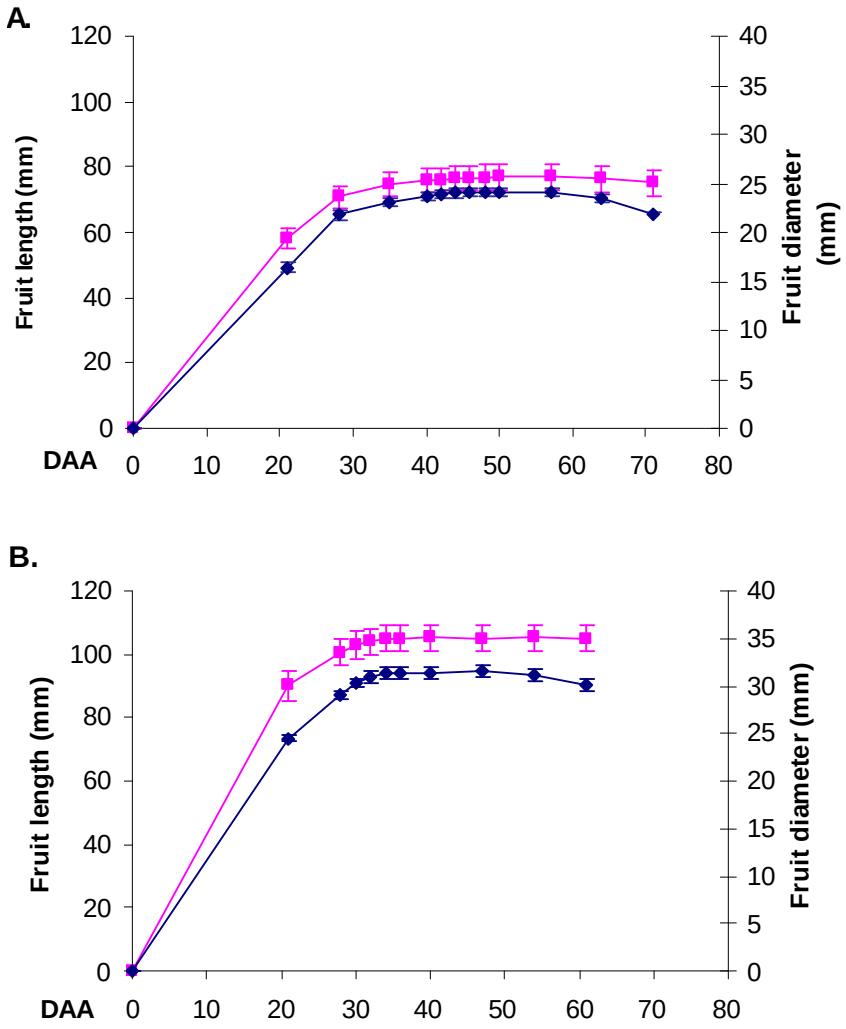

Figure 3.1 Change in length (■) and diameter (♦) of fruit from "Papri Queen" during development and ripening in winter (A) and summer (B). Crops were grown from March 2004 to August 2004 (A) and from October 2004 to March 2005 (B). Data are the means of eight replicates (± SE).

Regardless of whether fruit were grown in winter or summer the ripening process through six colour stages after LG was similar (26 and 28 days, respectively) and fruit ripened fully at 69.6 ± 0.6 and 62.4 ± 0.4 DAA, respectively (Table 3.2). Interestingly, fruit grown in winter changed colour from LG to dark green (DG) more slowly than fruit grown in summer (7 days compared to 5 days) but quicker

from DG to breaker (BR) (7 days compared to 12 days). From BR both fruit spent similar time to the final deep red and partially dried (DR&D) stage (11 to 11.5 days).

Table 3.2 The time course (days after anthesis) for fruit from "Papri Queen" to reach six colour stages. Fruit were left to ripen on the plant during winter (June/August 2004) and summer (Jan/March 2005). Data are the means of eight replicates (± SE).

Growing time	Deep green	Breaker	Breaker red	Light red	Deep red	Deep red and partially dried
Winter	51.2±0.6	55.5±0.6	58.3±0.8	63.3±0.7	66.0±0.7	69.6±0.6
Summer	39.3±0.2	46.8±0.7	51.3±0.3	55.8±0.5	58.80±0.6	62.4±0.4

3.3.1.2 Internal carbon dioxide concentration

CO_2 concentration of fruit from "Papri Queen" ripened on the plant varied from between 1.5 and 3 mL L^{-1} g^{-1}. There was a climacteric-like peak in CO_2 concentration (twofold increase) when fruit turned totally red (56 DAA) but this pattern was not observed in fruit ripened off the plant (Figure 3.2). When fruit were harvested at the LG stage, the CO_2 concentration was dramatically reduced (around sixfold) by just 5 days after harvest and was significantly lower than that of other fruit during ripening. When harvested at the deep green (DG) stage, the CO_2 concentration of fruit also slightly but significantly decreased (twofold) by 47 DAA and then did not significantly change. When fruit were harvested at the breaker (B) stage, although there appeared to be an increase in the CO_2 concentration by 51 DAA, it was insignificant. The CO_2 concentration was subsequently unchanged, but was significantly lower than that of fruit ripened on the plant and higher than that of fruit harvested at the LG and DG stages.

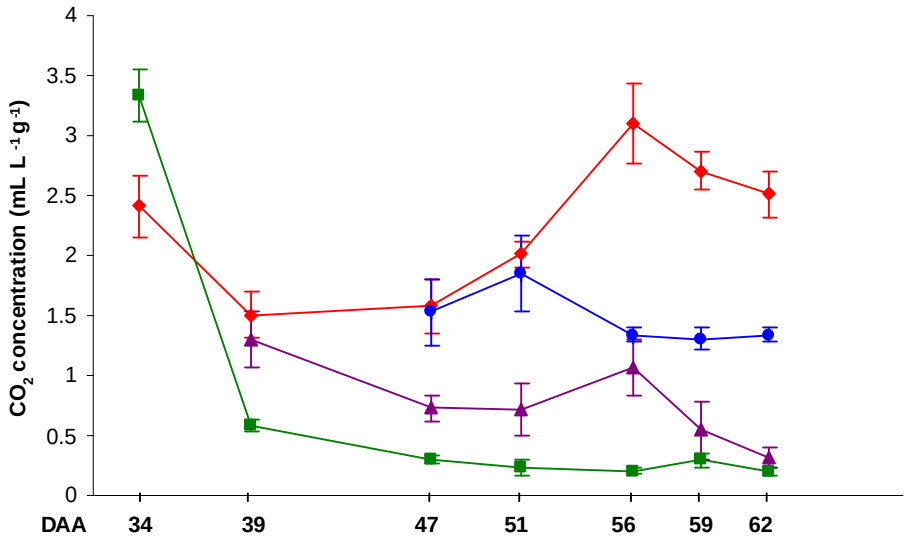

Figure 3.2 Change in internal CO_2 concentration of fruit from "Papri Queen" (mL L^{-1} g^{-1}) during ripening. Fruit were left to ripen on the plant (♦), harvested at the LG stage (■), at the DG stage (▲), and the B stage (●).Data are the means of six replicates (± SE) and the least significant difference ($P<0.05$) is 0.1949.

3.3.1.3 Internal ethylene concentration

At the early stages of ripening (from 34 to 47 DAA), C_2H_4 concentration was at an undetectable level in all fruit from "Papri Queen" regardless of ripening status or harvest date (Table 3.3). When red colour started to appear on the skin of fruit on the plant, the C_2H_4 concentration significantly increased to a detectable level. Although there was an insignificant drop at 59 DAA, C_2H_4 concentration stayed mostly unchanged throughout ripening. In contrast, in fruit harvested at green stages, C_2H_4 remained either undetectable or not significantly different to zero. When fruit were harvested at the B stage, the C_2H_4 concentration significantly increased by 4 days after harvest but sharply dropped afterwards. It was detectable, but not statistically different to zero.

Table 3.3 Internal ethylene concentration of fruit from "Papri Queen" (µL L^{-1}) during ripening. Fruit were either left to ripen on the plant or were harvested at different colour stages (LG-DG-B) and ripened at room temperature. Data are the means of six replicates (± SE). The least significant difference (P<0.05) is 0.083.

Treatment	**Time (DAA)**						
	34	**39**	**47**	**51**	**56**	**59**	**62**
Ripened on the plant	0±0	0±0	0±0	0.145± 0.057	0.138± 0.033	0.065± 0.017	0.179± 0.034
Harvested at LG	0±0	0±0	0±0	0±0	0±0	0±0	0±0
Harvested at DG		0±0	0.049± 0.025	0±0	0.083± 0.036	0.014± 0.005	0±0
Harvested at B			0±0	0.185± 0.093	0.064± 0.016	0.065± 0.021	0.054± 0.018

3.3.1.4 ACCS activity

ACCS activity was relatively lower in the first half of ripening (from 34 to 47 DAA) in all fruit regardless of ripening status and harvesting date (Figure 3.3). When fruit were ripened on the plant, ACCS activity gradually increased to 59 DAA when fruit was deep red. However, ACCS activity dropped significantly when fruit were partially dried.

When fruit were harvested at the LG stage, the ACCS activity was almost unchanged for the early period of ripening (34 to 51 DAA). However, there was a slight but significant increase for the next period before a subsequent decline at 62 DAA. ACCS activity of fruit harvested at the LG stage was similar to that of fruit ripened on the plant at the beginning but generally lower during ripening.

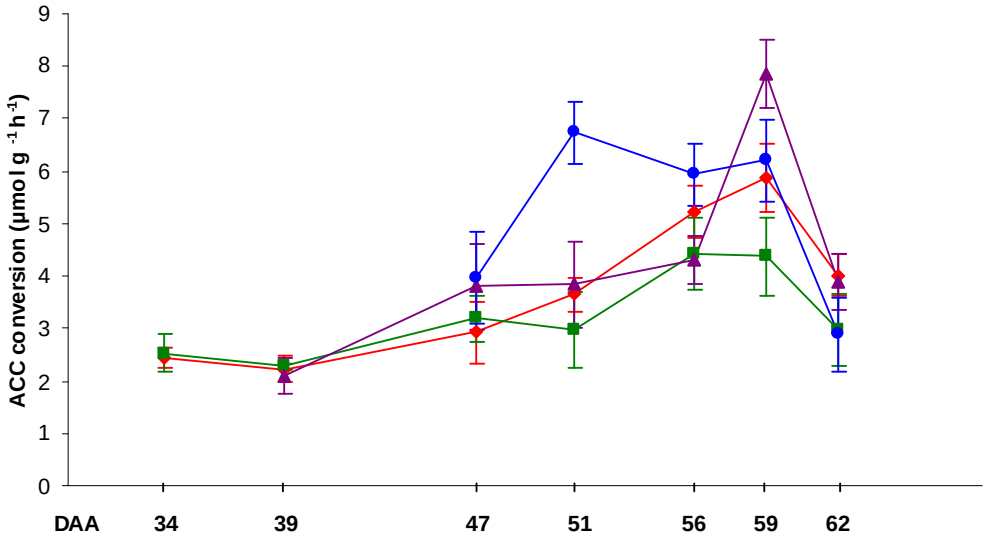

Figure 3.3 Change in ACCS activity of fruit from "Papri Queen" during ripening. The enzyme activity was calculated by the amount of ACC converted by 1 g of fresh tissue in 1 h (µmol g^{-1} h^{-1}) from fruit ripened on the plant (♦), harvested at the LG stage (■), at the DG stage (▲), and the B stage (●).Data are the means of six replicates (± SE) and the least significant difference (P<0.05) is 1.152.

When fruit were harvested at the DG stage, ACCS activity showed a similar trend to those fruit ripened on the plant with a twofold peak at 59 DAA followed by a decline towards 62 DAA. Apart from this peak, the enzyme activity of fruit harvested at the DG stage was not significantly different from that of fruit ripened on the plant.

The change in ACCS activity of fruit harvested at the B stage was to a similar extent as the other fruit except that the dramatic increase occurred more quickly at 51 DAA. The high level of enzyme activity was maintained until 59 DAA after which there was a significant decrease by 62 DAA.

3.3.1.5 ACCO activity

When fruit ripened on the plant, ACCO activity increased by around two or threefold when fruit colour turned from green to breaker red (Figure 3.4). However when reddening was completed (51 DAA), the activity dropped back to the initial level observed before ripening. When fruit were harvested at the LG stage, ACCO activity also significantly increased (approximately three or fourfold) by 47 DAA then

remained at that level until 56 DAA after which there was a slight reduction (59 DAA) followed by an increase to the previous level.

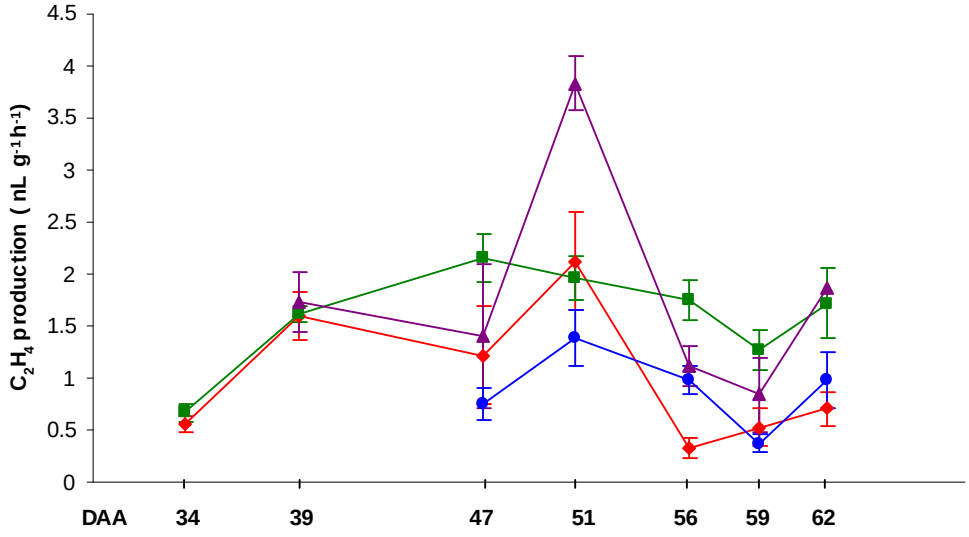

Figure 3.4 Change in ACCO activity of fruit from "Papri Queen" during ripening**.** Enzyme activity was calculated by the amount of C_2H_4 converted by 1 g of fresh tissue in 1 h (nL g^{-1} h^{-1}) from fruit ripened on the plant (♦), harvested at the LG stage (■), at the DG stage (▲), and the B stage (●).Data are the means of six replicates (± SE) and the least significant difference ($P<0.05$) is 0.3873.

When harvested at the DG stage, the ACCO activity of fruit followed the same trend as fruit ripened on the plant except that the ACCO activity was significantly higher from 51 DAA. Fruit harvested at the B stage also showed a similar trend except that the enzyme activity was significantly lower.

3.3.1.6 Extractable colour

The change in extractable colour of fruit from "Papri Queen" during ripening was significantly different between treatments, especially in the period of 47 to 62 DAA (Figure 3.5).

Fruit ripened on the plant showed a dramatic fourfold-increase in extractable colour between 47 and 56 DAA and another sharp increase at the final stage, from 59 to 62 DAA. In contrast, the extractable colour of fruit harvested at the LG stage remained

unchanged for most of ripening and the only significant increase was observed in the final stage (from 59 to 62 DAA). The significant increase in extractable colour of fruit harvested at the DG stage occurred earlier, from 51 to 56 DAA. Moreover, extractable colour of fruit harvested at the DG stage was far higher than that of fruit harvested at the LG stage, but lower than that of fruit ripened on the plant. The change in extractable colour of fruit harvested at the B stage was similar to that of fruit ripened on the plant except for a second significant increase at the final stage. At the final stage of ripening (62 DAA), only light green-harvested fruit had the extractable colour lower than the acceptable level of 140 ASTA units.

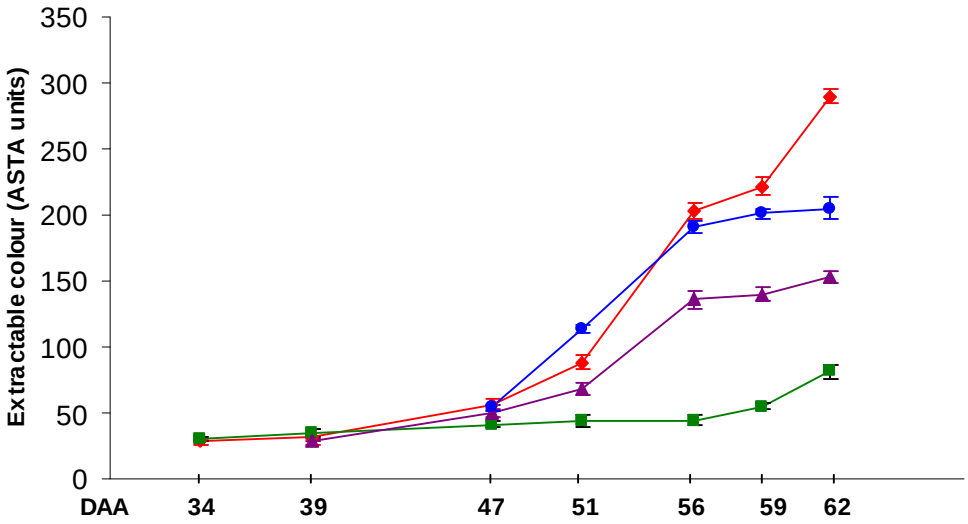

Figure 3.5 Change in extractable colour (ASTA units) of fruit from "Papri Queen" during ripening. Fruit were left to ripen on the plant (♦), harvested at the LG stage (■), at the DG stage (▲), and the B stage (●). The acceptable level is 140 ASTA units. Data are the means of six replicates (± SE) and the least significant difference ($P<0.05$) is 5.76.

3.3.1.7 Total soluble solid content

The change in total soluble solid content (TSSC) of fruit from "Papri Queen" differed significantly between treatments (Figure 3.6). TSSC of fruit ripened on the plant gradually increased in the early period of ripening (34 to 51 DAA). TSSC then dramatically increased and peaked at 56 DAA (when fruit were totally red) before a slight decline towards the end of ripening. In contrast, TSSC of fruit harvested at the LG and DG stage was unchanged throughout the experiment and significantly lower

compared to that of fruit ripened on the plant. TSSC of fruit harvested at the B stage remained unchanged for the first few days after harvest (from 47 to 56 DAA), but significantly increased from 56 to 59 DAA before a subsequent decline at 62 DAA. TSSC of fruit harvested at the B stage was higher than that of fruit harvested at green stages, but significantly lower than TSSC of fruit ripened on the plant by 62 DAA.

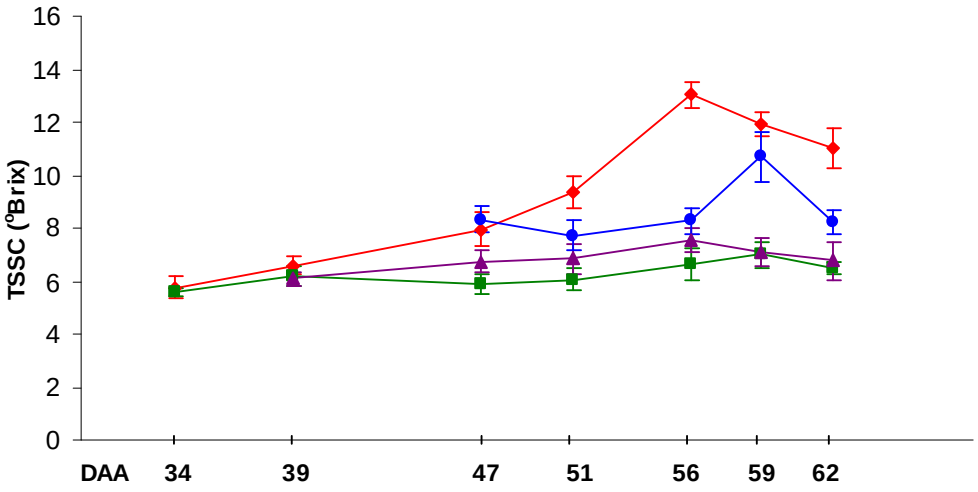

Figure 3.6 Change in TSSC (°Brix) of fruit from "Papri Queen" during ripening. Fruit were left to ripen on the plant (♦), harvested at the LG stage (■), at the DG stage (▲), and the B stage (●).Data are the means of six replicates (± SE) and the least significant difference (P<0.05) is 0.662.

3.3.2 "Aries" cultivar

3.3.2.1 Growth curve

Fruit from "Aries" had the fastest growth rate during the early period of development. From 20 DAA, the growth rate slowed and did not change from 40 DAA (Figure 3.7). Fruit that had reached their full size were considered as LG and commenced ripening at 38 DAA. The average length and diameter of fruit at this stage were 75.4 ± 2.6 mm and 67.8 ± 1.9 mm, respectively.

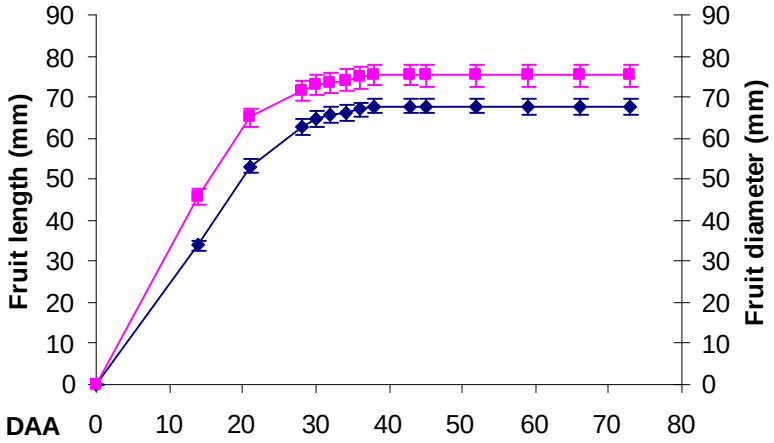

Figure 3.7 Change in length (■) and diameter (♦) of fruit from "Aries" during development and ripening. Crop was grown from November to March 2005. Data are the means of eight replicates (± SE).

From the LG stage, fruit colour developed to the deep red stage over approximately 36 days (Table 3.4) and the final stage DR&D was omitted because fruit turned soft and rotten and were not used for sampling. The six sampling times for fruit from "Aries", therefore, were determined as: 38, 43, 50, 58, 68, and 74 DAA.

Table 3.4 The time course (days after anthesis) for "Aries" to reach five colour stages. Fruit were left to ripen on the plant during February 2005. Data are the means of eight replicates (± SE).

Deep green	Breaker	Breaker red	Light red	Deep red
43.4±0.4	49.975±0.1	58.1±0.2	67.9±0.5	74.2±0.2

3.3.2.2 Internal carbon dioxide concentration

When fruit from "Aries" ripened on the plant, CO_2 concentration was reduced dramatically (approximately fivefold) at the early period of ripening (38 to 50 DAA). The CO_2 concentration then increased significantly with a climacteric-like peak at 68 DAA, but this peak was far lower than the level of CO_2 concentration at the onset of ripening (Figure 3.8). When fruit were harvested at the LG stage, CO_2 concentration gradually decreased to the lowest level at 58 DAA followed by a small second peak

during the end of ripening. The CO_2 concentration was significantly lower than that of fruit ripened on the plant, especially from 50 DAA.

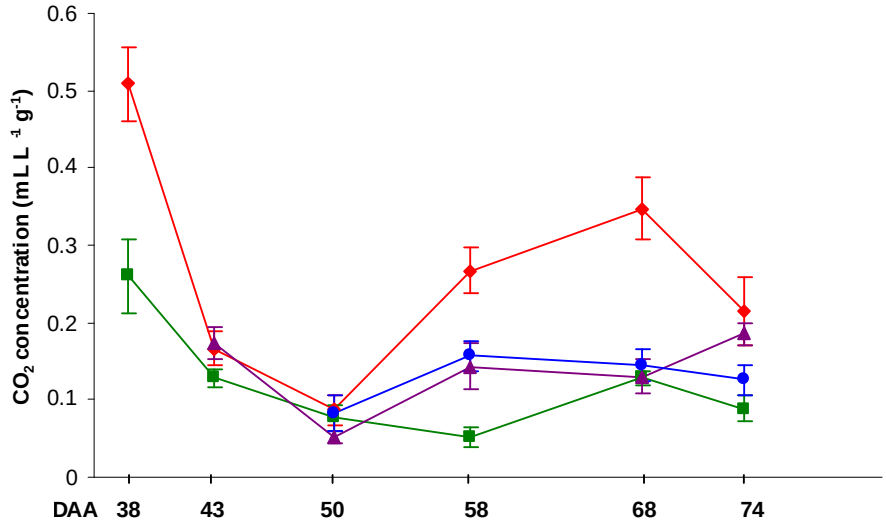

Figure 3.8 Change in internal CO_2 concentration of fruit from "Aries" (mL L^{-1} g^{-1}) during ripening. Fruit were either left to ripen on the plant (♦), or harvested at the LG stage (■), or at the DG stage (▲), or at the B stage (●).Data are the means of six replicates (± SE) and the least significant difference ($P<0.05$) is 0.0402.

CO_2 concentration of DG harvested fruit significantly declined (around threefold) by 50 DAA, but gradually increased during the latter part of ripening. At the final stage (74 DAA), the CO_2 concentration was not significantly different to that of fruit ripened on the plant and higher than that of other fruit. When fruit were harvested at the B stage, CO_2 concentration slightly but significantly increased and subsequently declined towards the end of ripening. CO_2 concentration was similar to that of fruit harvested at the DG stage except for the final stage (74 DAA), where it was significantly lower.

3.3.2.3 Internal ethylene concentration

When fruit from "Aries" ripened on the plant, C_2H_4 was hardly detectable and the only measurable concentration of C_2H_4 was detected when fruit were totally red at 68 DAA (Table 3.5). Similarly, fruit harvested at the LG stage produced no significant amount of C_2H_4. Some traces of C_2H_4 were detected at two different stages: immediately after harvest and the final stage of ripening, but they were not

statistically different from zero. Fruit harvested at the DG stage also produced a significant quantity of C_2H_4 at 68 DAA. However, due to variation, it was not considered significant at other stages.

Table 3.5 Internal ethylene concentration of fruit from "Aries" (μL L^{-1}) during ripening. Fruit were either left to ripen on the plant or harvested at different colour stages (LG-DG-B) and ripened at room temperature. Data are the means of six replicates (± SE).The least significant difference ($P<0.05$) is 0.094.

Treatment	**Time (DAA)**					
	38	**43**	**50**	**58**	**68**	**74**
Ripened on the plant	0±0	0±0	0±0	0.037± 0.020	0.138± 0.033	0.065± 0.017
Harvested at LG	0. 007± 0.003	0±0	0±0	0±0	0±0	0.075± 0.024
Harvested at DG		0.018± 0.015	0±0	0±0	0.197± 0.156	0.075± 0.031
Harvested at B			0.007± 0.002	0±0	0.037± 0.015	0.044± 0.015

3.3.2.4 ACCS activity

ACCS activity of fruit from "Aries" was significantly different between treatments (Figure 3.9). When fruit ripened on the plant, ACCS was low during the early period of ripening (38 to 50 DAA) but then dramatically increased (over threefold) between 50 and 58 DAA. Enzyme activity then gradually declined towards the end of ripening. The ACCS activity of fruit ripened on the plant was lower than that of fruit ripened off the plant during the first half of ripening but significantly higher during the second half of ripening (from 52 DAA). The change in ACCS activity of fruit harvested at the LG stage contrasted with that of fruit ripened on the plant. ACCS activity was doubled by 43 DAA but declined sharply after that to a level lower than before ripening.

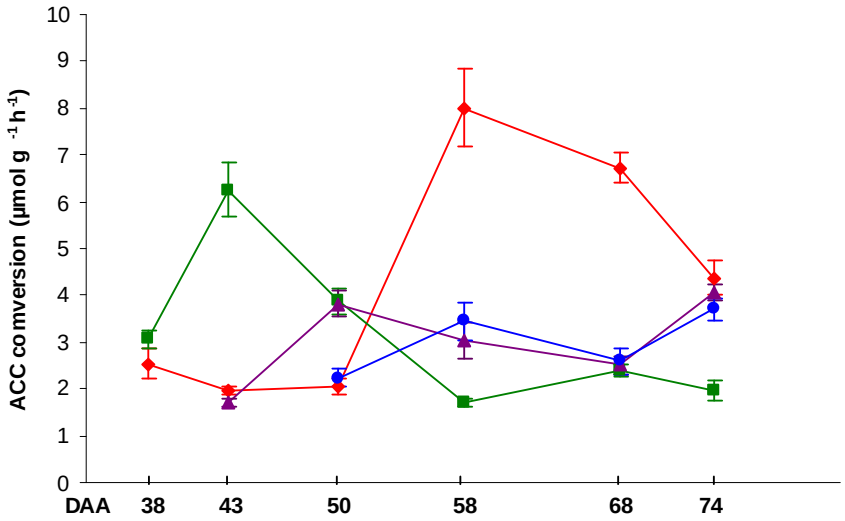

Figure 3.9 Change in ACCS activity of fruit from "Aries" during ripening. The enzyme activity was calculated by the amount of ACC converted by 1 g of fresh tissue in 1 h (µmol g^{-1} h^{-1}) from fruit ripened on the plant (♦), harvested at the LG stage (■), at the DG stage (▲), and the B stage (●).Data are the means of six replicates (± SE) and the least significant difference (P<0.05) is 0.3626.

The ACCS activity of fruit harvested at the DG stage also doubled after harvest (50 DAA), then gradually declined before an increase at 74 DAA. Except for the beginning of ripening (43 DAA), the enzyme activity was slightly higher than or equal to that of fruit harvested at the LG stage. The change in ACCS of fruit harvested at the B stage was very similar to that of fruit harvested at the DG stage with an initial increase at 58 DAA. Moreover, the enzyme activity was also at the same level except for the first stage at 50 DAA when it was significantly lower.

3.3.2.5 ACCO activity

When fruit from "Aries" ripened on the plant, ACCO activity showed a climacteric-like pattern during ripening (Figure 3.10) with a dramatic peak of approximately seven fold in ACCO activity when reddening occurred (50 to 68 DAA). Once the fruit were completely red, the enzyme activity dropped sharply back to the initial level observed at the onset of ripening. Except for this climacteric-like peak, ACCO activity of fruit ripened on the plant was usually lower than that of fruit ripened off the plant. The change in ACCO activity of fruit harvested at the LG, DG and B stage was characterised by a significant and dramatic increase after harvest and then a

gradual decrease for the remaining time. The only exception was the enzyme activity at the beginning of ripening, when fruit was just harvested. At 43 DAA, ACCO activity of fruit just harvested at the DG stage was lower than that of fruit harvested at the LG stage, which had ripened off the plant for several days. Similarly, ACCO of fruit just harvested at the B stage at 50 DAA was also lower than that of fruit harvested at the LG and DG stages which had several days of off-plant ripening.

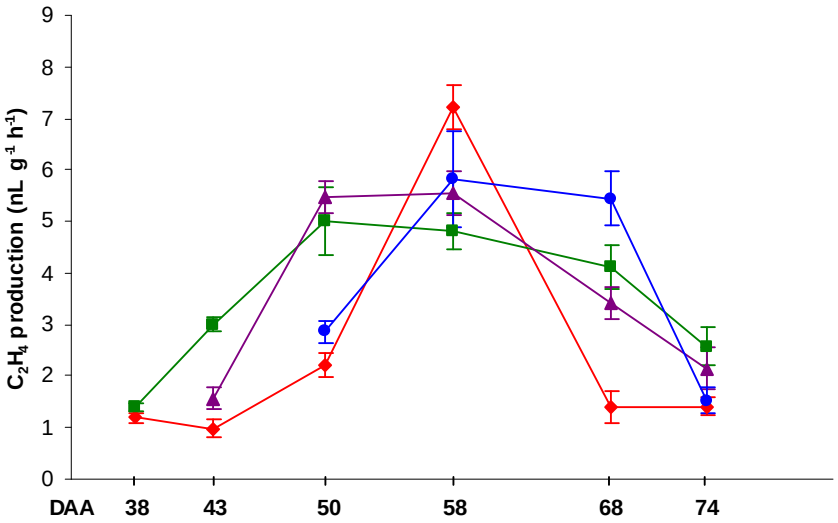

Figure 3.10 Change in ACCO activity in fruit from "Aries" during ripening**.** Enzyme activity was calculated by the amount of C_2H_4 converted by 1 g of fresh tissue in 1 h (nL g^{-1} h^{-1}) from fruit ripened on the plant (♦), harvested at the LG stage (■), at the DG stage (▲), and the B stage (●).Data are the means of six replicates (± SE) and the least significant difference ($P<0.05$) is 0.4157.

3.3.2.6 Extractable colour

Extractable colour of fruit from "Aries" was similar between treatments during the early phase of ripening (39 to 50 DAA) but significantly different for the remaining time (Figure 3.11). Extractable colour of fruit from all treatment failed to reach the level of 140 ASTA units at the end of ripening.

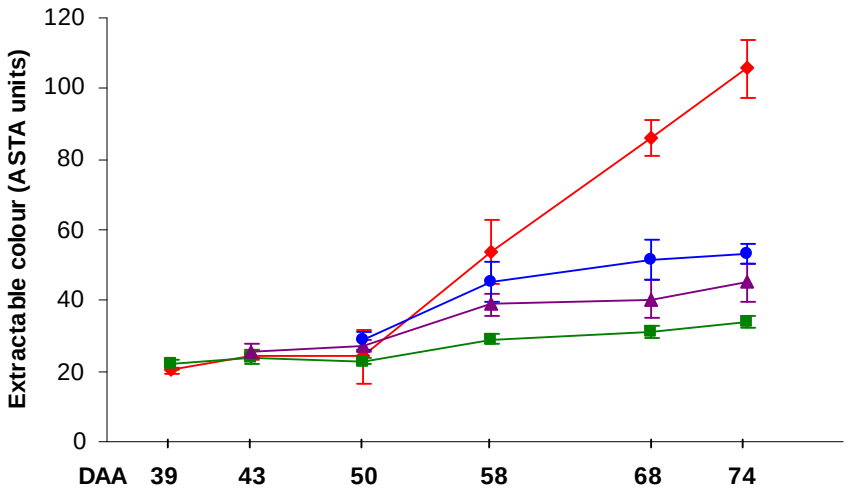

Figure 3.11 Change in extractable colour (ASTA units) of fruit from "Aries" during ripening. Fruit were left to ripen on the plant (♦), harvested at the LG stage (■), at the DG stage (▲), and the B stage (●).Data are the means of six replicates (± SE) and the least significant difference (P<0.05) is 4.363.

When fruit ripened on the plant, the extractable colour was mostly unchanged from 38 to 50 DAA but increased dramatically (over fivefold) afterwards and was higher than that of fruit ripened off the plant. The extractable colour of fruit harvested at the LG stage increased only slightly from 50 DAA and at the final stage (74 DAA) the extractable colour was just over 50% higher than the level at the beginning of ripening. The extractable colour of fruit harvested at the DG and B stages also gradually increased during ripening but to a greater extent such that fruit harvested at B had a higher extractable colour than DG and LG harvested fruit, respectively. However, fruit ripened off the plant generally had far lower levels of extractable colour than fruit ripened on the plant.

3.3.2.7 Total soluble solid content

When fruit from "Aries" ripened on the plant, there was a small peak in TSSC at 43 DAA and a higher peak at 68 DAA, when fruit turned totally red (Figure 3.12). TSSC of fruit harvested at the LG stage fluctuated at a level significantly lower than that of fruit ripened on the plant with the only significant increase observed at 58 DAA. The TSSC of fruit harvested at the DG stage was slightly reduced during ripening with a significant drop to 58 DAA. Fruit harvested at the B stage had a

gradual decrease in TSSC without any significant drop. The TSSC of fruit ripened off the plant was significantly lower than that of fruit ripened on the plant during the late period of ripening.

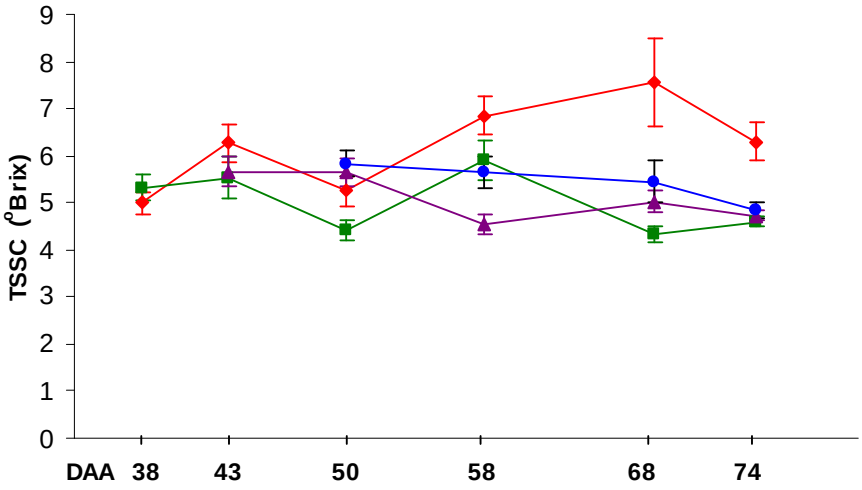

Figure 3.12 Change in TSSC (°Brix) of fruit from "Aries" during ripening. Fruit were left to ripen on the plant (♦), harvested at the LG stage (■), at the DG stage (▲), and the B stage (●).Data are the means of six replicates (± SE) and the least significant difference (P<0.05) is 0.4081.

3.3.3 "Caysan" cultivar

3.3.3.1 Growth curve

Fruit from "Caysan" had the fastest growth rate during the first 30 DAA but the rate slowed during the next 20 to 25 days and was almost unchanged afterwards (Figure 3.13) Fruit ceased their development and commenced ripening at 54 DAA. Fruit at this stage, the LG stage, were on average 88.0 ± 3.2 mm long and 18.0 ± 0.6 mm wide.

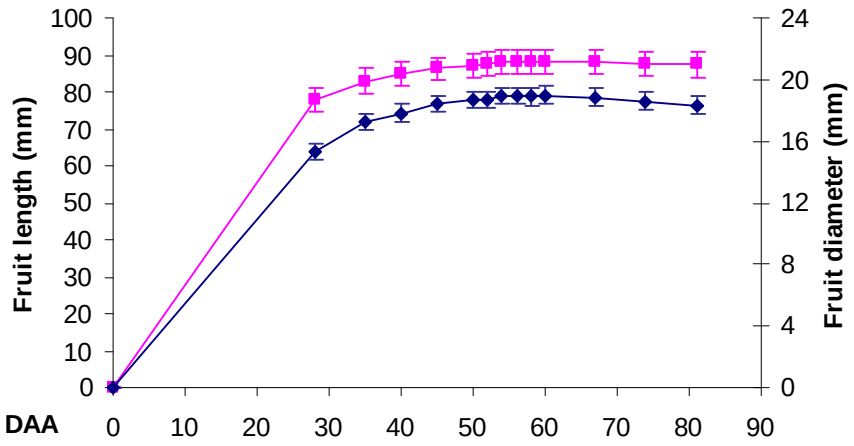

Figure 3.13 Change in length (■) and diameter (♦) of fruit from "Caysan" during development and ripening. Crop was grown from March to August 2004. Data are the means of eight replicates (± SE).

From the LG stage, fruit ripened on the plant to deep red and partially dried over approximately 28 days (Table 3.6). Fruit usually progressed from the light red, deep red and finally deep red and partially dried stage within a very short time (within one to two days). The stage of deep red was omitted.

Table 3.6 The time course (days after anthesis) for "Caysan" to five colour stages. Fruit were left to ripen on the plant during July/August 2004. Data are the means of eight (± SE).

Deep green	Breaker	Breaker red	Light red	Deep red and partially dried
61.6±0.5	71.3±0.7	75.4±0.5	78.8±0.6	82.3±0.2

3.3.3.2 Internal carbon dioxide concentration

When fruit from "Caysan" ripened on the plant, there was a climacteric-like peak (two or threefold) in CO_2 concentration in association with the completion of reddening of the fruit skin (Figure 3.14). In contrast, CO_2 concentration of fruit harvested at the LG stage significantly declined at the early period of ripening (from 54 to 71 DAA) and remained unchanged after that.

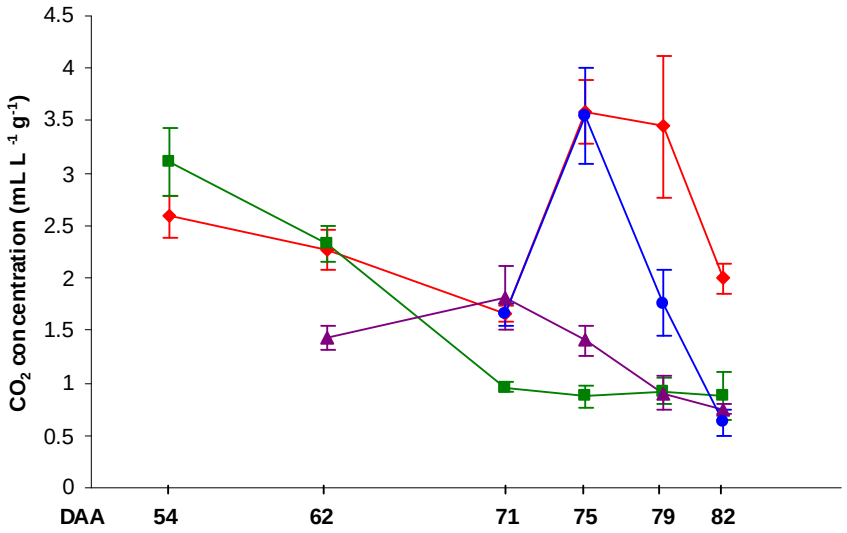

Figure 3.14 Change in internal CO_2 concentration of fruit from "Caysan" (mL L^{-1} g^{-1}) during ripening. Fruit were left to ripen on the plant (♦), harvested at the LG stage (■), at the DG stage (▲), and the B stage (●).Data are the means of six replicates (± SE) and the least significant difference ($P<0.05$) is 0.3441.

The CO_2 concentration of fruit harvested at the DG stage remained stable after harvest (62 to 71 DAA), but gradually declined from 71 DAA (towards the end of ripening). The CO_2 concentration of fruit harvested at the B stage increased after harvest, but the peak was far higher (twofold) and climacteric-like. The magnitude of the peak at 75 DAA was similar to that of fruit ripened on the plant, but the CO_2 concentration dropped immediately afterwards and was significantly lower during the final period of ripening.

3.3.3.3 Internal ethylene concentration

A significant and climacteric-like peak of internal C_2H_4 concentration in all fruit from "Caysan" regardless of treatments occurred at 75 DAA (Figure 3.15). Fruit ripened on the plant had a highest peak followed by that of fruit harvested at B, DG and LG stages.

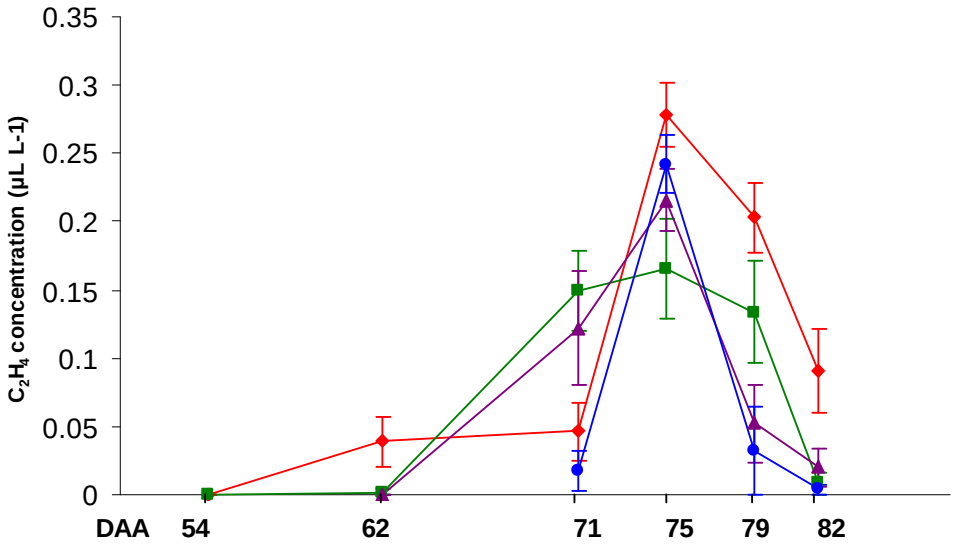

Figure 3.15 Change in internal C_2H_4 concentration of fruit from "Caysan" (µL L^{-1}) during ripening. Fruit were left to ripen on the plant (♦), harvested at the LG stage (■), at the DG stage (▲), and the B stage (●).Data are the means of six replicates (± SE) and the least significant difference ($P<0.05$) is 0.0137.

3.3.3.4 ACC synthase

ACCS activity of fruit from "Caysan" ripened on the plant increased slightly when fruit colour turned from green to breaker and significantly dropped at the breaker red stage (Figure 3.16). However, when reddening was completed, the enzyme activity increased with a significant peak of three or fourfold at 79 DAA (the light red stage). When fruit were harvested at the LG stage, ACCS peaked at 71 DAA and there was another increase at the final stage of ripening (82 DAA). Except for the final period of ripening, the enzyme activity was relatively higher than that of fruit ripened on the plant.

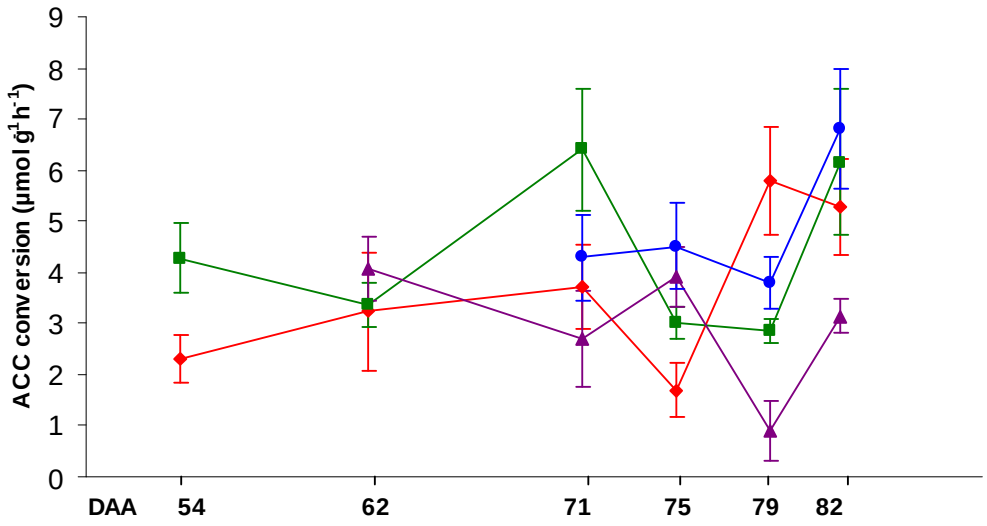

Figure 3.16 Change in ACCS activity of fruit from "Caysan" during ripening. The enzyme activity was presented by the amount of ACC converted by 1 g of fresh tissue in 1 h (µmol g^{-1} h^{-1}) from fruit ripened on the plant (♦), harvested at the LG stage (■), at the DG stage (▲), and the B stage (●).Data are the means of six replicates (± SE) and the least significant difference ($P<0.05$) is 0.801.

When fruit were harvested at the DG stage, the ACCS activity fluctuated at the early phase of ripening (62 to 75 DAA) but dropped significantly at 79 DAA before increased again at the final stage. ACCS activity of fruit harvested at the B stage also fluctuated from 71 to 79 DAA, increased at the final stage of ripening, and was higher than that of fruit harvested at the DG stage. ACCS activity increased in the final stage of ripening of fruit off the plant, while it declined in fruit ripened on the plant in the same period.

3.3.3.4 ACC oxidase

ACCO activity of fruit from "Caysan" increased by twofold when fruit colour changed from light to deep green on the plant (between 54 and 62 DAA). However, the enzyme activity gradually declined during ripening despite an insignificant increase at 79 DAA (Figure 3.17). The ACCO activity was lower than that of fruit harvested at LG and DG stages with 62 DAA the only exception.

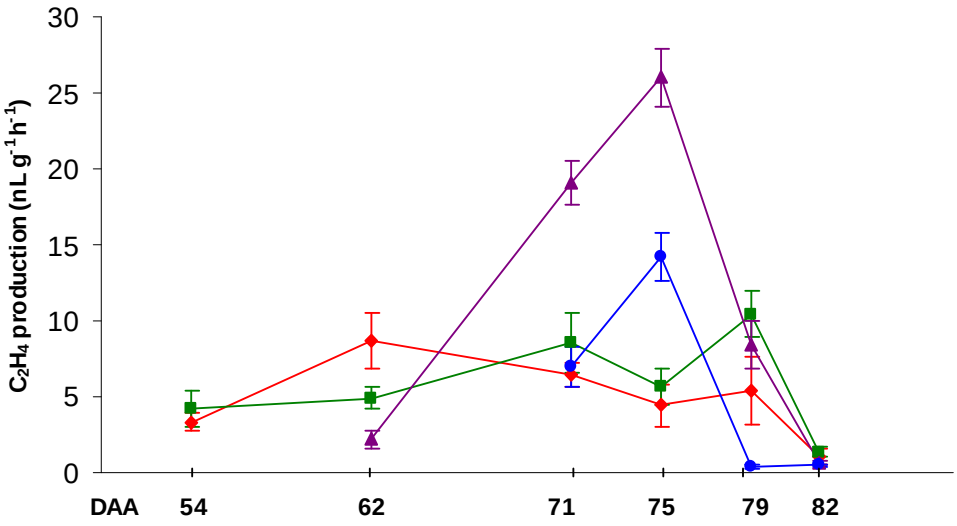

Figure 3.17 Change in ACCO activity in fruit from "Caysan" during ripening**.** Enzyme activity was calculated by the amount of C_2H_4 converted by 1 g of fresh tissue in 1 h (nL g^{-1} h^{-1}) from fruit ripened on the plant (♦), harvested at the LG stage (■), at the DG stage (▲), and the B stage (●).Data are the means of six replicates (± SE) and the least significant difference ($P<0.05$) is 2.572.

There were a couple of significant peaks in ACCO activity of fruit harvested at the LG stage: the first, a small one at 71 DAA, and the second a slightly higher one at 70 DAA. The extent of the increase was similar to that of fruit ripened on the plant (~ twofold). In contrast, ACCO activity dramatically increased (tenfold) after fruit were harvested at the DG stage, and peaked at 75 DAA. The change in ACCO of fruit harvested at the B stage was similar but the peak was significantly lower.

3.3.3.6 Extractable colour

Extractable colour of fruit from "Caysan" differed significantly during ripening, especially from 71 DAA (Figure 3.18). During the early phase of ripening, extractable colour of fruit ripened on the plant slightly increased and was not much higher than that of fruit ripened off the plant. However, from 71 DAA, extractable colour dramatically increased (near fivefold) and was significantly higher than that of fruit off the plant. In contrast, extractable colour of fruit harvested at the LG stage increased only by around 30% at the final stage of ripening.

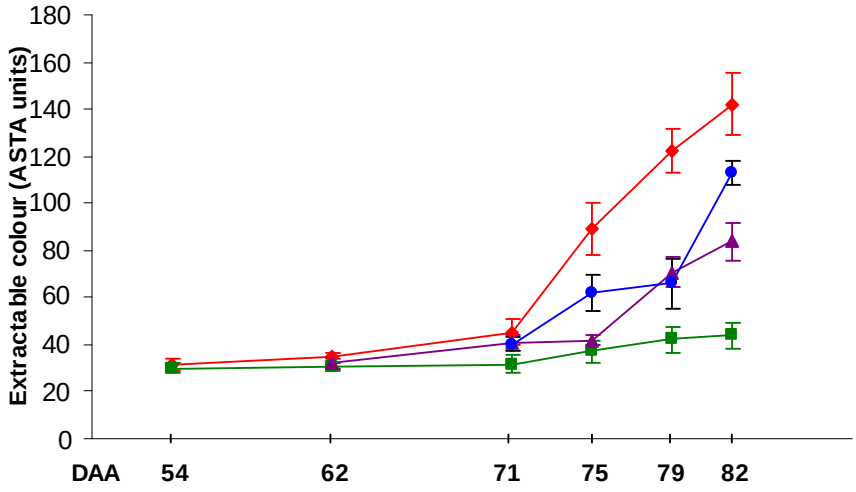

Figure 3.18 Change in extractable colour (ASTA units) of fruit from "Caysan" during ripening. Fruit were left to ripen on the plant (♦), harvested at the LG stage (■), at the DG stage (▲), and the B stage (●).Data are the means of six replicates (± SE) and the least significant difference (P<0.05) is 7.00.

Extractable colour of fruit harvested at the DG and B stages also increased during ripening and by 82 DAA there was a three and fourfold increase, respectively. At the final stage of ripening, only fruit ripened on the plant and breaker-harvested fruit had the extractable colour higher than the acceptable level of 110 ASTA unit for this type of pepper.

3.3.3.7 Total soluble solid content

The TSSC of fruit from "Caysan" increased significantly during the ripening process on the plant, especially after 71 DAA when reddening started (Figure 3.19). The TSSC of fruit ripened on the plant was usually higher than that of fruit ripened off the plant, especially for the final phase of ripening. TSSC of fruit harvested at the LG stage did not change through the whole ripening period and was far lower than that of fruit ripened on the plant or harvested at B.

When fruit were harvested at the DG stage, TSSC gradually increased and peaked at 75 DAA before decreasing towards the end of ripening. TSSC of fruit harvested at the B stage changed in a similar pattern.

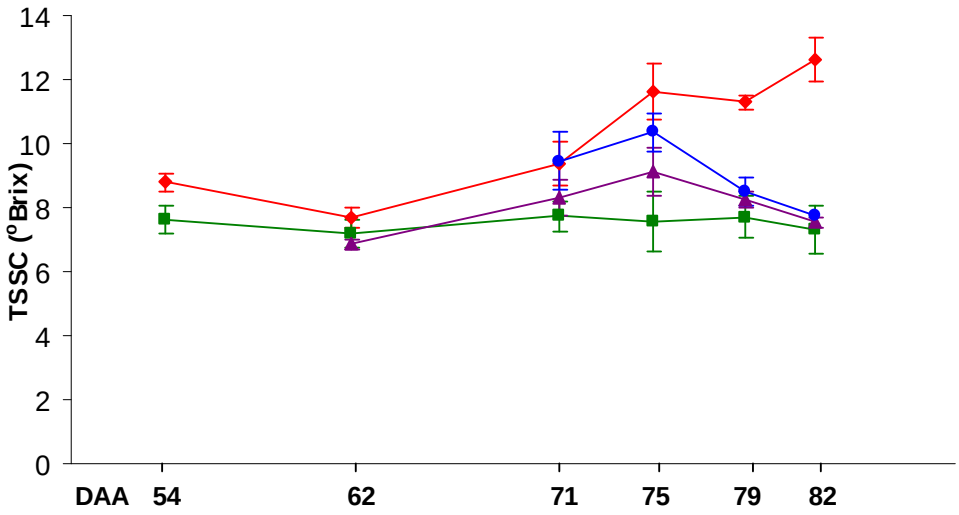

Figure 3.19 Change in TSSC (°Brix) of fruit from "Caysan" during ripening. Fruit were left to ripen on the plant (♦), harvested at the LG stage (■), at the DG stage (▲), and the B stage (●).Data are the means of six replicates (± SE) and the least significant difference (P<0.05) is 0.628.

3.4 Discussion

There was very limited involvement of C_2H_4 during ripening of capsicum and the change in activities of ACC synthase and ACC oxidase (two enzymes in C_2H_4 biosynthesis pathway) was not closely related to that of C_2H_4. However, it appeared that colour development in *cv.* "Papri Queen" was closely associated with C_2H_4 production while a climacteric-like peak of C_2H_4 could be observed in all fruit from *cv.* "Caysan". The levels of internal CO_2 concentration, extractable colour and TSSC were greater in fruit ripened on the plant followed by fruit harvested at the breaker, deep green and light green stage, respectively. Compared to the other two cultivars, "Papri Queen" and "Aries", "Caysan" appeared to be the closest to a climacteric type but all three could be considered non-climacteric.

When fruit from "Papri Queen" and "Aries" were ripened on the plant, CO_2 concentration showed a climacteric-like pattern with a peak of approximately twofold when reddening occurred. Even though similar observations were reported in the same or other types of capsicum (Krajayklang *et al.*, 2000; Villavicencio *et al.*,

2001), it is unlikely to be a typical climacteric increase. The observations that the highest peak of CO_2 was at the same level as that of fruit prior to ripening and that breaker-harvested fruit managed to ripen normally without a peak in CO_2 supports this argument. A climacteric increase in CO_2 is usually considered as a response to the production of C_2H_4 by climacteric fruit (Tucker, 1987). However, fruit from both "Papri Queen" and "Aries" produced very little C_2H_4. Fruit from "Papri Queen" produced C_2H_4 only when they developed red colour (when ripened on the plant or harvested at the B stage). C_2H_4 concentration of fruit from "Aries", regardless of whether ripened on or off the plant and regardless of harvest dates, was also very low and mostly undetectable or not statistically different from zero. The increase in CO_2 when fruit were ripened on the plant, therefore, was unlikely to be induced by C_2H_4 as typical of climacteric fruit. "Papri Queen" and "Aries" should be classified as non-climacteric as concluded by other authors for similar types of capsicum (Lurie *et al.*, 1986; Saltveit, 1997; Villavicencio *et al.*, 1999).

In contrast, fruit from "Caysan" had a peak in CO_2 concentration in both fruit ripened on the plant and harvested at the B stage which coincided with a peak of C_2H_4. Gross *et al.* (1986) also found a climacteric increase in CO_2 of fruit from cultivar Chooraehong (hot chilli type) harvested at the mature green stage despite a very low level of C_2H_4. Although C_2H_4 concentration of fruit from "Caysan" was low throughout ripening, both fruit ripened on and off the plant produced a climacteric-like peak at the same time (75 DAA). This peak was also observed in hot chilli *cv.* Chooraehong (Gross *et al.*, 1986) and chilli pepper *cv.* New Mexico (Biles *et al.*, 1993) but was absent in *cv.* Changjiao (Lu *et al.*, 1990). Moreover, even though green-harvested "Caysan" fruit failed to ripen and turn red, they were still able to produce a significant quantity of C_2H_4 and it was generally higher than that from "Papri Queen" and "Aries", respectively. It is likely that C_2H_4 may play a more important role in the ripening of "Caysan" than "Papri Queen" in which C_2H_4 appeared to be a result of ripening rather than a ripening induction factor. Because non- and climacteric fruit respond differently to exogenous C_2H_4 application (Biale and Young, 1981; McGlasson, 1985) , further work on this aspect may help to clarify the nature of the ripening characteristic of these three cultivars.

The first step from SAM to ACC is catalysed by ACC synthase (ACCS), and the second step from ACC to C_2H_4 is catalysed by ACC oxidase (ACCO) (Figure 1.3). Two different systems of C_2H_4 production during fruit ripening were previously reported (McMurchie *et al.*, 1972). In *system 1*, C_2H_4 produced in the pre-climacteric stage of climacteric fruit or in non-climacteric ones was at the basal low level due to the low activity of ACCS. When ripening progresses, the internal C_2H_4 level gradually increases with the increase of ACCO activity and when this internal level passes a certain threshold, ACCS is induced leading to the burst of C_2H_4 production called a climacteric peak. This system is referred to as *system 2* of C_2H_4 production and presents only in climacteric fruit (Oetiker and Yang, 1995).

For fruit from "Papri Queen", C_2H_4 was detected in red fruit during the late phase of ripening. Although ACC synthase activity increased significantly during this period, it did not increase C_2H_4 production as typical of *system 2*. Moreover, there was no clear difference in ACCS level between fruit that produced a significant quantity of C_2H_4 and fruit that did not. ACCO in all fruit remained at relatively stable levels during ripening except for some isolated increases. C_2H_4 appeared to be produced by *system 1* with a low basal level throughout ripening.

The changes in ACCS activity of fruit from "Aries" were significantly different from "Papri Queen". When fruit ripened off the plant, ACCS increased immediately after harvest then dropped to low levels during ripening and this increase was likely induced by wounding at harvest (Saltveit, 1997). In contrast, ACCS of fruit ripened on the plant increased and peaked when fruit turned red and this increase appeared to be induced by ripening-related factors. ACCS is encoded by a multigene family which is induced by different factors such as tissue wounding, auxins, and other ripening related signals (Oetiker and Yang, 1995; Tastuki *et al.*, 2003). However, all the changes in enzyme activity, especially in ACCS, the rate-limiting enzyme in fruit ripened on the plant, did not directly regulate C_2H_4 production because all fruit from "Aries" produced mostly undetectable levels of C_2H_4. C_2H_4, therefore, may not play any significant role during the ripening of "Aries" fruit.

All fruit from "Caysan" regardless of treatments produced a significant amount of C_2H_4 at 75 DAA but the changes in ACCS and ACCO of these fruit did not follow

this pattern. If the peak of C_2H_4 was climacteric-like, it would be a result of a burst in ACCS activity in *system 2*. However, ACCS activity at this stage did not increase as dramatically as C_2H_4 and so it is unlikely it belongs to the *system 2* typical for climacteric fruit.

Interestingly, the level of ACCO activity in fruit from "Caysan" was higher than that from "Papri Queen" and "Aries" cultivars in a similar manner to the level of internal C_2H_4 concentration. The capacity to produce C_2H_4 in this case may depend on the activity of ACCO rather than ACCS as all three cultivars produced *system 1* C_2H_4 which is typical for non-climacteric fruit.

The levels of internal CO_2 concentration and TSSC of fruit from all three cultivars were affected by ripening status (on or off the plant) and harvest date. At nearly all time points, fruit ripened on the plant produced a higher CO_2 concentration than those ripened off the plant. Among fruit ripened off the plant, fruit from "Papri Queen" and "Caysan" harvested at B usually had higher CO_2 concentration than that harvested at green stages while the pattern was not clear in "Aries". A similar pattern was observed for the change of TSSC.

The difference in CO_2 production between fruit harvested at different ripeness stages was also observed by other authors. Red harvested fruit from cultivar Maor (bell pepper type) also had a higher level of CO_2 production than that of green harvested fruit, and in both of them, CO_2 kept declining during storage (Lurie *et al.*, 1986; Lu *et al.*, 1990).The low CO_2 concentration of fruit off the plant may be due to the fact that fruit were not supplied with an external-carbon source by the mother plant as were fruit ripened on the plant. Dependence on the mother plant for assimilates during ripening is common in some typical non-climacteric fruits such as grape and strawberry (Seymour *et al.*, 1993) and this may be the case for capsicum. Although starch and sucrose may contribute to dramatic increases in hexose concentration during ripening, it is likely that there is a continuous import of photoassimilates from the plant (Hubbard and Pharr, 1992). Without assimilates supplied by the mother plant, harvested fruit may have to rely on their own starch reserve or sucrose for respiration. In this study, the TSSC of fruit ripened off the plant remained low, especially in fruit harvested at the green stages while TSSC of fruit ripened on the

plant kept increasing due to hexose accumulation by activities of starch and sucrose degradation enzymes such as invertase and sucrose phosphate synthase (Nielsen *et al.*, 1991). This low level of TSSC of green-harvested fruit may be due to the lack of both the external supply from the mother plant and the action of these enzymes. Of three studied cultivars, fruit from "Aries" had relatively lower levels of both TSSC and extractable colour than "Papri Queen" and "Caysan" it is likely due to the higher water content of fruit from "Aries".

For "Aries", the harvest date did not appear to significantly affect CO_2 concentration of harvested fruit although it was far lower than that of fruit ripened on the plant. This may suggest that for this cultivar, the lack of the external-carbon supply rather than the activity of related enzymes led to the low respiration activity. In contrast, for "Papri Queen" and "Caysan", the harvest date significantly affected the CO_2 concentration of fruit. The higher level of CO_2 concentration in fruit harvested at B compared to that of fruit harvested at LG and DG indicated that there should be some biochemical and physiological changes during the period fruit remained attached to the plant from LG to B that initiated respiration activity once fruit were harvested. These changes may be induced by unknown ripening-related signals rather than C_2H_4.

Generally, extractable colour of fruit from "Papri Queen" was far higher than that from "Caysan" and "Aries" although all fruit ripened on the plant naturally develop a deep dark red colour. Fruit harvested at B developed total red colour similar to fruit ripened on the plant but had lower levels of extractable colour. Fruit harvested at LG and DG failed to develop sufficient colour and usually could not turn totally red.

For all three cultivars, the early period of ripening saw very little increase in extractable colour in both fruit ripened on and off the plant although skin colour of fruit on the plant did change from light green to breaker. This change of skin colour was likely to be the result of a dramatic degradation/breakdown of chlorophyll (Biles *et al.*, 1993; Mosquera and Mendez, 1994; Amela *et al.*, 1996; Deli *et al.*, 2001) and subsequent unmasking of the pre-existing carotenoids such as β-carotene, violaxanthin, and lutein (Markus *et al.*, 1999). However, previous research has focused on changes in colour of fruit ripened on the plant only and not harvested

fruit. Therefore, examining the chlorophyll breakdown in harvested fruit may contribute some valuable information on the colour evolution of fruit ripened off the plant.

As ripening progressed, extractable colour increased but to a different extent among fruit ripened on and off the plant as well as among fruit harvested at different ripeness stages. This changing pattern in extractable colour was similar for all three cultivars, especially for "Papri Queen" and "Caysan", two cultivars used for spice processing. Fruit ripened on the plant developed a dramatic increase in colour as the result of the burst in *de novo* synthesis of red carotenoid: capsanthin, capsorubin and their compounds (Mosquera and Mendez, 1994; Mendez *et al*., 2000). In contrast fruit harvested at LG could only reach a level of extractable colour at least 40% short of the accepted level. The insufficiency in colour development of LG and to some extent of DG harvested fruit was also reported by other authors (Saltveit, 1997; Krajayklang *et al*., 2000). This failure may be caused by the incapacity of the cell to either break down chlorophyll or synthesise new carotenoid pigments.

In contrast, when harvested at the breaker stage, fruit developed colour in the same pattern as those ripened on the plant. At the time fruit were harvested, chlorophyll may be already degraded and the cells may have already undergone some changes [such as the transformation from chloroplast to chromoplast (Amela *et al.*, 1996)] and this may help the accumulation and synthesis of carotenoid later in the ripening process prior to the biosynthesis of new carotenoids.

3.5 Conclusion

Despite the morphological differences among three cultivars of capsicum studied in this chapter, their ripening behaviour was similar and typical for non-climacteric fruit. However, there was a significant difference in ripening behaviour between fruit ripened on and off the plant and among fruit harvested at different ripeness stages. The levels of internal CO_2 concentration, extractable colour and TSSC of fruit ripened on and off the plant usually followed a pattern: greatest in fruit ripened on the plant, followed by that of fruit harvested at B, DG and LG, respectively. Fruit

harvested at the LG stage failed to turn totally red and the low level of CO_2 concentration and total soluble solid content suggested the involvement of the external carbon supply from the mother plant in ripening of capsicum fruit.

During ripening, capsicum produced very low levels of C_2H_4 even in fruit ripened on the plant. The changes of ACC synthase and oxidase activity during ripening were not closely correlated with the changes in C_2H_4 concentration and not characteristic for climacteric fruit. Capsicum fruit, therefore, should be classified as non climacteric as C_2H_4 played very little role during ripening. The only noticeable observations were the peak of C_2H_4 (but in low levels and not considered as climacteric) in all fruit from “Caysan” at 75 DAA and the association of C_2H_4 and red colour development in fruit from “Papri Queen” which may suggest some involvement of C_2H_4 in colour development during ripening.

Chapter Four

Chapter Four - Effects of preharvest application on ripening of green harvested “Papri Queen”

4.1 Introduction

Preharvest treatment (particularly with plant growth regulators) is commonly used to alter ripening behaviour and improve postharvest quality of fruit. For capsicum fruit, preharvest treatments have mainly been used for colour improvement, thus better spice quality (Monselise, 1986; Rajput and Paruleke, 1998).

Several plant growth regulators have been used to improve the ripening process of the fruit crop but ethephon, an ethylene-generating compound, has been the most commonly used and effective for improving colour of fruit. Application of ethephon enhances chlorophyll degradation and carotenoid synthesis in many fruit crops (Sonkar and Ladaniya, 1999; Drake *et al.*, 2006). Besides C_2H_4, other plant growth regulators, especially auxins and abscisic acid (ABA) can also be used to promote ripening of fruit. It was found that ABA promoted ripening in climacteric fruits such as apple and banana mainly by enhancing C_2H_4 biosynthesis (Buesa and Vendrell, 1989; Riov *et al.*, 1990; Jiang *et al.*, 2000), whereas in non-climacteric fruits such as orange (citrus), ABA was shown to induce earlier degreening and to stimulate carotenoid biosynthesis. Jasmonic acid has also been shown to activate ACC oxidase activity, enhance C_2H_4 production in apple and stimulate β-carotene synthesis in tomato (Creelman and Mullet, 1997; Fan *et al.*, 1998).

Applying exogenous ethephon to capsicum fruit hastens the ripening process, but its effectiveness v”Aries” with cultivar, rate, number and timing of application and many other environmental and physiological factors (Cantliffe and Goodwin, 1975; Monselise, 1986). Application of ethephon at concentrations between 1,000 to 15,000 ppm improved colour of Tabasco pepper and increased the abscission of immature fruit which could be minimised by additional application of $Ca(OH)_2$

(Conrad and Sundstrom, 1987). However, when ethephon was applied to hasten ripening of paprika fruit, there was no significant effect on either the proportion of red fruit or the extractable colour of paprika powder (Cooksey *et al.*, 1994). Moreover, ethephon had very limited effect on colour development of green harvested fruit. Apart from ethephon, there has been no publication so far on the involvement of other plant growth regulators in the ripening process of capsicum fruit.

Green harvested capsicum fruit not only failed to develop sufficient red colour for spice processing but also had significantly lower internal CO_2 concentration and total soluble solid content (TSSC) than fruit ripened on the plant (Section 3.3). This is likely due to the lack of external carbon-supply from the mother plant during ripening. The effect of carbon-supply reduction on ripening of green harvested fruit can be studied by using the technique of cincturing (girdling) (Taiz and Zeiger, 2006). Cinctured fruit (fruit with cinctured stems) have no assimilates supplied from the mother plant and this may affect their ripening characteristics.

The reason why green harvested capsicum fruit fail to ripen normally is far from clear and attempts to improve ripening of green harvested fruit by preharvest application of plant growth regulators have been unsuccessful thus far. *The aims of this study, therefore, were to examine the effect of external carbon-supply from the mother plant to capsicum fruit on ripening behaviour as well as to examine the effect of preharvest application of various plant growth regulators at different concentrations on ripening behaviour of green harvested capsicum fruit.*

4.2 Materials and methods

4.2.1 Plant materials

Of the three cultivars used in the previous experiment to study ripening behaviour of fruit on and off the plant: “Papri Queen”, “Aries” and “Caysan” (see section 2.1), “Papri Queen” was selected to be used in the experiment examining the effect of preharvest treatments on ripening behaviour of green harvested fruit. This selection

was based on colour being the main attribute of this cultivar for spice powder and the need for colour improvement of green harvested fruit for spice processing.

The fruit used for experimentation described in this chapter were grown at the glass house at the University of Adelaide as described in section 2.2.2. “Papri Queen” crops were planted continuously from October 2004 to September 2006. All the preparation and fruit setting assessments were carried out as described in section 2.3.

4.2.2 Experimental design and methods

4.2.2.1 Effects of external carbon-supply

The experiment studying effects of sugar supply from the mother plant was designed as completely randomised with cincturing as the treatment factor. The experimental unit was an individual fruit and each treatment contained six fruit as six replicates. The experiment was repeated twice: first in Jan/Feb 2005 (summer trial) and second in July/August 2005 (winter trial).

Control fruit were left to ripen on the plant without treatment. Treated fruit had their stem cinctured at the LG stage (which was determined from the growth curve in section 3.3.1.1) and were allowed to ripen on the plant. Cincturing was carried out by using a sterilised scalpel to remove a small strip of tissues (approximately 1 cm long) around the stem. Removed tissues include dermal, ground tissues and especially, phloems. A small band of parafilm was used to cover the open wound created by cincturing to avoid infection (Figure 4.1).

During the ripening course, samples were taken three times corresponding to three colour stages of control fruit ripened on the plant: breaker, light red, and deep red and partially dried, which were determined from section 3.3.1.1. There was an extra sampling time for cinctured fruit in the second trial where cinctured fruit were left to ripen on the plant until, if possible, they reached the deep red and partially dried stage. Fruit were sampled to measure internal CO_2 concentration, extractable colour and total soluble solid content as described in section 2.3.

4.2.2.2 Effects of preharvest application of plant growth regulators

Initially, a preliminary trial was conducted as a "screening method" using different plant growth regulators at different concentrations, and different combinations, where possible. Based on the results of the preliminary trial, treatments with the most significant effect were then studied further.

Experiments studying effects of preharvest application of plant growth regulators was designed as completely randomized applying different plant growth regulators as treatment factors. The experimental unit was an individual fruit and each treatment contained six fruit as six replicates.

4.2.2.2.1 Method for applying plant growth regulator solution to fruit

The conventional method for preharvest application was spraying. However, the waxy skin of capsicum fruit may make it difficult for the solution to penetrate through and minimise the efficacy. Using the cincturing method (section 4.2.2.1) a new method was developed. When the stem was cinctured, an open circular area was created and the solution was applied into this area.

The ability of solution to penetrate through stem and fruit tissues was examined by applying 10 µL of common food dye solution (Red # 40) to the open area created by cincturing. Fruit were examined 24 h later and the dye solution could be seen to be transported to the distal end of fruit (Figure 4.2). This method, therefore, could be considered as effective and was used for all experimentation.

4.2.2.2.2 Preliminary trial

Fruit at the LG stage on the plant were treated with either distilled water as the control or different plant growth regulator solutions for treated fruit (Table 4.1). The concentrations of applied solutions were selected based on relevant references listed in Table 4.1. Solution (10 µL) was dropped by a pipette into the open area on the stem.

Figure 4.1 Cincturing of Capsicum fruit. The stem above the fruit was cinctured (right) and the open wound was covered with parafilm (left).

Figure 4.2 Penetration of dye solution through fruit tissue. Dye solution was supplied through an open wound created by cincturing. 24 h after application, dye solution had penetrated to the distal end of the fruit (arrows shown points of application).

Fruit were harvested 24 h later and stored individually in an aluminium foil tray covered with a polyethylene bag as described in section 2.3.1. Fruit were sampled at 25 days after harvest (equivalent to the time fruit went from the LG stage to deep red on the plant), and CO_2 concentration, extractable colour and total soluble solid content were measured as described in section 2.3.

Table 4.1 Description of preharvest treatments used in the preliminary trial. 10 µL of applied solutions was dropped into an open area on the stem created by cincturing.

Abbreviation	**Plant growth regulators**	**Volume and concentration**	**References**
Control	Distilled water	10 µL	
ETH-L		10 µL of 2,400 ppm	(Cooksey *et al.*, 1994)
ETH-M	Ethephon	10 µL of 4,800 ppm	(Boronat *et al.*, 2002)
ETH-H		10 µL of 7,200 ppm	
ETH-VH		10 µL of 48,000 ppm	
NAA-L		10 µL of 20 mg L^{-1}	
NAA-M	Naphthalene acetic acid (NAA)	10 µL of 100 mg L^{-1}	(Amoros *et al.*, 2004)
NAA-H		10 µL of 400 mg L^{-1}	
ABA-L		10 µL of $5x10^{-5}$ M	(Jiang and Joyce, 2003)
ABA-M	Abscisic acid (ABA)	10 µL of 10^{-4} M	(Masia *et al.*, 1998)
ABA-H		10 µL of $2x10^{-4}$ M	(Berhow, 2000)
SUC	Sucrose	10 µL of 5%	
JA	Jasmonic acid	10 µL of 100 mg.L^{-1}	(Fan *et al.*, 1998) (Watanabe *et al.*, 2001)
MiA	Ethephon + NAA	5 µL of 2,400 ppm 5 µL of 20 mg.L^{-1}	
MiB	Ethephon +ABA	5 µL of 2,400 ppm 5 µL of 10^{-4} M	
MiC	NAA +ABA	5 µL of 20 mg.L^{-1} 5 µL of 10^{-4} M	

Ethephon (2-chloroethylphosphonic acid) with the trading name of Ethrel® (2-chloroethylphosphonic acid 480 mg L^{-1}) was supplied by Bayer Australia (Pymble, NSW, Australia) and other plant growth regulators were supplied by Sigma-Aldrich Pty Ltd (Castle Hill, NSW, Australia).

To confirm the effect of ethephon treatment on CO_2 production, the respiration rate of control fruit (fruit treated with distilled water) or ethephon-treated fruit (48,000 ppm) was measured during the storage time. Each single fruit (six replicates for each treatment) was placed in a tightly closed 500-mL container for 1 h. 1 mL of gas sample was then withdrawn from the internal atmosphere for the quantification of CO_2 by gas chromatography as described in section 2.3.2. CO_2 quantification was carried out at 34, 39, 47, 51, 56 and 59 DAA corresponding to six colour stages of fruit ripened on the plant (section 3.3.1.1). The respiration rate was calculated as the amount of CO_2 per g of fresh fruit 1 h (mM g^{-1} h^{-1}).

4.2.2.2.3 Effects of preharvest application of ethephon

Based on the results of the preliminary trial (section 4.2.2.2.2), the following treatments were applied to fruit at the LG stage on the plant (Table 4.2).

Table 4.2 Description of preharvest treatments used in the trial that studied effects of ethephon. 10 µL of ethephon solution was dropped into an open area on the stem.

Name of treatments	Name of plant growth regulator	Volume and concentration
Control	Distilled water	10 µL
ETH-L	Ethephon	10 µL of 2,400 ppm
ETH-M	Ethephon	10 µL of 4,800 ppm
ETH-H	Ethephon	10 µL of 7,200 ppm
ETH-VH	Ethephon	10 µL of 48,000 ppm
ETH-SH	Ethephon	3 x 10 µL of 48,000 ppm

10 µL of solution was dropped by a pipette into the open area (3 x 5 x 1mm approximately) on the stem. Fruit were harvested 24 h later and stored individually in an aluminium foil tray covered with a polyethylene bag as described in section 2.3.1. Fruit were sampled at 0, 7, 14, 21 and 28 days after harvest. Half of the fruit tissue was dried for extractable colour measurement and the other half was ground in liquid nitrogen and used fresh for chlorophyll content determination. Extractable colour was measured as described in section 2.3 and total chlorophyll content was measured as described by Vernon (Vernon, 1976).

Fresh fruit tissues were quickly cut into small pieces with a scalpel and then ground with a mortar and pestle in liquid nitrogen. Around 1 to 2 g of ground sample was placed into a 50 mL volumetric flask and 80% acetone was filled to the volume and the flask was placed in the dark for 16 h. The extract solution was filtered using filter papers (Whatman 90 mm diameter) before being transferred to a 1 mL glass cuvette to measure the absorbance (A) at 649 and 665 nm using a spectrophotometer (SP8001, Metertech). Acetone was used as a blank and total chlorophyll content was calculated using following equation:

$$\textbf{Total chlorophyll (mg.L}^{-1}\textbf{)} = 6.45 \times A_{665} + 17.72 \times A_{649} \textbf{ (Vernon, 1976)}$$

4.3 Result

4.3.1 Effects of cincturing

Cinctured fruit were able to ripen and turn totally red similar to control fruit (Figure 4.3). However, the colour change in cinctured fruit was slower and in the second trial (in winter time), cinctured fruit reached the final stage of ripening (deep red and partially dried stage) 5 ± 0.25 days later than control fruit.

Figure 4.3 Cinctured fruit were able to turn breaker (left) and eventually red (right). Pictures were taken at 60 (left) and 70 (right) days after anthesis.

4.3.1.1 Extractable colour

When fruit ripened in summer, extractable colour of both cinctured and control fruit increased throughout ripening, but to a different extent (Figure 4.4A). The extractable colour of cinctured fruit was significantly lower than that of control fruit. However, at the final stage (62 DAA) cinctured fruit was considered fully red (Figure 4.4A) with extractable colour above the required level of 140 ASTA units.

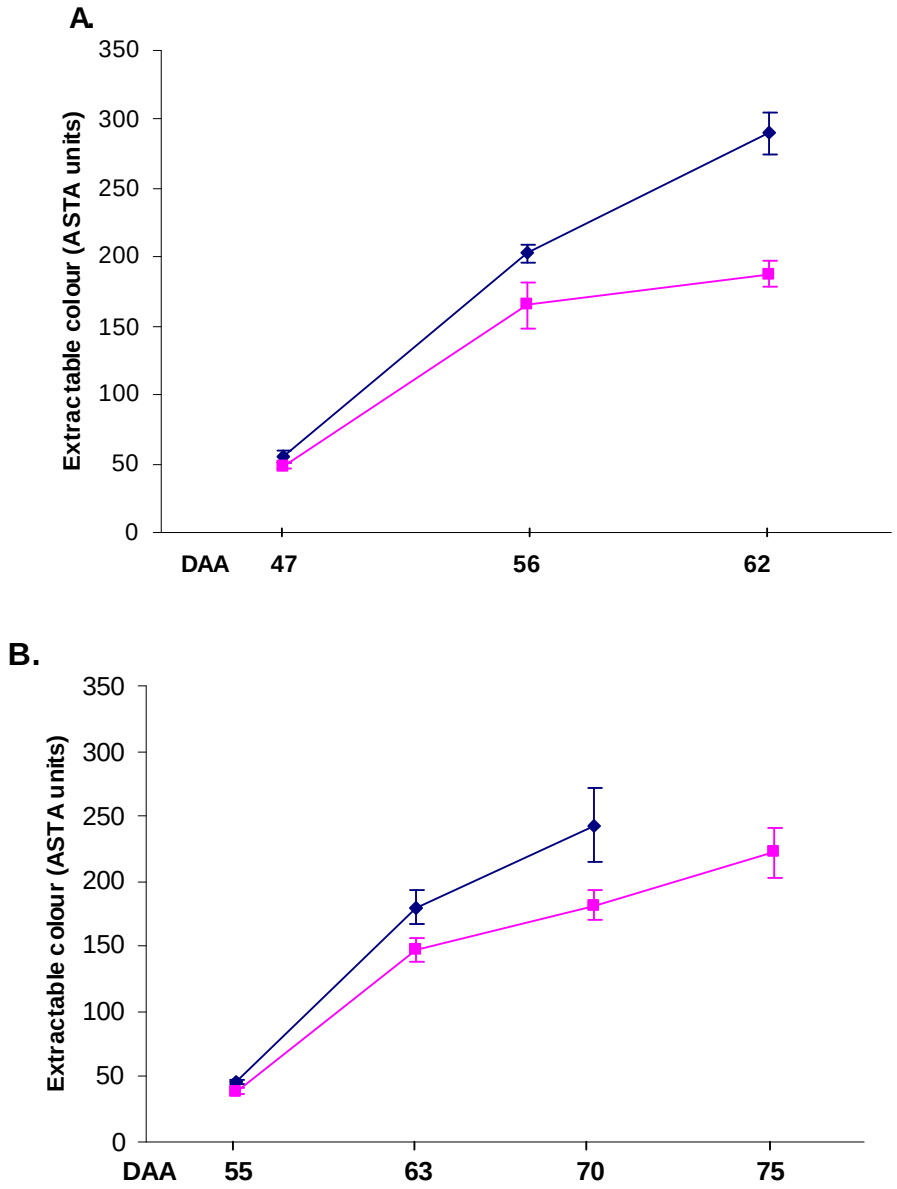

Figure 4.4 Change in extractable colour (ASTA units) of control fruit (♦) and cinctured fruit (■) ripened on the plant in summer (A) and winter (B). Crop was planted from October 2004 to February 2005 (A) and March to August 2005 (B). Data are the means of six replicates (± SE) and the least significant difference ($P<0.05$) is 21.47 for (A) and 28.92 for (B).

Similarly, when fruit ripened in winter, extractable colour of both cinctured and control fruit also increased throughout ripening (Figure 4.4B) but the increase in colour of cinctured fruit was slower. Notably, cinctured fruit reached the final deep red and partially dried stage 5 days later, and the extractable colour at this stage was not significantly different to that of control fruit at the same stage.

4.3.1.2 Internal CO_2 concentration

When fruit ripened in summer, the change in internal CO_2 concentration was similar in both cinctured and control fruit: it significantly increased from 47 to 56 DAA and then remained stable towards the end of ripening (Figure 4.5A). The CO_2 concentration of cinctured fruit was not significantly different to that of control fruit except for the final stage (62 DAA) when it was significantly higher.

When fruit ripened in winter, the change in internal CO_2 concentration of cinctured fruit showed a similar pattern but it peaked later than that in control fruit (Figure 4.5B). CO_2 concentration of control fruit peaked at 63 DAA (when fruit turned totally red) and then declined slightly to 70 DAA while for cinctured fruit it increased to 70 DAA to the same extent before declining to 75 DAA when fruit were deep red and partially dried. However, at 55 and 63 DAA the CO_2 concentration was significantly lower in cinctured fruit.

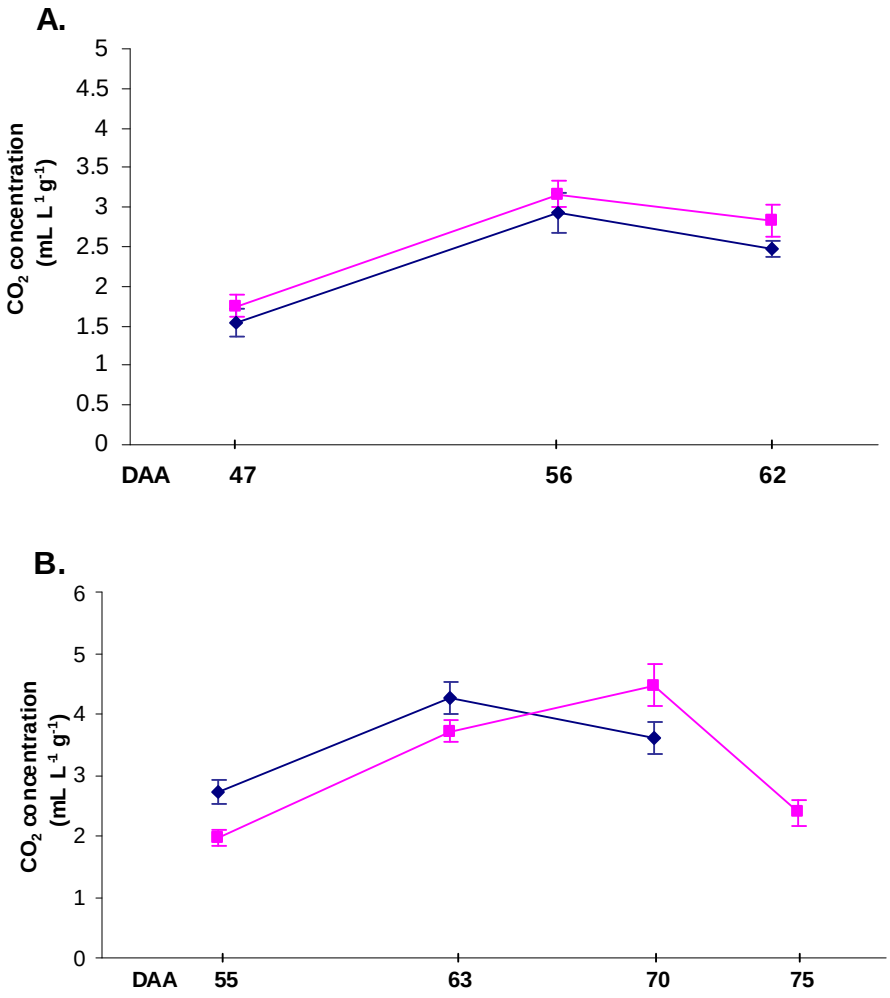

Figure 4.5 Change in internal CO_2 concentration (mL L^{-1} g^{-1}) of control fruit (♦) and cinctured fruit (■) ripened on the plant in summer (A) and winter (B). Crop was planted from October 2004 to February 2005 (A) and from March to August 2005 (B). Data are the means of six replicates (± SE) and the least significant difference ($P<0.05$) is 0.4125 for (A) and 0.3904 for (B).

4.3.1.3 Total soluble solid content

When ripened in summer time, TSSC of both cinctured and control fruit rapidly increased and was not significantly different during the early phase of ripening, from 47 to 56 DAA (Figure 4.6A). However, during the final phase of ripening, TSSC of both cinctured and control fruit remained stable and not significantly different.

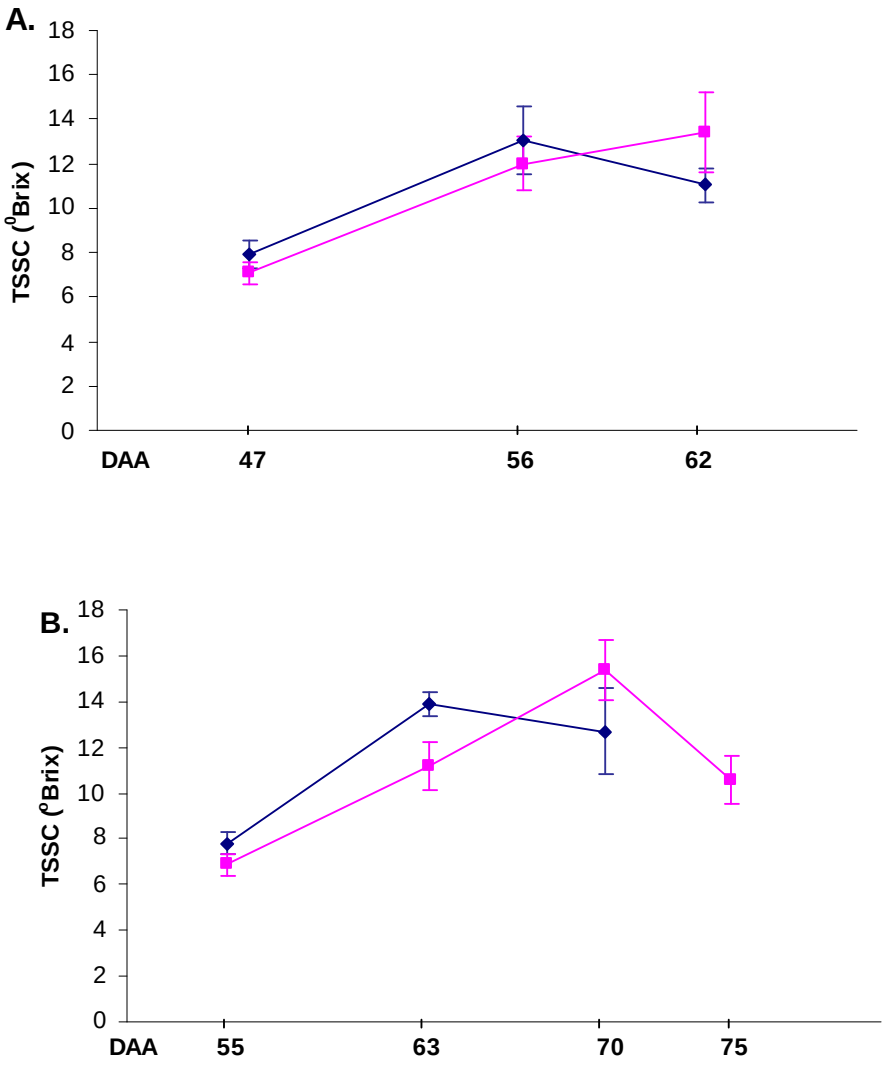

Figure 4.6 Change in TSSC (°Brix) of control fruit (♦) and cinctured fruit (■) ripened on the plant in summer (A) and winter (B). Crop was planted from October 2004 to February 2005 (A) and from March to August 2005 (B). Data are the means of six replicates (± SE) and the least significant difference (P<0.05) is 2.396 for (A) and 2.227 for (B).

When fruit ripened in winter, TSSC of both cinctured and control fruit increased to a similar extent between 55 and 63 DAA (Figure 4.6B). From 63 to 70 DAA, TSSC of cinctured fruit continued to increase while it was unchanged for control fruit. However, when cinctured fruit approached the deep red and partially dried stage at 75 DAA, TSSC was significantly reduced.

4.3.2 Effects of preharvest application of plant growth regulators

Only fruit treated with ethephon solution developed a totally red colour while fruit treated with other plant growth regulators failed to turn red like the control fruit.

4.3.2.1 Extractable colour

Fruit treated with 7,200 and 48,000 ppm ethephon solution had significantly higher extractable colour than control fruit (Figure 4.7). Although extractable colour of fruit treated with 48,000 ppm ethephon solution was the highest, it was still lower than the acceptable level of 140 ASTA units. It was interesting to note that all fruit treated with ethephon solution developed a totally red skin colour (Figure 4.8) despite their low level of extractable colour.

In contrast, fruit treated with sucrose had significantly lower extractable colour than the control and extractable colour of fruit from the rest of the treatments was not significantly different to the control.

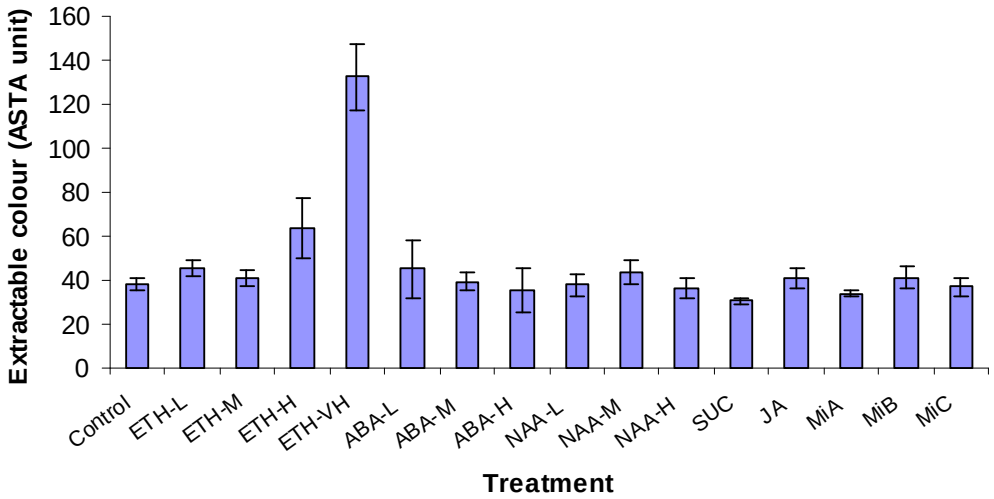

Figure 4.7 Extractable colour (ASTA) of fruit treated with different plant growth regulators (Table 4.1). Treatments were carried out one day before fruit were harvested at the LG stage. Measurements were carried out at 25 days after harvest. Data are the means of twelve replicates (± SE) from two experiments and the least significant difference ($P<0.05$) is 20.65.

Figure 4.8 The effect of preharvest application of ethephon on colour development of capsicum fruit. Fruit were treated with different concentrations of ethephon solution (0; 2,400; 4,800; 7,200 and 48,000 ppm) one day before harvest. All treated fruit developed a totally red skin colour at 25 days after harvest.

4.3.2.2 Carbon dioxide concentration

Fruit treated with three highest concentrations of ethephon (4,800; 7,200 and 48,000 ppm) had a significantly higher internal CO_2 concentration than control fruit (Figure 4.9). Although CO_2 concentrations of fruit treated with ethephon 2,400 ppm, 2 x 10^{-4} M ABA, NAA (20 and 100 mg L^{-1}) and JA solution appeared higher than the control fruit they were not statistically different. In contrast fruit treated with combination of ethephon and ABA (MiA) and of ethephon and NAA (MiB) had a significantly lower CO_2 concentration than control fruit. Fruit from the rest of the treatments had CO_2 concentrations not significantly different from the control fruit.

The respiration rate of both control fruit and fruit treated with ethephon 48,000 ppm solution decreased during the postharvest storage (Figure 4.10). However, the respiration rate of fruit treated with ethephon was significantly lower than that of control fruit at harvest (24 h after treatment). After 39 DAA, the respiration rate of ethephon-treated fruit was slightly but significantly higher than that of control fruit.

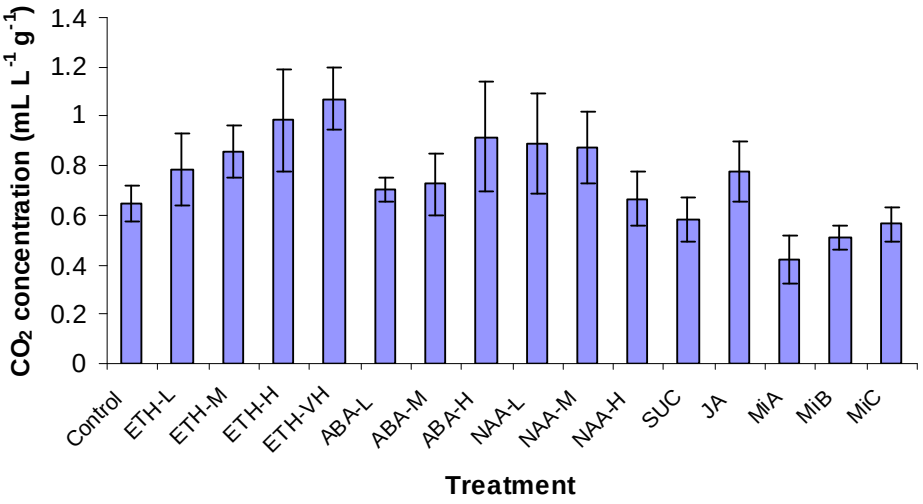

Figure 4.9 Internal CO_2 concentrations (mL L^{-1} g^{-1}) of fruit treated with different plant growth regulators (Table 4.1). Treatments were carried out one day before fruit were harvested at the LG stage. Measurements were carried out at 25 days after harvest. Data are the means of twelve replicates (± SE) from two experiments and the least significant difference (P<0.05) is 0.3492.

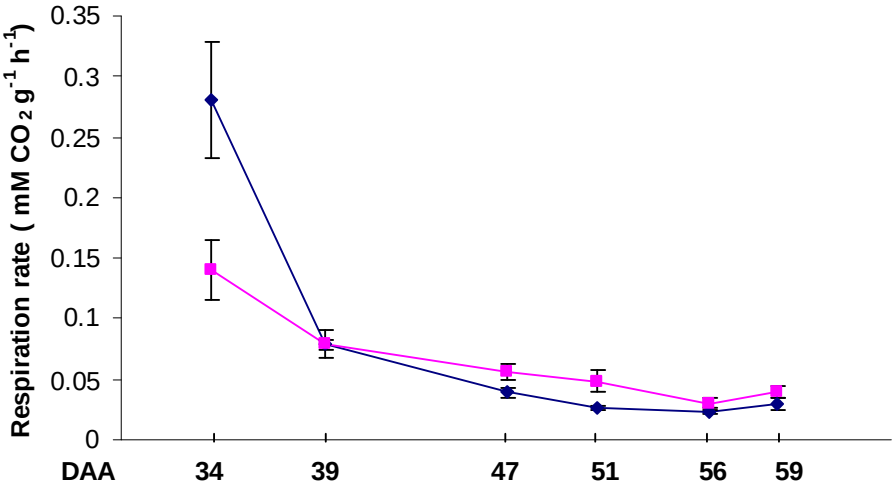

Figure 4.10 Change in respiration rate (m M CO_2 g^{-1} h^{-1}) of control and treated fruit during postharvest storage. Fruit were supplied with 10 µL of water (Control-♦) or ethephon solution 48,000 ppm (Treated-■) at 33 DAA, harvested 1 day later and stored at room conditions. Data are the means of six replicates (± SE) and the least significant difference (P<0.05) is 0.019.

4.3.2.3 Total soluble solid content

TSSC of fruit treated with 7,200 and 48,000 ppm ethephon, and JA solution was significantly higher than that of the control fruit (Figure 4.11). In contrast, fruit treated with 2,400 and 4,800 ppm ethephon solution, NAA 400 mg L^{-1} and a combination of ethephon and NAA (MiB) had lower TSSC than the control fruit. TSSC of fruit from the rest of the treatments was not significantly different from that of the control fruit.

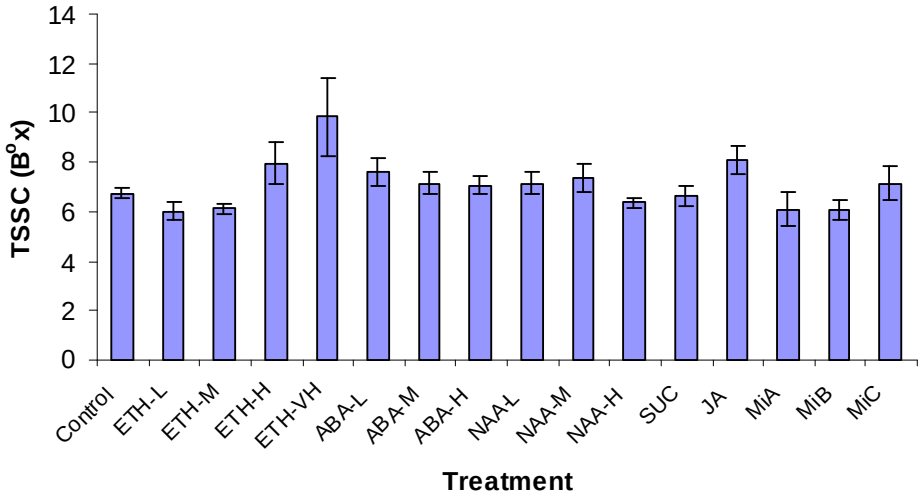

Figure 4.11 Total soluble solid content ($B^{o}x$) of fruit treated with different plant growth regulators (Table 4.1). Treatments were carried out one day before fruit were harvested at the LG stage. Measurements were carried out at 25 days after harvest. Data are the means of twelve replicates (± SE) from two experiments and the least significant difference ($P<0.05$) is 1.617.

4.3.3 Effects of preharvest application of ethephon

All fruit treated with ethephon developed red skin colour (Figure 4.8), especially fruit treated with 3 x 48,000 ppm ethephon solution which turned deep red and partially dried at the final stage (Figure 4.12). However, fruit treated with ethephon, especially at high concentration were severly infected with fungus at the stem and calyx areas (Figure 4.13).

Figure 4.12 Fruit treated with 3 x 48,000 ppm ethephon solution turned deep red and partially dried at 25 days after harvest.

Figure 4.13 Fruit treated with ethephon solution were infected with fungus at 25 days after harvest.

4.3.3.1 Extractable colour

There was no significant difference in extractable colour between ethephon-treated and control fruit during the first 7 DAH (Figure 4.14). Extractable colour of control fruit slightly increased to 28 DAH but at the final stage, it was lower than that of all treated fruit. At 14 and 21 DAH, fruit treated with 3 x 48,000 ppm ethephon solution had twofold higher extractable colour than the control fruit while extractable colour of fruit treated with other lower concentrations was not significantly different to the control fruit. However, at 28 DAH all treated fruit had significantly higher extractable colour than the control fruit. Treatment with 3 x 48,000 ppm ethephon

solution increased extractable colour threefold while treatments with other concentrations increased it by 30 to 50%.

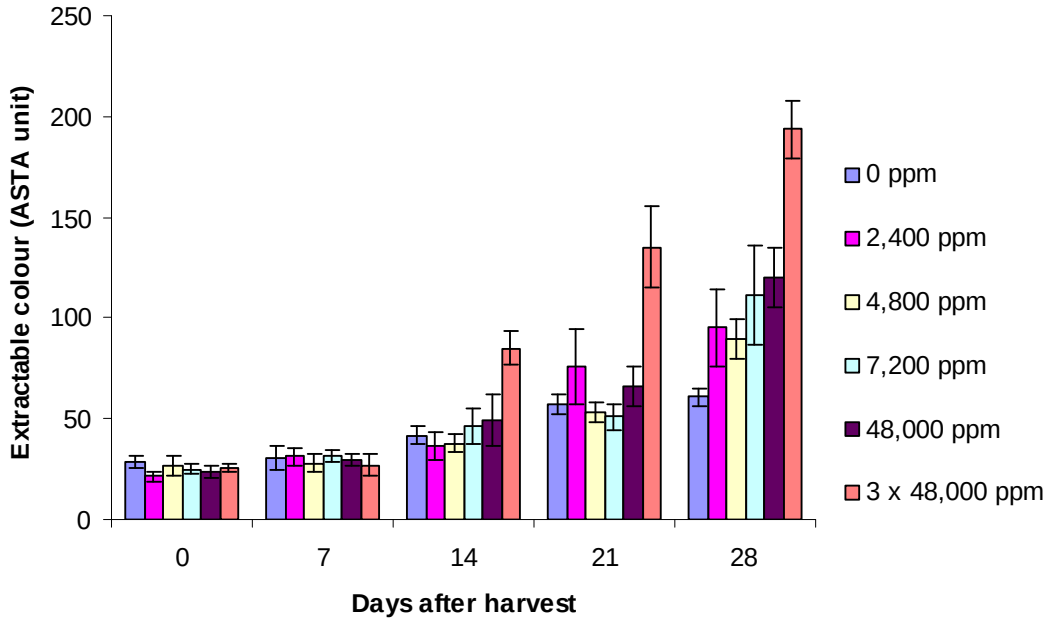

Figure 4.14 Change in extractable colour (ASTA unit) of fruit treated with different concentrations of ethephon solution: 0 (■); 2,400 (■); 4,800 (■); 7,200 (■); 48,000 (■) and 3 x 48,000 ppm (■).Treatments were carried out one day before fruit were harvested at the LG stage. Measurements were carried out at 0, 7, 14, 21 and 28 days after harvest. Data are the means of six replicates (± SE) and the least significant difference (P<0.05) is12.08.

4.3.3.2 Chlorophyll content

There was no significant difference in chlorophyll content between ethephon-treated and control fruit during the first 7 day after harvest (DAH) (Figure 4.15). From 14 DAH, chlorophyll content of both control and treated fruit decreased but the degradation rate was higher in treated fruit and it increased with the increase of ethephon concentration.

At 14 DAH, chlorophyll content of fruit treated with high concentration (from 7,200 to 3 x 48, 000 ppm ethephon solution) was significantly lower than that of the control fruit or of fruit treated with 2,400 and 4,800 ppm solution. At 21 DAH all treated fruit, regardless of solution concentration, had significantly lower chlorophyll content than the control fruit. The difference ranged between 50 and 80%. At the end

of ripening, 28 DAH, chlorophyll content of all treated fruit was reduced dramatically and was equivalent to only 10 % of chlorophyll content of the control fruit.

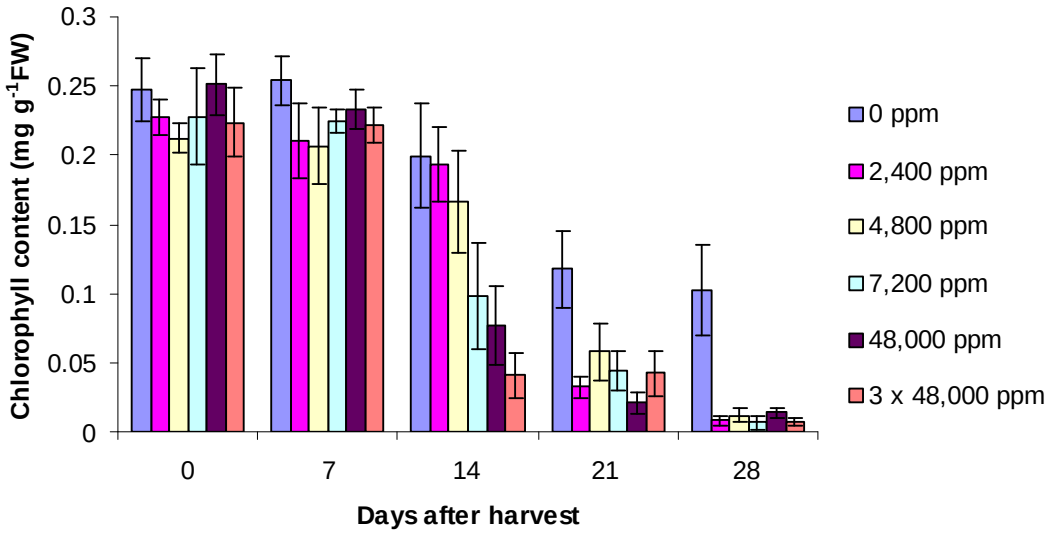

Figure 4.15 Change in chlorophyll content (mg g^{-1}FW) of fruit treated with different concentrations of ethephon solution: 0 (■); 2,400 (■); 4,800 (■); 7,200 (■); 48,000 (■) and 3 x 48,000 ppm (■).Treatments were carried out one day before fruit were harvested at the LG stage. Measurements were carried out at 0, 7, 14, 21 and 28 days after harvest. Data are the means of six replicates (± SE) and the least significant difference (P<0.05) is 0.0268.

4.4 Discussion

4.4.1 Effect of cincturing

Cincturing delayed ripening of fruit from "Papri Queen" on the plant. All the changes in CO_2 concentration, extractable colour and total soluble solid content were similar between cinctured and control fruit, but the changes in cinctured fruit occurred approximately 5 ± 1.3 days later.

Cincturing was carried out at the LG stage when fruit had ceased their growth and development and had commenced ripening. At this stage, fruit had completed accumulating starch and sucrose in preparation for the next phase of ripening when mainly hexoses were accumulated (Nielsen *et al.*, 1991). The similarity in CO_2

concentration between cinctured and control fruit during summer (Figure 4.5) may suggest that either fruit did not entirely depend on external carbon sources for respiration activity or otherwise fruit had to utilise their internal carbon source to compensate the loss of external supply. The lower level of CO_2 concentration and the later increase in CO_2 concentration of cinctured fruit ripening in winter time (Figure 4.5) indicated that fruit did partly use external carbon supply for respiration activity. With hexoses produced by starch and sucrose degradation, cinctured fruit should have plenty of respiration substrate to maintain the required energy for biochemical and physiological processes during ripening. Cincturing apple fruit led to a declining starch level and the increase in the activities of many enzymes in the glycolysis pathway is a means of meeting energy required for the ripening process (Beruter and Feusi, 1997).

The higher CO_2 concentration in both cinctured and control fruit ripening in winter compared to fruit ripened in summer may indicate that the energy level required for ripening was higher in winter. Moreover, temperature and light factors may have an impact on starch degradation enzyme activities during winter time and this in combination with the higher energy required may lead to the lower level of CO_2 concentration of cinctured fruit during the early stage of ripening.

Cincturing delayed but did not inhibit extractable colour development of fruit from “Papri Queen” (Figure 4.4). Cinctured fruit were able to turn fully red and partially dried at the end of ripening (Figure 4.3), although this process took around 5 days longer than in control fruit. Cinctured fruit had significantly lower levels of extractable colour during the early phase of ripening. This suggested that external carbon-supply may play an important role in carotenoid synthesis of capsicum fruit. Carotenoid synthesis in capsicum fruit starts with the isopropenoid pathway in which AcetylCoA acts as a precursor (Goodwin, 1970). The loss of external carbon-supply may lead to a reduction of AcetylCoA production, and thus decreasing carotenoid biosynthesis. Moreover, a limited source of carbon-supply might create competition for AcetylCoA utilisation such that respiration activity was a priority at the early phases and carotenoid synthesis at the later phases of ripening.

Using hexoses produced from reserve starch and sucrose as a carbon-supply during ripening led to lower hexoses accumulated by cinctured fruit and thus lower TSSC than control fruit. The difference was especially significant during the early phase of ripening and during winter, when energy required for ripening was high. However, the highest levels of both extractable colour and TSSC of cinctured fruit were not significantly lower than those of control fruit. That may indicate that although capsicum fruit did use external carbon-supply, this took only an insignificant proportion, and sugars and other phloem-derived factors did not play a critical role during fruit ripening.

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4.4.2 Effect of preharvest application of plant growth regulators

Preharvest application of plant growth regulators (as presented in this chapter) had very limited effects on ripening behaviour of green harvested fruit with the exception of ethephon. Treatments with 48,000 ppm ethephon solution significantly increased CO_2 concentration, extractable colour and TSSC of "Papri Queen" fruit at the final stage of ripening (25 DAH), but other concentrations had variable effects.

Treatment with ethephon solutions significantly increased CO_2 concentration of fruit at 25 DAH, especially with 7,200 and 48,000 ppm concentration. However, when the respiration rate of ethephon-treated fruit during ripening was investigated (Figure 4.10), there was no such an increase in respiration activities as a typical response of fruit to C_2H_4 treatment (Biale and Young, 1981). In addition, at 25 DAH, all fruit treated with ethephon solutions were severely infected with fungus (Figure 4.13) and that may have increased the CO_2 concentration of the fruit (Saltveit, 1997).

All fruit treated with ethephon, regardless of concentration, developed totally red colour on the surface (Figure 4.8). This could be considered a significant improvement because ethephon was ineffective as a degreening agent for capsicum fruit. In previous studies (Lockwood and Vines, 1971; Conrad and Sundstrom, 1987), preharvest spraying with ethephon solutions increased the percentage of red fruit at harvest by accelerating ripening of fruit when they were still attached to plants or induced abscission of immature young fruit (Kahn *et al.*, 1997). However, despite

full red colour of the fruit surface, extractable colour of all treated fruit did not pass the acceptable level of 140 ASTA units for spice processing. The extractable colour of treated fruit was increased in proportion with the increase of concentration of ethephon solution and it appeared to be concentration-dependent.

The difference in skin colour and extractable colour development of ethephon-treated fruit could be well understandable if chlorophyll degradation was studied. The red colour of the fruit surface may be the result of complete chlorophyll breakdown (control fruit still retained some green colour), but carotenoid biosynthesis, especially capsanthin and capsorubin, may not be enhanced to improve extractable colour. This could be better understood by planning further study of chlorophyll and extractable colour changes during ripening of ethephon-treated fruit (section 4.2.2.2).

ABA has been shown to be involved in ripening of some fruit: to enhance C_2H_4 production, accelerate red colour development in strawberry (Jiang and Joyce, 2003) or to induce earlier degreening in banana (Jiang *et al.*, 2000) and orange (Buesa and Vendrell, 1989). However, when ABA solution was applied to capsicum fruit, no significant change in ripening behaviour was observed, especially extractable colour.

NAA and other synthetic auxins have been reported to be involved in ripening of many fruit, mainly by inducing C_2H_4 production. The effect of NAA treatment on ripening was variable depending on many factors such as species, timing and rate of application. NAA promoted ripening in some fruit such as peach (Ohmiya, 2000) and apple (Yuan and Carbaugh, 2007), but it delayed the onset of ripening in grape berry (Davies *et al.*, 1997). Although NAA appeared to have a stimulatory effect on respiration activity of fruit, it had no significant effects on overall ripening behaviour.

Both ABA and auxins (NAA) have been reported to closely interact with C_2H_4 and could be involved in ripening through altering C_2H_4 production (McGlasson, 1985). However, as C_2H_4 has a minimal role in ripening of capsicum fruit, ABA and auxins may have no effect even though they could influence C_2H_4 production.

Although green harvested fruit had low CO_2 and TSSC levels and failed to ripen, supplying fruit with sucrose solution did not promote ripening of fruit. This may suggest that the low levels of CO_2 and TSSC were caused by the inactivation or inhibition of enzyme activities rather than the lack of the external carbon-supply source. Moreover, the results of the cincturing trial indicated that the loss of this external source may not be the sole reason for the failure to ripen of green harvested fruit. Sucrose supply may have had an effect on the expression of carbohydrate-related genes that are regulated by sugar signals as observed in citrus and apple (Li *et al.*, 2003; Beruter and Feusi, 1997) but sugar supply appeared to have very little effect on capsicum and thus did not promote ripening of green fruit.

Although JA has been reported to enhance ACCO activity in apple (Creelman and Mullet, 1997) and stimulate β-carotene biosynthesis in tomato (Fan *et al.*, 1998), it had no significant effect on extractable colour of treated fruit.

Interestingly, preharvest application of some combinations of plant growth regulator also appeared to have very limited effects on ripening of capsicum fruit. It is likely that plant growth regulators other than C_2H_4 had very limited roles during ripening of capsicum fruit. Alternatively, they may have some close interactions with C_2H_4 but were not able to exert any significant effects due to the limited involvement of C_2H_4 during ripening of capsicum fruit.

4.4.3 Effect of preharvest application of ethephon

It is obvious that preharvest application of ethephon had significant effects on both chlorophyll content and extractable colour of fruit from “Papri Queen”. However, there was a time lapse before any considerable effects could be observed in treated fruit. Interestingly, the response to ethephon treatments was not similar in chlorophyll degradation and extractable colour development. Both were induced but chlorophyll degradation appeared to be concentration-independent while extractable colour did not.

The degradation of chlorophyll appeared to be only induced from 14 DAH and appeared to be proportional with ethephon concentrations. However, after this initial period, chlorophyll degraded in all treated fruit regardless of concentration and at the final stage, treated fruit retained almost no significant amount of chlorophyll. That is possibly why all treated fruit had a totally red surface.

It is interesting to note that chlorophyll was not significantly degraded during the early phase of ripening, especially between 0 and 7 DAH. *Chlase* 1 gene, which encodes chlorophyllase, the first enzyme in the chlorophyll degradation pathway (Figure 2.1), was heavily up-regulated after only 3 hours of C_2H_4 treatment in citrus fruit (Wilk *et al.*, 1999). However, this enzyme was located in the inner membrane of the chloroplast envelope and may require a chlorophyll carrier to shuttle chlorophyll from the thylakoids to its action site (Hortensteiner, 1999). The transition from chloroplast to chromoplast and disorganisation of thylakoids occurring during fruit ripening may assist the contact between enzyme and substrates, and therefore hasten the degradation of chlorophyll (Mendez and Mosquera, 2002). That may explain why *Chlase* was up-regulated but chlorophyll degradation was not induced during the first 7 DAH. That also suggested that not only the up-regulation of *Chlase* was involved in inducing chlorophyll degradation but also other proteins and ripening-related pathways which were also regulated by C_2H_4. The up-regulation of *Chlase* may be concentration-independent, but the other factors that help chlorophyll to reach the enzyme site may not and it may explain why chlorophyll degradation during the period between 7 and 14 DAH appeared to be dependent on ethephon concentration. However, when the substrate was in reach of the enzyme, it was completely degraded by increasing enzyme activity regardless of the concentration applied.

Similar to the degradation of chlorophyll, the development of extractable colour appeared to be induced no sooner than 7 DAH. However, during the remaining period of ripening, only one concentration of ethephon application, 48,000 ppm, significantly enhanced extractable colour of treated fruit. The totally red colour of the fruit surface, therefore, should be the result of the complete degradation of chlorophyll and the achievement of extractable colour should mainly depend on the synthesis of carotenoids, especially the red carotenoids specified for capsicum species: capsanthin and capsorubin.

The synthesis of carotenoids in capsicum fruit contains several steps with the participation of many different enzymes (Figure 2.2) (Mosquera and Mendez, 1994). Many studies have reported the induction of different carotenoid synthesis in fruit treated with C_2H_4 or C_2H_4-generating compounds (Marty *et al.*, 2005; Rodrigo and Zacarias, 2007), but few in capsicum fruit. The results presented in this research clearly indicated that the biosynthesis of carotenoid was induced when fruit were supplied with a sufficient amount of C_2H_4. However, the mechanism of C_2H_4 action and the amount necessary to induce carotenoid biosynthesis were far from clear and further studies are required to answer those questions.

Another interesting issue is the mechanism of C_2H_4 action during ripening of harvested capsicum fruit regarding the notion of climacteric and non-climacteric fruit. Non-climacteric fruit respond to C_2H_4 treatments in a one-off mechanism: when C_2H_4 was removed, ripening parameters return back to the level before treatment. In contrast, whenever ripening is induced in climacteric fruit, it is irreversible. This difference is mainly due to the capacity of climacteric fruit to produce C_2H_4 autocatalytically. Fruit in this study were treated with a one-off application of ethephon and the responses in chlorophyll content and extractable colour only could be observed from 14 DAH. These responses appeared to resemble the characteristic of climacteric fruit but whether or not C_2H_4 regulates the whole ripening process or just some individual events is unknown.

4.5 Conclusion

Cincturing did not inhibit but only delayed ripening of fruit from “Papri Queen”. Cinctured fruit were able to fully ripen with similar quality to control fruit. The loss of external carbon-source from the mother plant, therefore, was not the main reason why green harvested fruit failed to ripen normally.

Preharvest application of plant growth regulators other than C_2H_4 had very minor effects on inducing ripening of green harvested fruit in the scale of this study. Preharvest application of C_2H_4 was proven to be effective in inducing ripening of

green harvested fruit. Fruit treated with ethephon solutions, especially with 48,000 ppm, had higher levels of CO_2 concentration, extractable colour and TSSC than control fruit. C_2H_4 induced external colour development of green harvested fruit mainly by inducing the complete degradation of chlorophyll thus resulting in the totally red colour of the fruit surface. However, only a high amount of C_2H_4 could induce sufficient extractable colour development and the mechanism of action was unclear, especially given the late stage during storage at which effects became visible.

Chapter Five

Chapter Five - Effects of postharvest application on ripening of green harvested “Papri Queen”

5.1 Introduction

Postharvest treatments and handling have long been effectively used to control ripening behaviour of fruit. Several ripening processes in fruit such as colour development, texture change and chemical composition could be optimised for desirable qualities by applying suitable postharvest treatments and storage conditions. For capsicum fruit, extending the shelf-life is highly desirable for fresh consumption purposes while better extractable colour is extremely important for spice processing.

The failure of green harvested fruit to ripen normally and develop sufficient colour for spice processing was reported in a previous chapter of this study (section 3.3.1.6) as well as in other studies (Saltveit, 1997; Krajayklang *et al.*, 2000). Several postharvest treatments, therefore, have been intensively developed to improve colour development of green harvested fruit.

One of the most common methods for degreening fruit and enhancing colour development is exposure to ethylene (C_2H_4) gas. In this method, fruit are exposed to air containing C_2H_4 in a closed atmosphere for a period of time after harvest. This method has been commercially successful when applied to banana, muskmelon, citrus and many others (Burg and Burg, 1965; Wilk *et al.*, 1999). However, very limited successes have been recorded when C_2H_4 has been applied to capsicum fruit (Lockwood and Vines, 1971; Knavel and Kemp, 1973; Krajayklang *et al.*, 2000). Not only colour but also other ripening characteristics like CO_2 and C_2H_4 production did not respond significantly to C_2H_4 (section 4.3.2.2.) or propylene treatment (Lu *et al.*, 1990; Saltveit, 1997). This lack of response is one of the reasons why capsicum fruit were generally classified as non-climacteric. However, the ineffectiveness of C_2H_4

treatment may be due to the difficulty for C_2H_4 to penetrate through the thick cuticle of the fruit (Lockwood and Vines, 1971).

An alternative method to enhance colour development in capsicum fruit was to dip fruit into ethephon (2-cloroethylphosphonic acid, an ethylene-generating compound) solution. However, it has not been able to promote the red colour of green harvested fruit and the ability of ethephon to penetrate through the waxy skin of capsicum fruit was questionable.

Besides C_2H_4e and ethylene-generating compounds, other plant growth regulators have been found to be effective in enhancing ripening of many fruit: ABA in strawberries, bananas, apples (Buesa and Vendrell, 1989; Riov *et al*., 1990), auxin in apples and peach (Yuan and Carbaugh, 2007; Ohmiya, 2000), but to my knowledge there has not been such a study on harvested capsicum fruit reported.

Because attempts to promote colour development of green harvested fruit so far have not been very successful and the investigation of the involvement of plant growth regulators other than C_2H_4 have been limited for capsicum fruit, *the aim of the research presented in this chapter was to examine effects of postharvest application of various plant growth regulators on ripening of green harvested capsicum fruit.*

5.2 Materials and methods

5.2.1 Cultivar selected and plant materials

Among the three cultivars selected to study ripening behaviour of fruit on and off the plant: “Papri Queen”, “Aries” and “Caysan” (Section 2.1), “Papri Queen” was selected to examine the effect of postharvest application of plant growth regulators. This selection was based on the fact that colour was the main attribute of this cultivar for spice powder and improvement of colour was the main objective of this study.

The fruit used for experimentation described in this chapter were grown at the glass house at the University of Adelaide as described in section 2.2.2. “Papri Queen”

crops were planted continuously from October 2004 to September 2006. All preparation and fruit setting assessments were carried out as described in section 2.3.

5.2.2 Experimental design and methods

To study the effects of postharvest application of plant growth regulators, different plant growth regulators were randomly applied as treatment factors. The experimental unit was an individual fruit and each treatment contained six fruit as six replicates and the experiment was repeated twice.

Initially, a number of plant growth regulators were trialled and treatments with the most outstanding effects were then examined further.

The conventional method for postharvest application has been dipping or gas exposure. However, in both methods of application it may be difficult for plant growth regulators to penetrate through fruit tissues. Because the method used in the previous chapter (section 4.2.2.2) was demonstrated to be effective, the method of supplying solution to harvested capsicum fruit was similar. By using a scalpel to remove the outer layer of the stem including the phloem, a small hole (10x3x1 mm, approximately) on the stem was created into which the solution could be dropped to penetrate through the fruit tissue.

5.2.2.1 Effects of postharvest application of plant growth regulators

Fruit were harvested at the LG stage and an open hole (Figure 5.1) for applying solutions was created on the fruit stem by hand using a scalpel. 10 µL of plant growth regulator solutions (Table 5.1) was dropped into this hole for 10 consecutive days after harvest. Fruit were stored individually in an aluminium foil tray covered with a polyethylene bag at room temperature as described in section 2.3.1. Fruit were sampled at 25 days after harvest and internal CO_2 concentration, extractable colour and total soluble solid content were measured as described in section 2.3. The experiment was repeated twice.

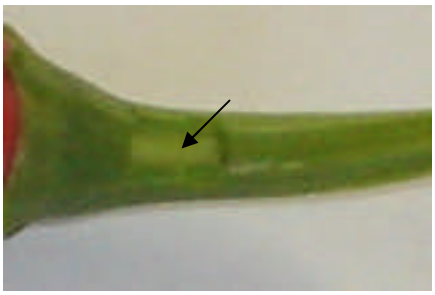

Figure 5.1 Postharvest application of plant growth regulator. A small open hole was created in the fruit stem for supplying solution.

Table 5.1 Description of postharvest application of plant growth regulators. 10 µL of applied solution was dropped into the open area on the stem for 10 consecutive days after harvest (using the same concentrations used in the previous chapter, section 4.2.2.2.2)

Name of treatments	Name of plant growth regulators	Concentration of solutions
Control	Distilled water	10 µL
ETH	Ethephon	10 µL of 2,400 ppm
NAA	Naphthalene acetic acid (NAA)	10 µL of 20 mg L^{-1}
ABA	Abscisic acid (ABA)	10 µL of $5x10^{-5}$ M
SUC	Sucrose	10 µL of 5%
JA	Jasmonic acid	10 µL of 100 mg L^{-1}

Ethephon (Ethrel® solution 2-chloroethylphosphonic acid 480mg L^{-1}) was supplied by Bayer Australia (Pymble, NSW, Australia) and other plant growth regulators were supplied by Sigma-Aldrich Pty, Ltd (Castle Hill, NSW, Australia).

5.2.2.2 Effect of postharvest application of ethephon

Based on the results of the preliminary trial (sections 5.2.2.2.2 and 5.3.1), the following treatments were applied to harvested fruit for the subsequent trial (Table 5.2).

Table 5.2 Description of postharvest application of ethephon. 10 µL of applied solution was dropped into the open area on the stem for 10 consecutive days after harvest.

Name of treatments	Name of plant growth regulator	Concentration/volume of solution
Control	Distilled water	10 µL
ETH-L	Ethephon	10 µL of 2,400 ppm
ETH-H	Ethephon	10 µL of 7,200 ppm
ETH-VH	Ethephon	10 µL of 48,000 ppm

Solutions (10 µL) were dropped by a pipette into the open hole on the fruit stem for 10 consecutive days after harvest. Fruit were stored individually in an aluminium foil tray covered with a polyethylene bag at room temperature as described in section 2.3.1. Fruit were sampled at 0, 7, 14, 21 and 28 days after harvest. Fruit tissues were either dried for extractable colour measurement, or ground in liquid nitrogen and used fresh for chlorophyll content. Extractable colour was measured as described in section 2.3, total chlorophyll content was measured as described by Vernon (Vernon, 1976) and in section 4.2.

5.3 Results

5.3.1 Effect of postharvest application of plant growth regulators

Only fruit treated with ethephon solution developed totally red skin colour. Fruit treated with other plant growth regulators did not have significantly different skin colour to the control fruit.

5.3.1.1 Extractable colour

In the preliminary trial, extractable colour of fruit treated with ethephon (2,400 ppm) was more than twice that of the control fruit but still lower than the acceptable level of 140 ASTA units (Figure 5.2).

In contrast, fruit treated with JA had lower extractable colour than the control fruit while postharvest treatments with other plant growth regulators (ABA, NAA and sucrose) did not significantly change extractable colour of fruit.

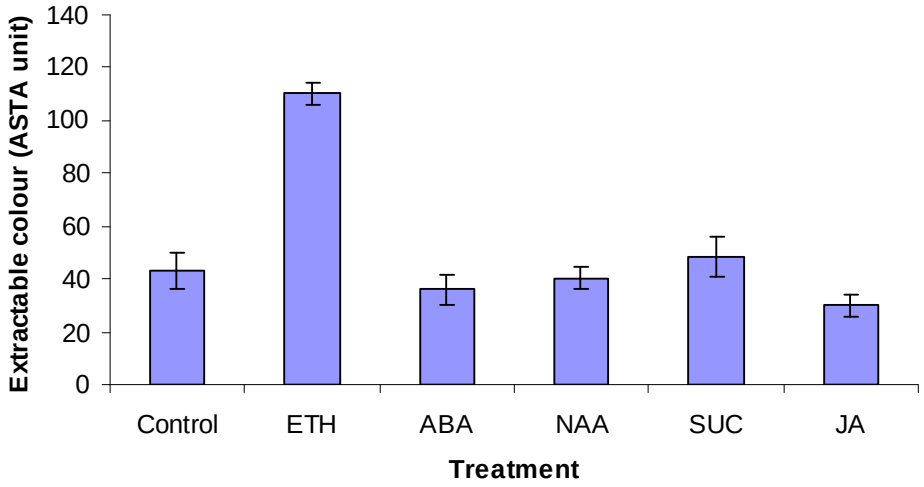

Figure 5.2 Extractable colour (ASTA unit) of fruit treated with different plant growth regulators (Table 5.1) for 10 days after harvest. Measurements were carried out at 25 days after harvest. Data are the means of twelve replicates (± SE) from two experiments and the least significant difference ($P<0.05$) is 9.49.

5.3.1.2 Carbon dioxide concentration

CO_2 concentration of fruit treated with all plant growth regulators including C_2H_4, ABA, NAA, sucrose and JA was not statistically different from that of the control fruit (Figure 5.3).

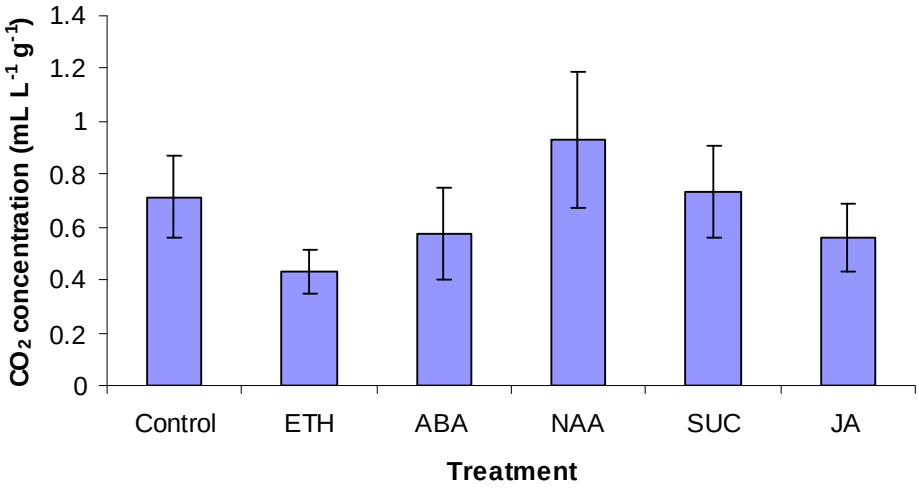

Figure 5.3 CO_2 concentrations (mL L^{-1} g^{-1}) of fruit treated with different plant growth regulators (Table 5.1) for 10 days after harvest. Measurements were carried out at 25 days after harvest. Data are the means of twelve replicates (± SE) from two experiments and the least significant difference ($P<0.05$) is 0.3388.

5.3.1.3 Total soluble solid content

Fruit treated with ethephon and JA had similar TSSC to control fruit while fruit treated with NAA, ABA and SAC had slightly but significantly lower TSSC than the control fruit (Figure 5.4).

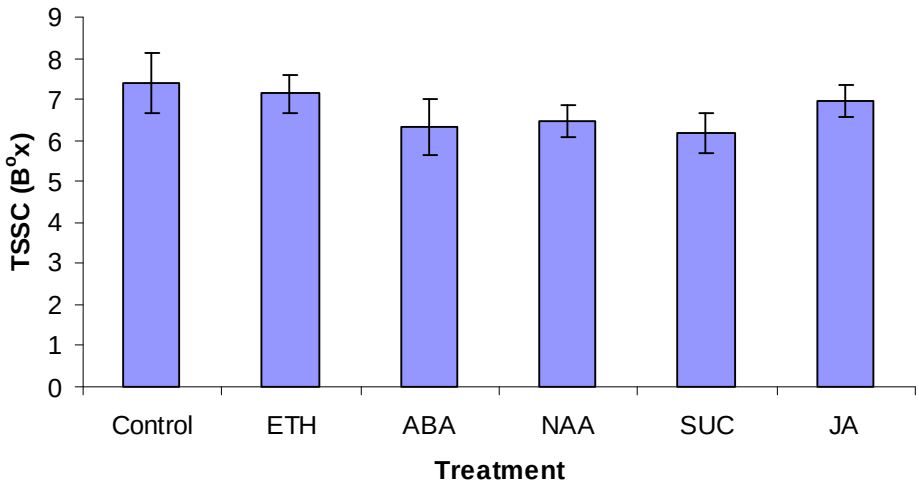

Figure 5.4 TSSC (B°x) of fruit treated with different plant growth regulators (Table 5.1) for 10 days after harvest. Measurements were carried out at 25 days after harvest. Data are the means of twelve replicates (± SE) from two experiments and the least significant difference (P<0.05) is 1.087.

5.3.2 Effect of postharvest application of ethephon

All fruit treated with ethephon, regardless of concentration, developed better skin colour than the control fruit and fruit appearance was deep red and partially dried at the final stage, especially fruit treated with 48,000 ppm of ethephon (Figure 5.5).

Figure 5.5 The effect of postharvest application of ethephon on colour development of capsicum fruit. Fruit were treated with different concentrations of ethephon solution (0; 2,400; 7,200 and 48,000 ppm) for 10 consecutive days. All treated fruit developed a totally red skin colour and were partially dried at 25 days after harvest.

5.3.2.1 Extractable colour

The extractable colour of both control and treated fruit did not change for the first 7 DAH (Figure 5.6). However, from 14 DAH, extractable colour of fruit treated with ethephon was higher than that of the control fruit. The difference in extractable colour between control and treated fruit increased with the increase of concentration. By the end of ripening (28 DAH) this difference was such that extractable colour was 50 %, 90% and 120% higher in fruit treated with 2,400; 7,200 and 48,000 ppm, respectively, than the control fruit. Moreover, extractable colour of fruit treated with 7,200 and 48,000 ppm ethephon solution was higher than the acceptable level of 140 ASTA units by 28 DAH.

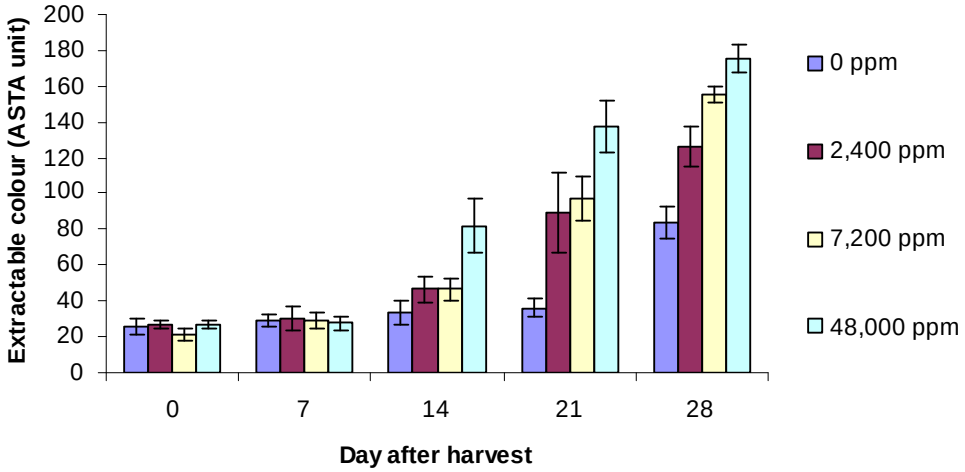

Figure 5.6 Change in extractable colour (ASTA unit) of fruit treated with different concentrations of ethephon solution: 0 (■); 2,400 (■); 7,300 (■) and 48,000 (■). Measurements were carried out at 0, 7, 14, 21 and 28 days after harvest. Data are the means of six replicates (± SE) and the least significant difference ($P<0.05$) is 10.83.

5.3.2.2 Chlorophyll content

There was no significant difference in chlorophyll content between fruit treated with ethephon and control fruit during the first 7 DAH (Figure 5.7). Chlorophyll content of control fruit remained at this level until 21 DAH before a 50 % reduction at 28 DAH. In contrast, chlorophyll content of treated fruit, regardless of concentrations, was reduced (over 50%) by 14 DAH while chlorophyll content of control fruit did not differ. By the end of ripening (28 DAH), treated fruit had lost almost all of their chlorophyll content while control fruit lost around 50% chlorophyll content compared to the level at the beginning of ripening.

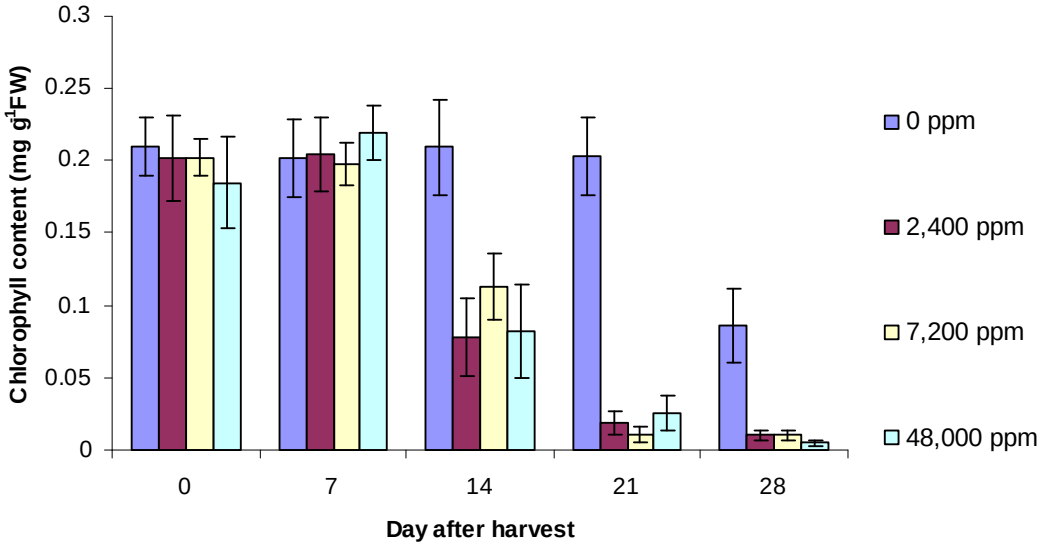

Figure 5.7 Change in chlorophyll content (mg g^{-1}FW) of fruit treated with different concentrations of ethephon solution: 0 (■); 2,400 (■); 7,200 (■) and 48,000 (■). Measurements were carried out at 0, 7, 14, 21 and 28 days after harvest. Data are the means of six replicates (± SE) and the least significant difference (P<0.05) is 0.0265.

5.4 Discussion

Postharvest application with plant growth regulators other than ethephon had minor effects on ripening behaviour of green harvested "Papri Queen" fruit under the conditions of this study. Application of ethephon solution increased extractable colour of green-harvested capsicum fruit but not other ripening characteristics. Application of high concentration of ethephon (7,200 and 48,000 ppm) induced sufficient colour development and extractable colour of treated fruit exceeded the acceptable level of 140 ASTA units.

Treatment with ethephon significantly increased both external skin colour and extractable colour of harvested fruit (Figure 5.3 and 5.5). This increase justifies the effectiveness of the method used to apply solutions and the ability of ethephon to penetrate through fruit tissue if applied directly. The ineffectiveness of C_2H_4 treatments on capsicum fruit reported by other authors (Lockwood and Vines, 1971; Knavel and Kemp, 1973; Krajayklang *et al.*, 2000) may, therefore, be due to the

difficulty for C_2H_4 gas and applied solutions to diffuse through the thick cuticle of fruit.

Although postharvest treatment with ethephon significantly increased fruit colour, other ripening behaviours such as internal CO_2 concentration and TSSC were not considerably promoted. C_2H_4 may act on chlorophyll degradation/carotenoid biosynthesis independently to other ripening processes. In climacteric fruit, C_2H_4 initiates the whole ripening process rather than inducing any individual event while in non-climacteric fruit exogenous C_2H_4 treatment has only a one-off effect (an increase in respiration) and can not initiate and promote ripening (Biale and Young, 1981). The involvement of C_2H_4 in ripening of capsicum fruit appeared to fall into neither categories. The dependence of extractable colour on the concentration of ethephon exhibited by capsicum fruit was typical for non-climacteric fruit without the capacity to produce C_2H_4 autocatalytically. As previously reported with citrus fruit (Rodrigo and Zacarias, 2007), C_2H_4 may play an integral role in carotenoid synthesis of non-climacteric fruit (citrus, capsicum) despite their non-climacteric behaviour during ripening.

Both chlorophyll degradation and extractable colour development of green harvested fruit from “Papri Queen” were promoted with ethephon treatment but no significant effects could be observed within 14 days of the first application (Figure 5.6 and 5.7). Moreover, extractable colour development but not chlorophyll degradation appeared to be concentration dependent. Although treated fruit were supplied with ethephon for the first 10 days after harvest, there was no chlorophyll breakdown during this period. Chlorophyll was heavily degraded only after 14 DAH and almost completely disappeared at the end of ripening leading to the totally red skin colour of fruit treated with ethephon. In contrast, control fruit still retained approximately 50% of its chlorophyll content.

C_2H_4 has been widely reported to be effective as a degreening agent by up-regulating genes involved in the chlorophyll degradation pathway (Figure 2.1) including chlorophyllase and mg-dechelatase (Purvis and Barmore, 1981; Wilk *et al.*, 1999; Costa *et al.*, 2005). However, the delay in chlorophyll degradation during the early period after treatment indicated that other factors could be involved in this pathway

such as the contact between substrate (chlorophyll) and enzyme (chlorophyllase) or the activity of other enzymes in the pathway such as phaeophorbide α oxygenase. Chlorophyll is located in the thylakoids while chlorophyllase is located in the inner membrane of the chloroplast envelope (Hortensteiner, 1999) and the transformation from chloroplast to chromoplast during ripening is said to be the key factor for the degradation of chlorophyll. If this transformation did not occur during the early period after treatment, chlorophyll could not be easily degraded even though related enzyme activities may be increased by ethephon treatments. Alternatively, the activity of phaeophorbide α oxygenase which is reported to be increased only at the breaker stage of ripening (Moser and Matile, 1997) may not be up-regulated by C_2H_4 resulting in the delay of chlorophyll degradation. The mechanism of C_2H_4 action on chlorophyll degradation in capsicum fruit will be well understood if the activity of different enzymes in chlorophyll degradation pathway and the expression of the genes encoding for these enzymes are studied.

As for chlorophyll degradation, the effect of postharvest ethephon treatments on extractable colour of green harvested fruit could be observed only after 14 DAH and appeared to be concentration dependent for the following period. The colour development was induced earlier (at 14 DAH) if a higher concentration (48,000 ppm) was used. When fruit were ripened naturally on plants, extractable colour did not start to increase until the same period as treated fruit (section 3.3.1.6) and this may also be associated with the transformation from chloroplast to chromoplast and the accumulation of chromoplast-specific carotenoids (Bouvier *et al.*, 1994). This delay period was possibly required for the transformation to be initiated leading to the degradation of chlorophyll and biosynthesis of chromoplast-specific carotenoids.

The biosynthesis and accumulation of several different carotenoids have been previously reported to be promoted by C_2H_4: *phytoene synthase-1* and *phytoene desaturase* genes in apricot fruit (Marty *et al.*, 2005); *phytoene synthase, ζ carotene desaturase* and *β-carotene hydroxylase* genes in citrus fruit (Rodrigo and Zacarias, 2007). However, as previously indicated (section 4.4) such studies were limited on capsicum fruit, especially on the accumulation of capsanthin and capsorubin, two chromoplast-specific carotenoids of the capsicum genus. The delay in colour development and the mechanism of C_2H_4 action can be better understood if the

expression of different enzymes in the carotenoid biosynthesis pathway in capsicum fruit is studied.

Postharvest treatments with other plant growth regulators (ABA, NAA, JA and sucrose) did not significantly alter ripening behaviour of green harvested fruit. Although there were some variations in CO_2 concentration, extractable colour and TSSC of treated fruit, they were not significant. Most of these plant growth regulators were believed to be involved in ripening of fruit by affecting the biosynthesis of C_2H_4 and thus ripening behaviour (McGlasson *et al.*, 1978). However, as previously indicated, C_2H_4 plays a very limited role during ripening of "Papri Queen" fruit, and thus other plant growth regulators may have limited roles in inducing ripening of green-harvested capsicum fruit.

5.5 Conclusion

Postharvest application of plant growth regulators except ethephon had limited effects on ripening of green harvested fruit from "Papri Queen" under the conditions of this study. Postharvest application of ethephon did promote extractable colour development but not CO_2 concentration and TSSC at the final stage of ripening. This provided further evidence that during ripening of green-harvested capsicum fruit, respiration and TSSC are not induced by C_2H_4 as colour development is.

Postharvest supply of 10 µL of ethephon solution for 10 consecutive days, regardless of concentration, promoted complete chlorophyll breakdown resulting in the total red colour of the fruit surface at the end of ripening. However, only application of 7,200 and 48,000 ppm ethephon solution induced extractable colour development of green harvested fruit so that it was higher than the acceptable level of 140 ASTA units. The improvement of colour appeared to depend on concentration as typical for non-climacteric fruit such as capsicum.

There was a time lapse from ethephon treatment until its effects were significantly exerted. From these results, a number of questions have been raised about the mechanism of C_2H_4 action. Why did it take a long time to be effective? Which

carotenoid was accumulated by ethephon treatment? What is a sufficient amount of ethephon to induce carotenoid accumulation? And does C_2H_4 induce carotenoid biosynthesis in capsicum fruit in a manner similar to that naturally occurring during fruit ripening?

Chapter Six

Chapter Six - Effect of ethephon treatment on the expression of carotenoid synthesis genes

6.1 Introduction

Ripening appears to be genetically controlled in many fruit as the alteration in expression of ripening-related genes brings changes to fruit colour, texture, and composition. In an attempt to understand how the different biochemical and physiological changes during ripening are coordinated, many ripening-related genes have been cloned and their expression during ripening has been studied (Picton *et al.*, 1995).

Ethylene (C_2H_4) regulates ripening in many fruit, especially climacteric ones (Biale and Young, 1981; McGlasson, 1985). C_2H_4 may be involved through initiation, modulation, regulation and co-ordination of expression of many genes necessary for ripening (Jones *et al.*, 2003; De Paepe *et al.*, 2004). Gene expression of ACC synthase, the rate-limiting enzyme in C_2H_4 biosynthesis pathway, has been shown to be up-regulated with the presence of C_2H_4 leading to autocatalytic production of ethylene in climacteric fruit (Jones *et al.*, 2003; Kim, 2006; Owino *et al.*, 2006; Park *et al.*, 2006). The Chlorophyllase *(Chlase)* gene, which encodes the first enzyme in the chlorophyll degradation pathway (Figure 1.1), and many carotenoid biosynthesis genes are also up-regulated by C_2H_4 resulting in fruit colour change during ripening (Wilk *et al.*, 1999; Costa *et al.*, 2005; Marty *et al.*, 2005).

Genes encoding many different enzymes in the carotenoid biosynthesis (Figure 2.2) pathway have been characterised including phytoene desaturase *Pds* (Hugueney *et al.*, 1992), ζ-carotene desaturase *Zds* (Breitenbach *et al.*, 1999), lycopene β cyclase *Lcy* (Hugueney *et al.*, 1995), zeaxanthin epoxidase *Zepd* (Bouvier *et al.*, 1996) and capsanthin-capsorubin synthase *Ccs* (Bouvier *et al.*, 1994). Most of these genes were

up-regulated during fruit ripening on the plant but, the expression of these genes when fruit were ripened off the plant has not been reported thus far.

Research presented in previous chapters established that pre- or postharvest application of ethephon promoted colour development of green-harvested fruit in a concentration-dependent manner. This development of colour was associated with chlorophyll degradation but enhancement of carotenoid synthesis may also have contributed. Fruit treated with low concentrations of ethephon developed totally red colour at the surface but produced low levels of extractable colour. However, when a very high concentration of ethephon was applied, extractable colour of treated fruit exceeded the acceptable level of 140 ASTA units. *The aims of the research presented in this chapter, therefore, were to characterise the expression of the carotenoid synthesis genes, Lcy and Ccs, during ripening (both on and off the plant) and after ethephon treatment.*

6.2 Materials and method

6.2.1 Plant materials

The cultivar “Papri Queen” was used to study gene expression of fruit ripened on and off the plant and was planted from March to September 2006 in the glass house at the University of Adelaide as described in section 2.2.2. Tissue samples used to study gene expression of fruit treated with ethephon were collected from previous studies on the effect of pre- and postharvest application of ethephon in section 4.2.2.2.3 and 5.2.2.3.

6.2.2 Chemicals

The stock solutions, including their composition and concentration used in this chapter are presented in Table 6.1.

Table 6.1 Composition and concentration of solutions used in this chapter

Solution	Composition
DNA extraction buffer (pH 8)	100 mM Tris-HCl, 100 mM NaCl, 10 mM ethylenediamine tetraacetic acid (EDTA), 1% (w/v) sarcosyl, 1% (w/v) poly(vinylpolypirrolidone)(PVPP)
TE buffer (pH 8)	10 mM tris (hydroxymethyl)aminomethane (Tris) (pH 7.4), 0.1 mM EDTA (pH8)
TAE buffer (pH 7.2)	40 mM Tris base, 20 mM CH_3COONa, 1 mM EDTA
10 x Northern buffer (pH 7)	200 mM 4-morpholinoproanesulfonic acid (MOPS), 50mM sodium acetate, 10 mM EDTA
RNA loading buffer	13.3% (v/v) 10x Northern buffer, 23.3% (v/v) formaldehyde, 66.7% (v/v) Formamide, 1.3% (w/v) Ethidium bromide,13.3% loading dye
10x SSC (pH 7)	1.5 M NaCl, 0.15 M sodium citrate
DNA denaturation buffer	1.5M NaCl, 0.5M NaOH
Hybridisation solution	250 mM Na_2HPO_4 pH 7.2, 7% (w/v) Sodium docedyl sulphate (SDS), 1 mM EDTA
LB medium (pH 7)	1% (w/v) bacto-tryptone, 0.5% (w/v) bacto-yeast extract, 1% (w/v) NaCl
LB agar (pH7)	1% (w/v) bacto-tryptone, 0.5% (w/v) bacto-yeast extract, 1% (w/v) NaCl, 1.5% (w/v) bacto-agar
Washing solution 1	2x SSC, 0.1% (w/v) SDS
Washing solution 2	1x SSC, 0.1% (w/v) SDS
Washing solution 3	0.5x SSC, 0.1% (w/v) SDS
Washing solution 4	0.2x SSC, 0.1% (w/v) SDS

6.2.3 Experimental design

The expression of two genes *Lcy* and *Ccs* were examined when fruit from "Papri Queen" were ripened on and off the plant at three different ripeness stages. Fruit were harvested at 44, 56 and 70 DAA (equal to LG, B and DR&D stage) to study gene expression of on-plant ripening while for off-plant ripening, fruit were harvested at 44 DAA and left to ripen at room temperature and sampled at 56 and 70 DAA. At sampling times, a longitudinal quarter of fruit was quickly cut into small pieces and frozen in liquid nitrogen. Frozen fruit tissues were then ground into a fine powder using a mortar and pestle and liquid nitrogen before storage at -80^{o}C until required.

To study the effects of ethephon treatments on the expression of *Lcy* and *Ccs*, tissue samples were collected from fruit treated with ethephon including preharvest application at 0; 48,000 and 3 x 48,000 ppm and postharvest application at 0; 2,400; 7,200 and 48,000 ppm at five different times 0, 7, 14, 21 and 28 days after harvest (DAH). Fruit tissues were also cut, frozen in liquid nitrogen, ground and stored at -80^{o}C until required.

6.2.4 Probe preparation

6.2.4.1 Design of gene-specific primers

The forward primer (Lc1-TTGTGGATCTTGCTGTGGTCGGTG) and reverse primer (Lc2- CAAACCAGGACGAGCAACAAGTGAGG) for *Lcy* were designed using the published sequence of *Lcy* for cultivar Yolo Wonder (GenBank accession X86221-NCBI) (Hugueney *et al.*, 1995) targeted to a 674 bp region starting with base pair 507 and ending at base pair 1180.

The forward primer (Cc1- CTCATGGGTTGATACTGATCTGGACGG) and reverse primer (Cc2- TCTTCACCCTCAGATATGGCTCATTACCTA) were designed using the published sequence of *Ccs* for cultivar Yolo Wonder (GenBank accession X76165-NCBI) (Bouvier *et al.*, 1994) targeted to a 614 bp region starting with base pair 276 and ending at base pair 889. All oligo analysis was carried out using Vector NTI Advance 10 software (Invitrogen, USA).

6.2.4.2 Genomic DNA extraction

Frozen ground sample was transferred to a pre-cooled 10 mL test tube to which DNA extraction buffer (Table 6.1) was added at the rate of 1 mL for every 1 mL volume of ground material. The tube was closed tightly and vortexed at low speed until all materials were thawed. For every 1 mL of buffer solution used, 1 mL of phenol: chloroform: isoamyl alcohol solution (25:24:1 v/v) was then added. The tube was vortexed and mixed for 15 min using an orbital mixer. The sample was then centrifuged at 6000 rpm for 15 min and the upper aqueous phase was transferred into a new 1.5 mL microfuge tube.

Additional chloroform extraction was carried out two or three more times for further removal of protein and to ensure better quality of DNA. Chloroform (0.5 volume) was added to 1 volume of this aqueous phase and the mixture was vortexed and centrifuged at 13,000 rpm for 10 min. The upper phase was then transferred to a new 1.5 mL microfuge tube and for every 1mL of this liquid, 90 µL of 3M sodium acetate (pH 5.2) and 900 µL isopropanol were added. The tube was then inverted on an orbital mixer for precipitation and centrifuged at 13,000 rpm for 10 min to pellet DNA. After centrifugation, the solution was poured off and the DNA pellet was washed with 70% ethanol. Finally the pellet was dried and resuspended in 20 to 30 µL of TE buffer (Table 6.1). The DNA amount was quantified using a spectrophotometer (SP8001, Metertech) with 1 absorbance unit at 260 nm (A_{260}) equivalent to the DNA concentration of 50 µg mL^{-1}. The DNA in TE buffer was stored at -20^{o}C until required.

6.2.4.3 PCR amplification with designed primers

Desired DNA fragments were amplified using GoTaq® Green Master Mix (Promega, WI, USA) and the designed gene-specific primers (section 6.2.4.1). PCR reaction mix contained 12.5 µL GoTaq, 2.5 µL each of the forward and reserve primer, 2 µL genomic DNA as template and nuclease-free water up to 25 µL. Conditions for the PCR reaction were 5 min for denaturation at 94^{o}C, 25 cycles of 30 s denaturation at 94^{o}C, 30 s annealing at 59^{o}C, and 90 s extension at 72^{o}C, a final extension of 7 min at 72^{o}C and stored at 4^{o}C until further use.

6.2.4.4 Gel electrophoresis for PCR products and visualisation

PCR products and a molecular size marker (DNA 1kb ladder, GeneWorks, Australia) were loaded into electrophoresis gels in horizontal mini tanks (Owl, NH, USA) containing electrophoresis buffer (1x TAE buffer, Table 6.1). Agarose gels (1% w/v) were prepared in 1X TAE buffer containing 0.2 µg mL^{-1} ethidium bromide. Gels were electrophoresed at approximately 120 V. PCR products were visualised on the

electrophoresis gel and photographed under a UV transilluminator (BioDoc-it, CA, USA).

6.2.4.5 Purification of DNA from the electrophoresis gel

Gel slices containing the desired DNA bands of expected size were excised by hand with a scalpel while visualising under the UV transilluminator. DNA samples were then purified using a QIAquick Gel Extraction Kit (Qiagen, Australia) according to the manufacturer's instructions.

6.2.4.6 DNA Ligation

Purified DNA fragments were ligated into pDrive cloning vector which contains *LacZ* α-peptide for white/blue colony selection as per the manufacturer's instructions (Qiagen). The reaction mixture (10 µL) contained 1 µL of pDrive cloning vector (50 ng μL^{-1}), 4 µL of purified PCR product and 5 µL of 2x Ligation Master Mix (Qiagen). The reaction mixture was mixed, incubated at 16^{o}C overnight and stored at -20^{o}C until required.

6.2.4.7 Cell transformation

LB medium and LB agar (Table 6.1) were freshly prepared and autoclaved prior to cell transformation. LB agar was left to cool to 55^{o}C before adding 1 mL ampicillin stock solution (100 mg mL^{-1}), 2 mL X-gal stock solution (40 mg mL^{-1}) and 0.5 mL IPTG stock solution (100 mM). The agar solution was mixed and poured into plates that were allowed to set, inverted and stored at 4^{o}C if not immediately used. Plate preparation was carried out under sterile conditions.

An appropriate amount of heat-shock competent *E.coli* cells (DH5α^{TM}-supplied by Invitrogen, prepared according to the manufacturer's protocol) required for transformation was thawed on ice immediately before the transformation. Ligation mixture (10 µL) was added to 100 µL of competent cells and the reaction tube was mixed gently and incubated on ice for 5 min. The tube was then heated in a heating

block at 42 ^{o}C for 45 s without shaking. The tube was incubated in ice for 2 min before adding 400 µL LB medium and incubation at 37^{o}C with shaking at 170 rpm for 1h. For good separation of colonies, 50 µL of the transformation mixture was plated onto one plate and the rest was plated onto another. The plates were then incubated at room temperature until the transformation mixture was absorbed into the agar. Plates were inverted and incubated at 37^{o}C overnight to allow colonies to develop for blue-white selection.

6.2.4.8 Confirmation of transformation

Five single white colonies were picked for positive screening. Each single colony was numbered and transferred to the new "reference" plate using a pipette tip. This tip was then dipped into a PCR tube containing 5 µL GoTaq (Promega), 0.5 µL each of the sequencing primers T7 and SP6 designed for pDrive (T7-TAATACGACTCACTATAGGG and SP6-GATTTAGGTGACACTATAG) and 4 µL nuclease-free water. Conditions for the colony PCR reaction were 5 min for denaturation at 94^{o}C, 25 cycles of 30 s denaturation at 94^{o}C, 30 s annealing at 55^{o}C, and 60 s extension at 72^{o}C, a final extension of 7 min at 72^{o}C and storage at 4^{o}C until further used.

The products of the colony PCR and a molecular size marker were loaded into an electrophoresis gel as described in section 6.2.2.5. The gel was visualised and photographed. Positive colonies were selected for sequencing based on the expected size of the DNA fragment (854 bp for *Lcy* and 794 bp for *Ccs*).

6.2.4.9 Sequencing of cloned DNA fragment

Positive colonies which had been confirmed through colony PCR were picked from the reference plate and inoculated into 3 mL LB medium containing 3 µL ampicillin stock solution. The liquid culture was incubated overnight at 37^{o}C in a shaking incubator at 100 rpm. Plasmid DNA was harvested from 5 mL of the overnight culture using Wizard® Plus SV Minipreps DNA Purification System (Promega) as per the manufacturer's instructions. Purified plasmid DNA was quantified with a

spectrophotometer and approximately 500 ng of DNA was put into a 200 µL tube containing 1 µL each sequencing primers SP6 and T7 (3.2 M) and nuclease-free water up to a total of 15 µL. Samples were sent to AgGenomics (Vic, Australia) for sequencing.

6.2.4.10 PCR amplification for probes

After the cloned PCR product sequence was confirmed, probes were prepared for further Southern and Northern analysis. Purified plasmid DNA obtained from minipreps was used as a template for PCR amplification using designed gene-specific primers as described in section 6.2.2.4. PCR products were loaded onto an aragose gel and electrophoresed as described in section 6.2.2.5. Desired cloned DNA fragments were cut from the gel and purified as described in section 6.2.2.6 and used for Northern and Southern analysis.

6.2.5 Northern analysis

6.2.5.1 RNA extraction

Total RNA was extracted using TRIzol® Reagent supplied by Invitrogen (USA) following the supplier's protocol with some modifications. Frozen ground tissue was transferred to a 10 mL tube containing TRIzol solution (10 mL of solution per 1 gram tissue), the mixture was mixed for 1 min by vortexing and stored at room temperature for 5 to 10 min. Chloroform was added at a rate of 0.2 mL per 1 mL TRIzol used and the mixture was shaken vigorously for 15 s and stored at room temperature for a further 2 to 5 min. The mixture was then centrifuged at 6000 rpm for 20 min at 4^{o}C and the aqueous phase containing RNA was transferred to a clean tube with the addition of 0.5 volume of chloroform, mixed and centrifuged as described above. This step was repeated twice for further removal of protein and DNA and to ensure better RNA quality.

Isopropanol (0.5 mL per 1 mL TRIzol used) was added to the supernatant phase containing RNA for precipitation, mixed and left at room temperature for 5 to 10 min before being centrifuged at 6000 rpm for 20 min at 4^{o}C to pellet RNA. After

centrifugation, the isopropanol solution was poured off and the RNA pellet was washed with 70 % (v/v) ethanol. The RNA pellet was then air dried and resuspended in 15 to 20 µL of nuclease free TE buffer (Table 6.1). The RNA solution was quantified using a spectrophotometer with 1 unit of A_{260} equivalent to 40 µg mL^{-1} RNA. The RNA solution was kept at -80^{o}C and used within 1 week of extraction.

6.2.5.2 Denaturing RNA gel electrophoresis

Approximately 5 µg RNA was added to 15 µL premixed loading buffer (Table 6.1) and denatured at 65^{o}C for 10 min. Following denaturation, RNA samples were put on ice for 2 min before being loaded onto a denaturing RNA gel. The denaturing RNA gel was prepared from 1.2 % (w/v) agarose, 5 % (v/v) formaldehyde in 1 x northern buffer (Table 6.1). The gel was run, quickly visualised and photographed as described in section 6.2.2.5 and 6.2.2.6.

6.2.5.3 Gel transfer and membrane hybridisation

RNA on the denaturing RNA gels was transferred to Hybond N+ membranes (Amersham Bioscience, NJ, USA) using a capillary blotting technique with 10 x SSC solution (Table 6.1) for at least 15 h (Davis *et al.*, 1986). The membranes were then UV-crosslinked in a GS Gene Linker® UV chamber (Bio-Rad, CA, USA) and stored in dry conditions at -80^{o}C until required.

6.2.5.4 Membrane hybridisation

Membranes were pre-hybridised for at least 1 h in 25 mL hybridisation solution (Table 6.1) at 65^{o}C in a bottle in a hybridisation oven. Probes (section 6.2.2.11) were radio-labelled with ^{32}P and purified using Ready-To-Go DNA Labelling Beads and ProbeQuant G-50 Micro Columns (Amersham Bioscience, NJ, USA) following the manufacturer's instructions. The labelled probes were denatured at 100^{o}C for 10 min and added to the bottle containing membranes and hybridisation solution. Membranes were hybridised at 65^{o}C for at least 15 h.

Following hybridisation, membranes were washed twice with 50 mL washing solution 1 (Table 6.1) for 30 min in a hybridisation oven followed by another wash with washing solution 2 (Table 6.1) for 20 min. The radioactive background was checked with a Geiger counter and washes were carried out until the background was minimised (less than two counts). The membranes were exposed to Hyperfilms (Amersham Bioscience, NJ, USA) at -80^{o}C for 5 to 7 days before being developed in CP1000 developr (AGFA, Germany).

6.2.6 Southern analysis

6.2.6.1 Digestion of genomic DNA

Genomic DNA was extracted as described in section 6.2.2.3. Genomic DNA was digested with four different restriction enzymes: EcoRV, DraI, XhoI and NcoI. All enzymes and suitable buffers were supplied by New England Biolabs, Inc. The reaction tube contained 15 to 20 µg of genomic DNA, 2 µL of enzyme solution, 2 µL supplied buffer and distilled water up to 20 µL. The reaction mixture was incubated at 37^{o}C for 5 to 7 h.

6.2.6.2 Gel electrophoresis and preparation

After enzymatic cleavage, the reaction mixtures with loading dye were loaded into 1% agarose gels and the gels were run, visualised and photographed as described in section 6.2.2.5 and 6.2.2.6. The gels were then submerged in DNA denaturation buffer (Table 6.1) and incubated for 30 min with gentle agitation.

6.2.6.3 Gel transfer

After incubation, DNA on the gels was transferred to Hybond XL membranes (Amersham Bioscience, NJ, USA) with DNA denaturation solution (Table 6.1) for at least 15 h as described in section 6.2.5.3. The membranes were then UV-crosslinked in a GS Gene Linker® UV chamber (Bio-Rad, CA, USA) and stored in dry conditions at -80^{o}C until required.

6.2.6.4 Membrane hybridisation

Membranes were hybridised using radio-labelled probes and exposed to Hyperfilms for development as described in section 6.2.5.4.

6.3 Results

6.3.1 Probe sequences

The sequence for the probe cloned from genomic DNA of *Capsicum annuum cv*. "Papri Queen" using primers Lc1 and Lc2 was aligned with the cDNA sequence for *Lcy* gene from *cv.* Yolo Wonder (Figure 6.1). There were only two sequence differences (at position 702 and 721).

Likewise, the sequence for the probe cloned from genomic DNA of *Capsicum annuum cv.* "Papri Queen" using primers Cc1 and Cc2 was aligned with the cDNA sequence for the *Ccs* gene from *cv.* Yolo Wonder (Figure 6.2). There was only one sequence difference (at position 696).

Both *Lcy* and *Ccs* genes of fruit from "Papri Queen" cultivar contain no intron. These two sequences were, therefore, considered to be ideal as probes for the *Lcy* and *Ccs* genes, respectively.

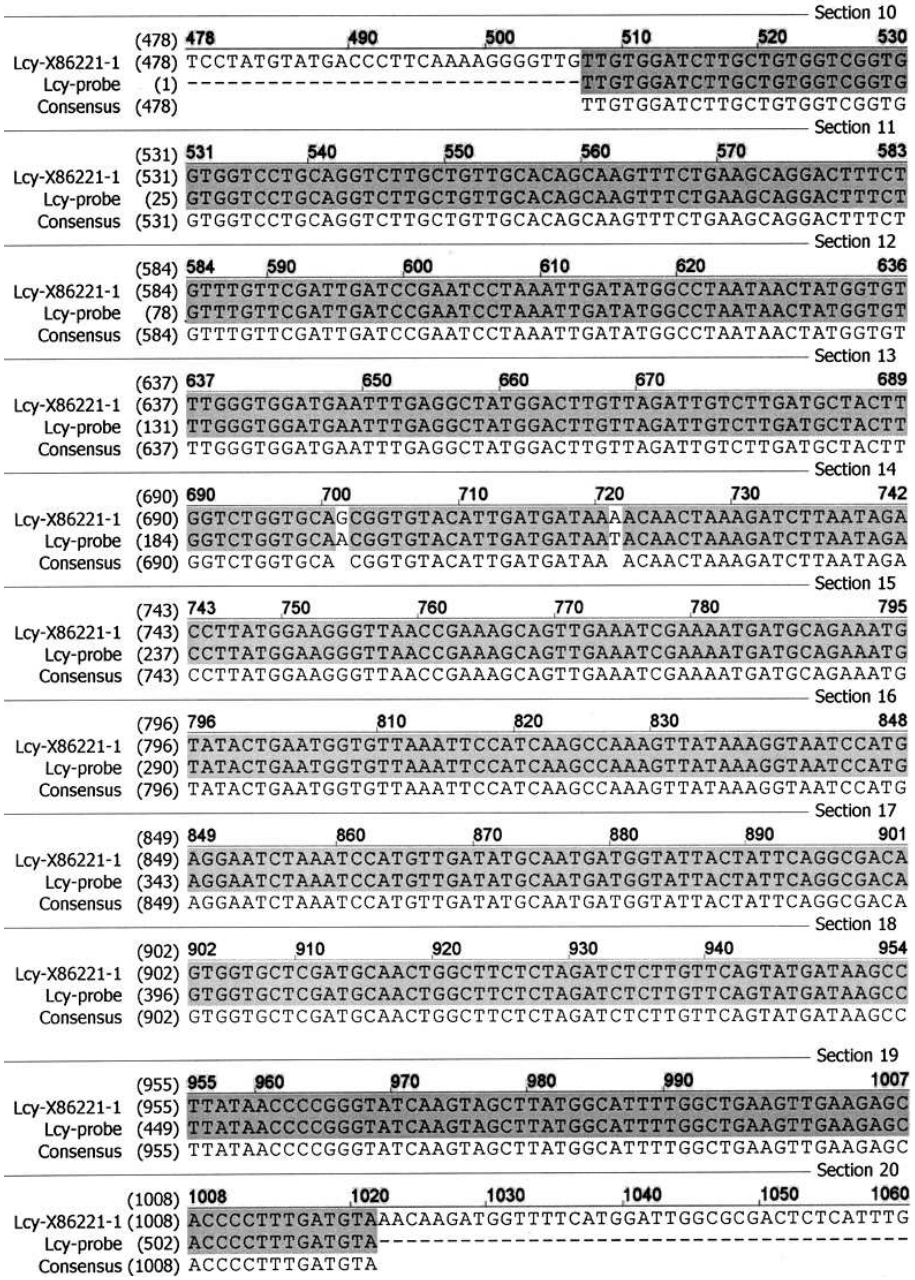

Figure 6.1 Alignment between cDNA for *Lcy* from *Capsicum annuum cv*. Yolo wonder (Lcy-X86221-1) and the probe prepared from *Capsicum annuum cv.* "Papri Queen" using Lc1 and Lc2 primers. Only the region between base pair 478 and 1060 of accession X86221 (GenBank, NCBI) is shown. The consensus sequences are highlighted in grey.

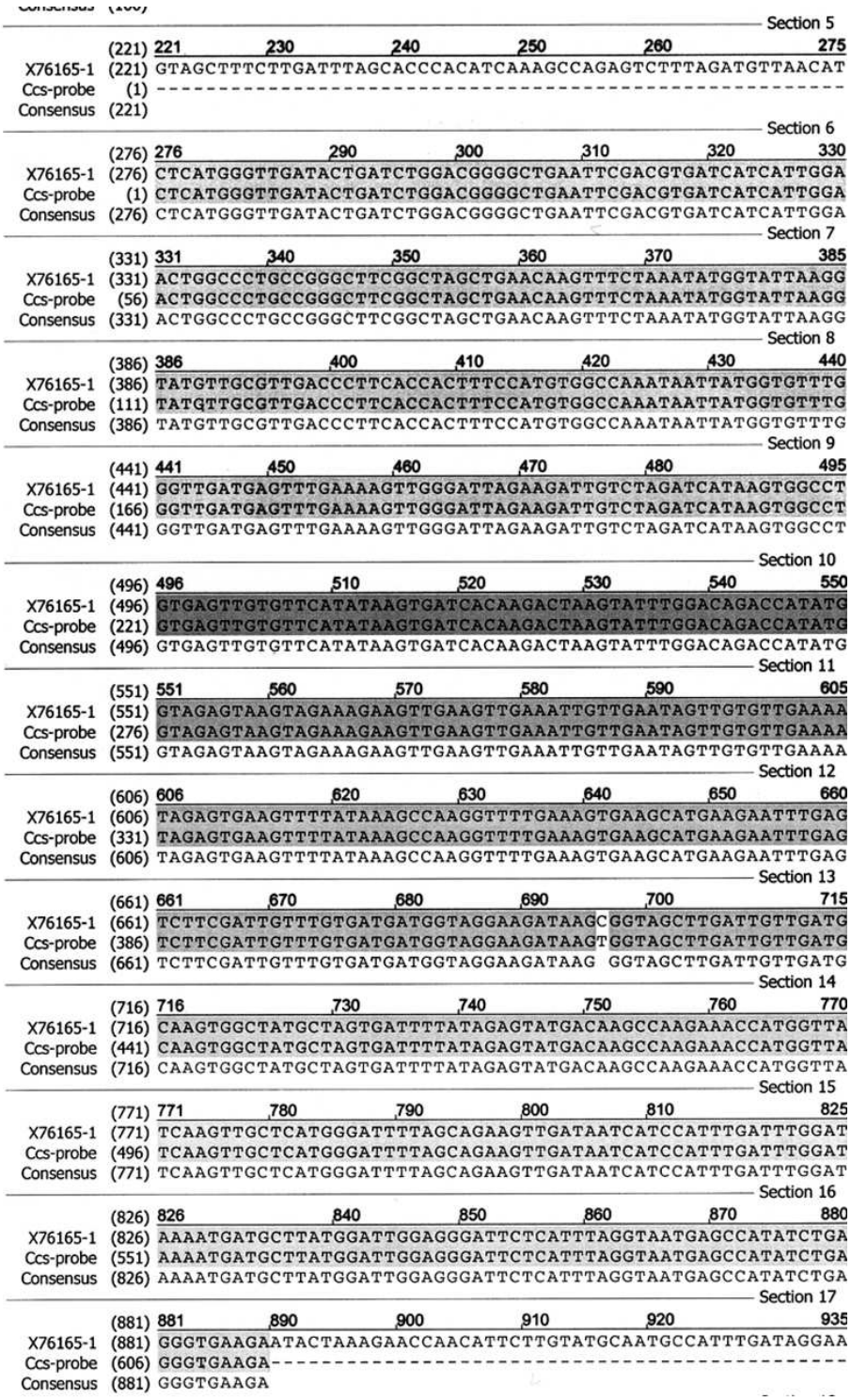

Figure 6.2 Alignment between cDNA for *Ccs* from *Capsicum annuum cv*. Yolo wonder (CCs-X76165-1) and the probe prepared from *Capsicum annuum cv*. "Papri Queen" using Cc1 and Cc2 primers. Only the region between base pair 261 and 936 of *Ccs* (accession number X76165, GenBank, NCBI) is shown. Consensus sequences are highlighted in grey.

6.3.2 Expression profile of *Lcy*

There was no expression of *Lcy* in fruit ripened on and off the plant (Figure 6.3). When fruit were treated with different ethephon concentrations in both pre- and post harvest application, the expression of *Lcy* was also undetectable (Figure 6.4 and 6.5).

To ensure the probe was functional, Southern analysis was performed and the presence of the *Lcy* gene in the genome of fruit from “Papri Queen” cultivar was confirmed (Figure 6.6).

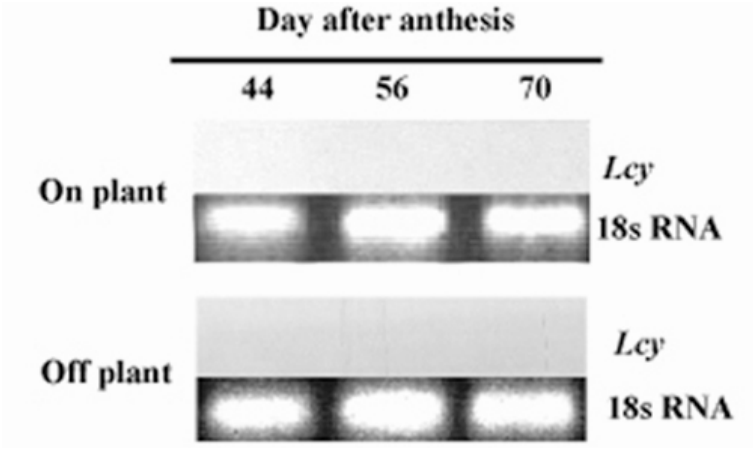

Figure 6.3 *Lcy* gene expression in fruit from "Papri Queen" ripened on and off the plant. Total RNA was isolated fruit fruit riepend on and off the plant at three different ripeness stages: 44, 55 and 70 DAA. RNA was separated on a denatured gel and transferred to a nylon membrane. The radiolabelled cloned for cDNA for *Lcy* (Figure 6.1) was used as a hybridisation probe. 18s RNA is shown as loading control.

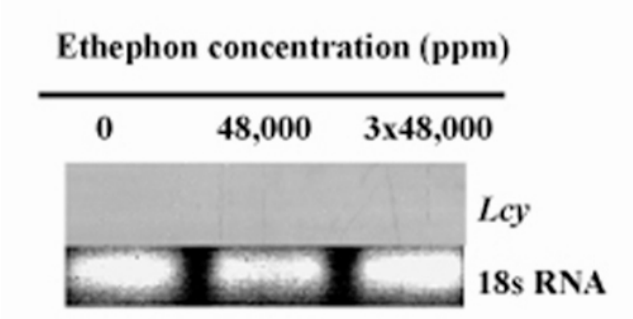

Figure 6.4 *Lcy* gene expression in fruit treated with preharvest ethephon. Total RNA was isolatedfrom fruit treatd with different concentrations of ethephon solution: 0; 48,000 and 3 x 48,000 ppm at 28 days after harvest. RNA was subjected to northen analysis. 18s RNA is shown as loading control.

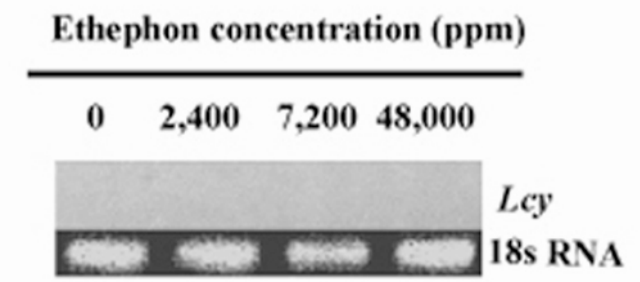

Figure 6.5 *Lcy* gene expression in fruit treated with postharvest ethephon. Total RNA was isolatedfrom fruit treatd with different concentrations of ethephon solution: 0; 2,400; 7,200 and 48,000 ppm at 21 days after harvest. RNA was subjected to northen analysis. 18s RNA is shown as loading control.

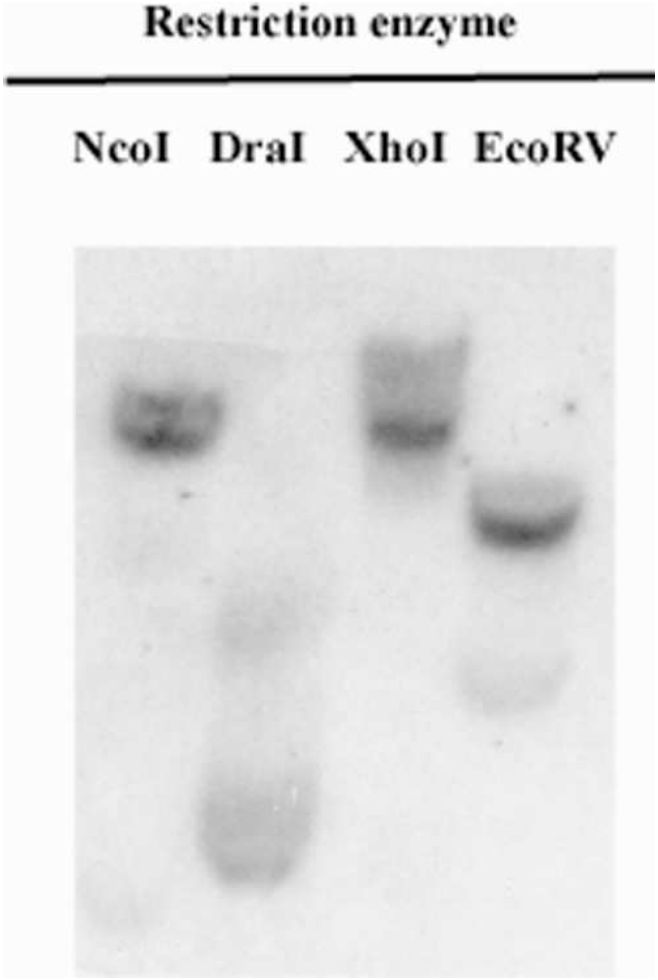

Figure 6.6 Southern analysis of gDNA for *Lcy* gene. gDNA was isolated from fruit tissue and digested by four different restriction enzymes: NcoI, DraI, XhoI and EcoRV. Digested DNA was sepereted on denaturation gel and transferred to a nylon membrane. The radiolabelled cloned cDNA for *Lcy* (Figure 6.1) was used as a hybridisation probe.

6.3.3 Expression profile of *Ccs*

Generally the expression of *Ccs* was up-regulated during ripening of fruit from "Papri Queen" and by ethephon treatments.

6.3.3.1 Fruit ripened on and off the plant

Ccs was not expressed at the LG stage at 44 DAA (Figure 6.7) but its expression increased as ripening progressed particularly on the plant. However, the expression was greater for fruit ripened on the plant than that of fruit ripened off the plant at both 55 and 70 DAA.

6.3.3.2. Preharvest application of ethephon

There was no expression of *Ccs* in control and fruit treated with ethephon at 0 and 7 days after harvest (DAH) (Figure 6.8). From 14 DAA for treated and 21 DAH for control fruit, *Ccs* started to express but to different extents. The expression was strongest for fruit treated with 3 x 48,000 ppm ethephon, followed by that of fruit treated with 48,000 ppm and control fruit, respectively. Interestingly, the levels of expression for treated fruit were similar between 21 and 28 DAH.

6.3.3.3 Postharvest application of ethephon

There was no expression of *Ccs* in control and fruit treated with ethephon at 0 and 7 days after harvest (DAH) (Figure 6.9).

At 14 DAH, the *Ccs* gene was up-regulated in both control and treated fruit but to a lesser extent in control fruit. There was no significant difference in *Ccs* expression among fruit treated with different concentrations of ethephon. At 21 DAH, the expression of *Ccs* in control fruit did not significantly change while it was strongly up-regulated in treated fruit. The expression of *Ccs* in treated fruit was also stronger than that at 14 DAH and was not significantly different among fruit treated with different concentrations.

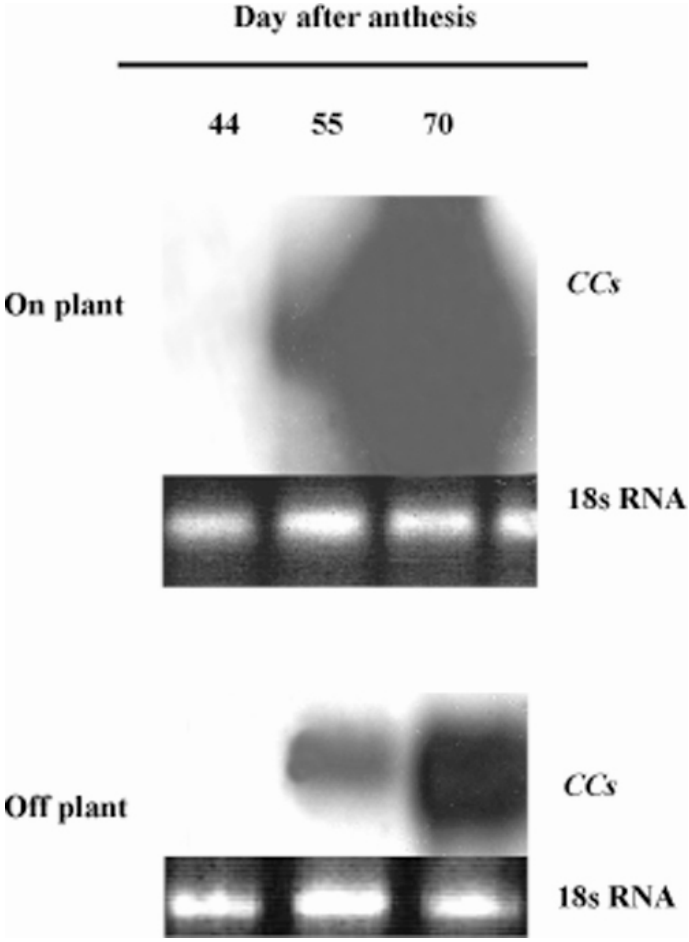

Figure 6.7 *Ccs* gene expression in fruit from "Papri Queen" ripened on and off the plant. Total RNA was isolated fruit fruit riepend on and off the plant at three different ripeness stages: 44, 55 and 70 DAA. RNA was separated on a denatured gel and transferred to a nylon membrane. The radiolabelled cloned for cDNA for *Ccs* (Figure 6.2) was used as a hybridisation probe. 18s RNA is shown as loading control.

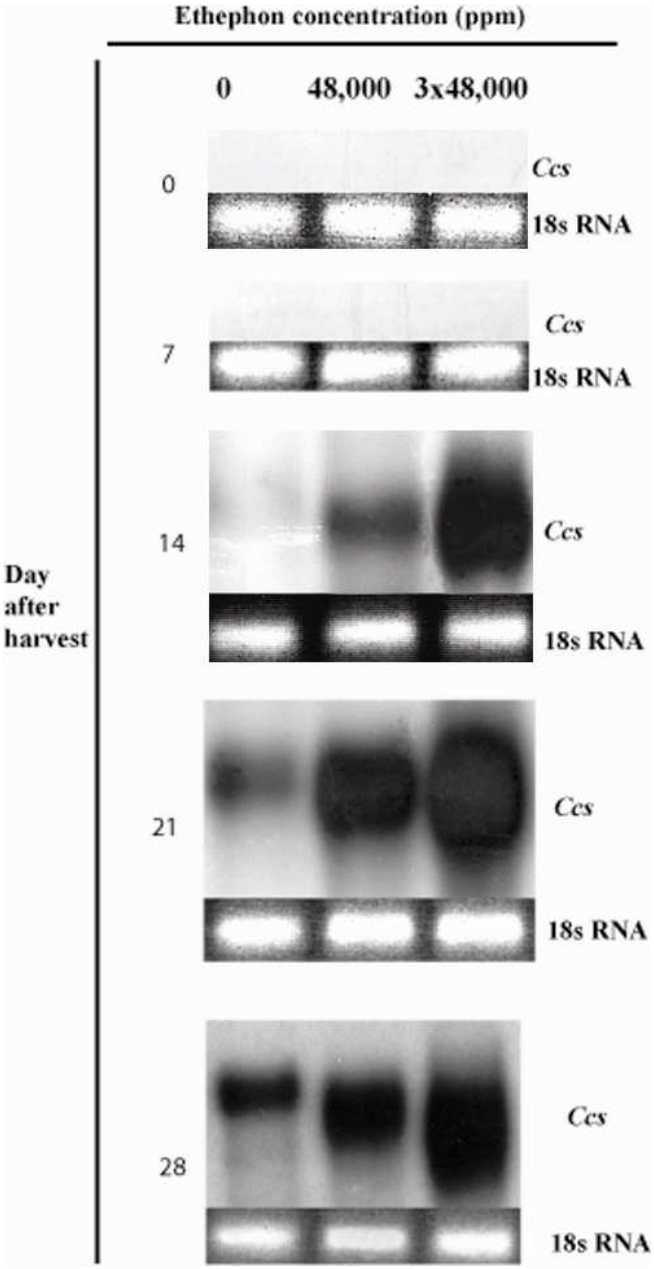

Figure 6.8 *Ccs* gene expression in fruit treated with preharvest ethephon. Total RNA was isolatedfrom fruit treatd with different concentrations of ethephon solution: 0; 48,000 and 3 x 48,000 ppm at five different times during ripening: 0, 7, 14, 21 and 28 days after harvest. RNA was subjected to northen analysis. 18s RNA is shown as loading control.

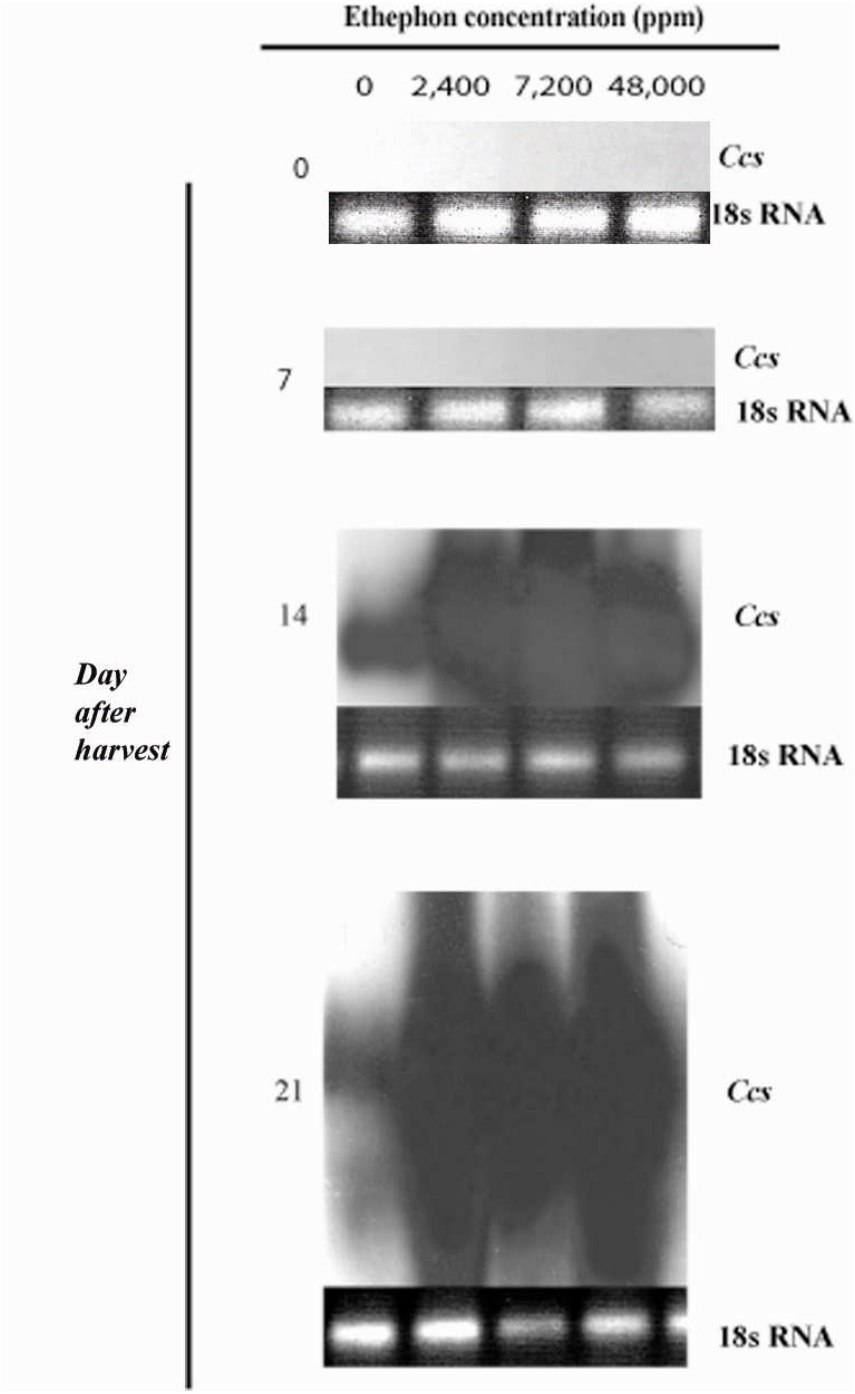

Figure 6.8 *Ccs* gene expression in fruit treated with postharvest ethephon. Total RNA was isolatedfrom fruit treatd with different concentrations of ethephon solution: 0; 2,400; 7,200 and 48,000 ppm at four different times during ripening: 0, 7, 14 and 21 days after harvest. RNA was subjected to northen analysis. 18s RNA is shown as loading control.

6.4 Discussion

Carotenoid biosynthesis gene expression was affected by whether fruit ripened on or off the plant and by ethephon treatments. The expression profiles characterised in this study also indicated the greater role for *Capsanthin capsorubin synthase (Ccs)* than *Lycopene-β-cyclase (Lcy)* during ripening of Capsicum fruit.

β-carotene exists in the chloroplast of young green capsicum fruit as a light-harvesting pigment (Taiz and Zeiger, 2006). β-carotene is synthesised from lycopene by the action of lycopene-β-cyclase which is encoded by *Lcy*, a gene constitutively expressed during fruit development (Hugueney *et al.*, 1995). However, no expression of *Lcy* was detected at any ripening stage of fruit from "Papri Queen" in this study. *Lcy* has also been found to not be up-regulated during ripening of capsicum *cv.* Yolo Wonder (Hugueney *et al.*, 1995), tomato (Pecker *et al.*, 1996) and papaya (Skelton *et al.*, 2006). *Lcy*, therefore, may be highly up-regulated during fruit growth to massively synthesise β-carotene for the accumulation of other carotenoids later on and thus, *Lcy* is not necessarily up-regulated during the ripening stage. However, it is unlikely to be the case for "Papri Queen" cultivar in this study. When paprika fruit start to ripen, not only did the level of β-carotene increase, but other carotenoids which require β-carotene as a precursor such as antheraxanthin, violaxanthin, capsanthin, capsorubin were *de-novo* synthesised (Mosquera and Mendez, 1994; Mendez *et al.*, 2000; Deli *et al.*, 2001; Mendez and Mosquera, 2002). This would suggest that lycopene β-cyclase should be synthesised during ripening (or at least be active).

Many authors have reported the similarity in nucleotide sequence and conserved motif between *Lcy* and *Ccs* which encodes capsanthin-capsorubin synthase, an enzyme which catalyses the formation of capsanthin and capsorubin in capsicum fruit (Hugueney *et al.*, 1995; Pecker *et al.*, 1996; Ronen *et al.*, 1999). *Ccs* has been reported to demonstrate the enzymatic activity of lycopene-β-cyclase when expressed in *E.coli* and its transcript has been shown to be more abundant than the *Lcy* transcript during ripening of capsicum (Hugueney *et al.*, 1995). This suggests that the increase in β-carotene level observed during ripening of capsicum fruit may be due to the action of both lycopene-β-cyclase and capsanthin-capsorubin synthase

(Hugueney *et al.*, 1995). However, the role of *Lcy* during ripening of capsicum fruit would be better characterised if the actual enzyme activity is be measured.

Ccs was up regulated when fruit from "Papri Queen" ripened both on and off the plant. On the plant, the level of *Ccs* mRNA increased with ripening and reached the highest level at the final stage when fruit turned deep red and partially dried. *Ccs* was also up-regulated when the bell pepper Yolo Wonder cultivar ripened but the mRNA level did not increase from the intermediate ripening stage (breaker) to the fully red stage (Bouvier *et al.*, 1994). This may help to explain the difference in extractable colour between the sweet bell pepper type ("Aries" and Yolo Wonder cultivars) and the paprika type ("Papri Queen" cultivar). The paprika type, which is mainly used for spice processing, has a far higher extractable colour than the sweet bell pepper type (section 3.4) and this may be due to the strong expression of *Ccs* at the final (red) stage leading to the massive accumulation of capsanthin and capsorubin.

When ripening advanced both on and off the plant, *Ccs* was up-regulated such that the *Ccs* mRNA level at the final stage (70 DAA) was higher than that at previous stages (56 DAA). However, this mRNA level was far lower than that of fruit ripened on the plant at the same stage leading to the differences in colour development between fruit ripened on and off the plant. Although *Ccs* was up-regulated when fruit went through the ripening process off the plant, the expression may still not reach the required level to ensure sufficient colour development.

Ethephon treatments (both pre- and postharvest) induced significant up-regulation of *Ccs* in capsicum fruit. The up-regulation of *Ccs* in the presence of C_2H_4 was detected by both Northern analysis (Figure 6.8 and 6.9) and RT-PCR technique (data not shown). Several carotenoid synthesis genes including phytoene synthase, phytoene desaturase, ζ-carotene desaturase and carotene cyclase (Marty *et al.*, 2005; Rodrigo and Zacarias, 2007) have been found to be up-regulated by C_2H_4 but to my knowledge, no study on *Ccs* has been reported thus far. Capsicum fruit have been classified as non-climacteric with very little involvement of C_2H_4 during ripening (Pretel *et al.*, 1995). However, observations from the previous experiment on ripening behaviour on and off the plant suggested the association of C_2H_4 with colour

development of “Papri Queen” fruit (section 3.4) which was confirmed by the increase in *Ccs* expression in the presence of ethephon.

The C_2H_4-induced expression of *Ccs* was concentration-dependent which was typical for non-climacteric fruit with a lack of autocatalytic capacity (Biale and Young, 1981). There was a time lapse of about seven days between ethephon application and the response in *Ccs* mRNA level which may suggest that C_2H_4 did not directly induce the up-regulation of *Ccs*. This is supported by the finding that the typical ethylene-responsive element (GCCGCC) has not been found in the promoter region of *Ccs* (Deruere *et al.*, 1994; Bouvier *et al.*, 1998). Hence the mechanism of C_2H_4 action through C_2H_4 binding factors previously reported (Bleecker and Kende, 2000; Chen *et al.*, 2005; Etheridge *et al.*, 2006) is unlikely applicable to the *Ccs* gene in capsicum fruit.

An alternative mechanism of C_2H_4 action, therefore, needs to be considered and it probably involves another ripening-related signal. *Ccs* expression was induced by the ripening-related signal and this signal could be weakened by inhibition or inactivation when fruit were harvested at the light green stage resulting in weaker expression of *Ccs* and subsequent lack of colour development. When fruit were treated with ethephon (either pre- or postharvest) and ripened off the plant, C_2H_4 may induce the transduction of this ripening-related signal which in turn induces the up-regulation of *Ccs*. When fruit were ripened on the plant, it took 12 days (from the light green stage at 44 DAA to the breaker stage at 56 DAA) for the signal to induce *Ccs* expression. Similarly, when fruit were treated with ethephon, it also took 14 days (after fruit were harvested at 44 DAA) for C_2H_4 to induce the expression of *Ccs*. When the promoter region of *Ccs* was studied in transgenic tomato plants, it was strongly up-regulated by ripening as early as at the mature green stage which is typical for climacteric fruit (Kuntz *et al.*, 1998). In contrast, for capsicum, a non-climacteric fruit, *Ccs* was up-regulated as early as at the breaker stage. The transduction of a putative ripening-related signal in the early phase of ripening, therefore, may be the distinctive feature between climacteric and non-climacteric fruit.

6.5 Conclusion

The expression of two carotenoid synthesis genes *Lcy* and *Ccs* was different during ripening of fruit from "Papri Queen". *Lcy* which encoded lycopene β cyclase did not express when fruit ripened or were treated with ethephon. In contrast, *Ccs* was up-regulated by both ripening and ethephon treatment. The expression of *Ccs* increased in fruit both on and off the plant when ripening progressed but the expression in fruit off the plant was far lower than that of fruit on the plant. That difference may contribute to the failure of green harvested fruit to develop sufficient colour. When harvested fruit were treated with ethephon, *Ccs* expression was up-regulated in an ethephon concentration-dependent manner from 14 days after harvest. C_2H_4 is unlikely to directly induce the expression of *Ccs* but may induce some ripening-related factors which in turn induce the expression of *Ccs.*

Chapter Seven

Chapter Seven - General discussion

7.1 Introduction

Capsicum fruit, due to their rich colour and pungency, are important for spice production. Fruit commence ripening at different times depending on their position on the plant and when capsicum crops are once-over harvested fruit are usually at different ripeness stages. In order to achieve good quality of spice powder, harvested fruit should turn red before processing so that induction of colour development in harvested fruit is essential. However, green-harvested capsicum fruit have been widely reported to not ripen normally (not turn fully red) and the factors responsible for this are unclear.

There have been many attempts to induce ripening of green-harvested fruit without much success. Preharvest spraying of ethephon solution increased the number of red fruit at harvest but also induced immature abscission (Cantliffe and Goodwin, 1975; Wien, 1997). Moreover, preharvest application of ethephon did not have a significant effect on extractable colour of capsicum fruit (Cooksey *et al.*, 1994). Similarly, postharvest application of C_2H_4 (or propylene, an analogue of C_2H_4) did not induce colour development of green-harvested fruit (Lockwood and Vines, 1971; Krajayklang *et al.*, 2000) as well as other ripening characteristics such as respiration and C_2H_4 production (Lu *et al.*, 1990; Saltveit, 1997). An effective method to improve extractable colour of green-harvested fruit, therefore, is essential.

Ripening behaviour of capsicum fruit was reported to be variable among different cultivars. Although capsicum fruit were generally classified as non-climacteric (Saltveit, 1997; Villavicencio *et al.*, 1999) some types of hot chillies have shown some climacteric-like behaviours (Gross *et al.*, 1986). Moreover, fruit ripened on the plant also exhibited some climacteric-like peaks in CO_2 and C_2H_4 production

(Krajayklang *et al.*, 2000; Villavicencio *et al.*, 2001) which were absent in fruit ripened off the plant.

This study, therefore, was aimed at providing a better understanding of ripening behaviour on and off the plant of three different cultivars of capsicum and improving colour development of green harvested fruit.

7.2 Outcomes of the study

Despite the morphological and biochemical differences among three cultivars studied in this project, fruit exhibited typical non-climacteric behaviour during ripening. Although fruit ripened on the plant underwent a dramatic change in colour, internal CO_2 concentration and total soluble solid content (TSSC), it was not accompanied by the burst in C_2H_4 production which is typical for climacteric fruit. All three cultivars of capsicum produced minimal C_2H_4 during ripening both on and off the plant. Fruit from the "Papri Queen" cultivar produced C_2H_4 only when fruit turned red while C_2H_4 levels in fruit from "Aries" were mostly undetectable. Fruit from the "Caysan" cultivar which was found to show some climacteric behaviour in a previous report (Gross *et al.*, 1986) produced more C_2H_4 than the others and a peak of C_2H_4 could be observed in all fruit ripened on and off the plant. However, when ACC synthase and ACC oxidase activity were considered, this increase was found to be unlikely the result of a climacteric increase in ACC synthase which is usually characteristic for climacteric fruit (Oetiker and Yang, 1995). Generally, the activity of the two enzymes in the C_2H_4 biosynthesis pathway showed no correlation with the level of C_2H_4 during ripening which suggests that capsicum fruit should be classified as non-climacteric with very little role of C_2H_4 during ripening. However, even though C_2H_4 production was minimal it occurred during colour development in fruit from the "Papri Queen" cultivar which may suggest the potential of C_2H_4 application to induce colour development.

Fruit harvested at the light green stage also had a lower level of extractable colour, internal CO_2 concentration and TSSC compared to fruit ripened on the plant or

harvested at the later stages (dark green and breaker). The external carbon-source from the mother plant, therefore, may play a role in ripening of green harvested fruit. However, cinctured fruit (fruit without external nutricient supply from the mother plant) were able to ripen normally and turn fully red (albiet at a slower rate than control fruit) which suggested that the lack of the external-carbon supply during ripening was not the main reason for the failure to ripen. Because C_2H_4 and external-carbon and other phloem derived supply appeared not to play a key role in ripening, other factors transported through xylem tissues are likely to play a role in ripening initiation but the mechanism of action was far from clear. There may be other ripening-related signals transmitted to the cells which initiate the physiological and biochemical changes of fruit on the plant during the period between the light green and breaker stage. Alternatively, these changes may be induced by a combination of many different factors such as C_2H_4, sugar/phloem-derived and xylem-derived signal, especially the possible involvement of other plant growth regulators including abscisic acid, auxins. Further research is required to provide a better understanding of the nature of ripening induction in capsicum fruit.

Despite the ineffectiveness of C_2H_4 treatment that has been previously reported (Lockwood and Vines, 1971; Knavel and Kemp, 1973), C_2H_4 has been proven to effectively induce colour development of fruit from “Papri Queen” in this study. C_2H_4 has been found to enhance the degradation of chlorophyll and the biosynthesis of carotenoids when fruit were treated with ethephon (both pre- and postharvest). However, the induction was not visible until 14 DAH which indicated its effect may not be direct.

Fruit treated with ethephon lost most of their chlorophyll at the end of ripening (at 25 DAH) but the enhancement of the degradation has not been observed until 14 DAH. C_2H_4 may directly up-regulate *chlorophyllase (Chlase)*, the first enzyme in the chlorophyll degradation pathway (Figure 1.2), as has been observed in citrus fruit (Wilk *et al.*, 1999). However, this up-regulation may not lead to the immediate degradation of chlorophyll during the early phase of ripening because the substrate, chlorophyll, cannot reach the reaction site for the enzyme to be active. When fruit commence the following phase of ripening, the transformation of chloroplasts into

chromoplasts and the disorganisation of thylakoids (Hortensteiner, 1999; Mendez and Mosquera, 2002) may help the enzyme to contact its substrate and become active resulting in massive degradation of chlorophyll. Alternatively, other enzymes in the pathway such as pheophorbide α oxygenase, which are not induced by C_2H_4, become active only during the later phase of ripening (Moser and Matile, 1997) so that chlorophyll should not completely degraded until later.

C_2H_4 effectively promoted extractable colour of green-harvested capsicum fruit in a concentration-dependent manner. The increase in extractable colour is likely due to the up-regulation of *capsanthin-capsorubin synthase (Ccs)* which encodes for the enzyme forming capsanthin and capsorubin, two carotenoids specific for red colour of capsicum fruit. Similar to the C_2H_4-induced degradation of chlorophyll, the up-regulation of *Ccs* was not observed until 14 DAH which indicated it may not be a direct effect and that other signal transduction factors may be involved. This indicates that when fruit are ripened on the plant, colour development may be induced by ripening-related factors (other than C_2H_4) which is possibly inhibited or inactivated when fruit are harvested at the green stage. C_2H_4 application to fruit at this stage may help to reactivate or recover these factors which in turn induce colour development.

Although C_2H_4 induced colour development of capsicum fruit in this study, it did not induce other ripening behaviours including internal CO_2 and total soluble solid content. C_2H_4 has been shown to have limited roles in ripening of non-climacteric fruit and the response to exogenous C_2H_4 treatment is the increase in CO_2 production (Biale and Young, 1981). In contrast, C_2H_4 induces ripening of many climacteric fruit and application of C_2H_4 brings changes to many ripening parameters including CO_2 and C_2H_4 production, fruit colour, taste and texture (McGlasson, 1985). The role of C_2H_4 in ripening of capsicum fruit, therefore, may be different to that of both non- and climacteric fruit previously characterised. In addition, the colour development may not be closely linked to the carbon metabolism process during ripening of capsicum fruit.

7.3 Future directions

Lcy encoding for lycopene-β-cyclase does not express during ripening and its role in colour development of capsicum fruit is unclear. Although it is suggested that capsanthin-capsorubin synthase has the enzymatic activity of lycopene-β-cyclase, the *Ccs* gene does not express until the later stages of ripening. The accumulation of β-carotene during fruit development and the early phase of ripening is unlikely to be the result of capsanthin capsorubin synthase action. Examining *Lcy* expression during fruit development and the activity of its enzyme during fruit ripening can provide necessary information to characterise the role of *Lcy* during ripening of capsicum fruit.

Chlorophyll degradation was induced by C_2H_4 but the induction could not be observed until 14 DAH. Questions regarding the action of C_2H_4 on chlorophyll degradation, therefore, still remain. Firstly, which genes in the chlorophyll degradation pathway are up-regulated by C_2H_4 leading to the completion of chlorophyll degradation? Secondly, is the mechanism of C_2H_4 action similar to that observed in *Ccs* expression or not? Comparing the expression of chlorophyll degradation and carotenoid biosynthesis in response to C_2H_4 treatment may provide valuable information on the transduction of the C_2H_4 signal during the early stages of ripening.

Finally, signal transduction during the early stage of ripening and the possible involvement of other ripening-related factors should be examined to better understand the mechanism of C_2H_4 action. Characterisation of different elements in this ripening-related signal transduction is a great challenge which, if successful, will help to clarify better the nature of ripening in non-climacteric fruit.

7.4 Conclusion

From the results of this stuidy, a proposed model for ripening behaviour of capsicum fruit and the possible effect of C_2H_4 has been developed (Figure 7.1). When fruit are ripened on the plant, a ripening signal sent by the mother plant activates physiological/biochemical processes (such as sugar accumulation, respiration and chlorophyll degradation). This signal also activates some ripening-related factors which act as an intermediate at the later stage. When fruit are harvested at the breaker stage, these processes have been induced and the active ripening-related factors in turn activate some other physiological/biochemical processes such as capsanthin/capsorubin accumulation. As a result, breaker-harvested fruit can ripen normally as fruit on the plant.

In contrast, when fruit are harvested at the light green stage, there is no ripening signal sent from the mother plant and many physiological/biological processes are subsequently not fully activated. Light green-harvested fruit, therefore, fail to ripen. When fruit are treated with ethephon, C_2H_4 may activate some physiological/biochemical processes (such as chlorophyll degradation) but not others (such as sugar accumulation and respiration). C_2H_4 also activates the ripening-related factors that are required for the activation of some other processes at the later stage of ripening (such as the accumulation of capsanthin/capsorubin). C_2H_4-treated fruit, therefore, develop sufficient colour development but do not change substantially in other processes.

Finally, the study has provided better understanding of the ripening physiology of three different cultivars of capsicum grown in Australia. Although the method of the ethephon application to improve colour development used in this study is not practical in the industry, it helps to provide the fundamentals for further research in improvement of the method of ethephon application on an industrial scale.

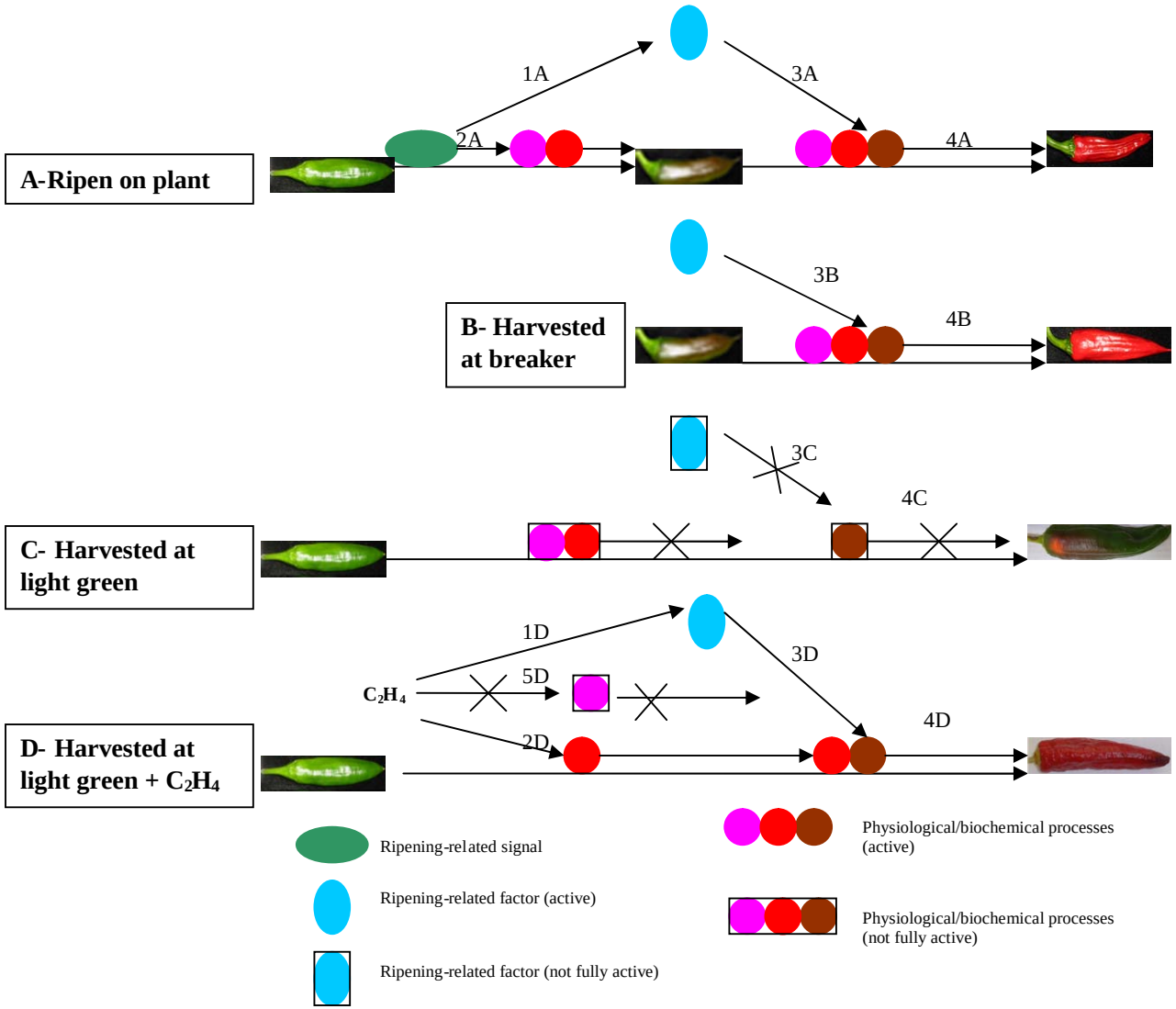

Figure 7.1 Proposed ripening behaviour of capsicum fruit on and off the plant and the effect of ethylene.

A. When fruit are ripened on the plant, the ripening signal sent by the mother plant activates some physiological/biological processes (1A) and some ripening-related factors (2A) which in turn activate other processes during the later phase of ripening (3A). Fruit ripen normally as a result of these physiological and biochemical changes (4A).

B. When fruit are harvested at the breaker stage, the physiological/biochemical processes and ripening-related factors which have been activated during the previous stage help fruit to ripen normally (3B and 4B).

C. When fruit are harvested at the light green stage, there is no ripening signal. Physiological/biochemical changes, therefore, are not fully activated. Similarly, ripening-related factors are not activated and cannot induce changes at the later phase of ripening (3C). Fruit fail to ripen as a result (4C).

D. When C_2H_4 is supplied to light green-harvested fruit, C_2H_4 can activate some of the physiological/biochemical processes (2D) and the ripening-related factors (1D) which in turn activate other processes at the later phase of ripening (3D). However, there are some processes that C_2H_4 cannot induce (5D). As a result, treated fruit developed normal red colour but other behaviour do not substantially change (4D).

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Appendices

1. Full length sequence of cDNA for Lcy from Capsicum annuum cv. Yolo Wonder

1 cttaattata gaaatactta agatatatca ttgcccttta atcatttatt tttaactctt
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2. Full length sequence of cDNA for Ccs from Capsicum annuum cv. Yolo Wonder

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3. Posters

Ethylene Effects on Expression of Colour Development Genes in Green-Harvested Pepper Fruit

THE UNIVERSITY OF ADELAIDE AUSTRALIA


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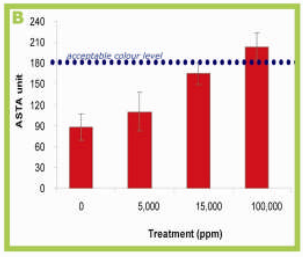
OUR RESEARCH FOCUS

- Capsicums do not ripen when harvested green: why?
- Do any postharvest treatments induce ripening?

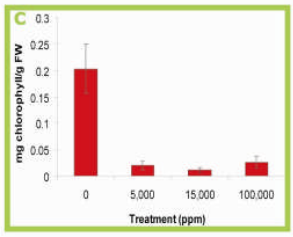
Ethylene treatment enhances surface colour development in paprika pepper (cv. Papri queen) BUT extractable colour is only suitable for spice production at 100,000 ppm ethylene

The effect of ethylene treatment on ripening (A) and extractable colour (B). Harvested fruit were treated with 10μL of ethephon (at different concentrations) through a hole in the stem for 10 days after harvest. 21 days after harvest, fruit were dried, ground and the extractable red colour measured as per Woodbury (1997, *Official Method of Analysis, 16th Edn.*). Colour is acceptable to the spice industry at 180 American Spice Trade Association (ASTA) Units.



Ethylene treatment induces chlorophyll degradation and capsanthin-capsorubin synthase (CCS) gene expression

To help answer the question of why colour does not fully develop, we have measured chlorophyll levels (C) and examined the gene expression (D) of two carotenoid pathway enzymes - lycopene cyclase (LCY) and capsanthin-capsurobin synthase (CCS). LCY catalyses the conversion of lycopene to carotene. CCS completes the colour development pathway to ensure synthesis of capsanthin and capsorubin in *C. annum* chromoplasts. No LCY gene expression was observed in harvested fruit.



CONCLUSIONS & FURTHER RESEARCH

- Ethylene is unlikely to be involved in *C. annum* ripening
  - Extremely high and sustained levels of ethephon (100,000 ppm) are required to induce colour development
  - We have previously observed that green-harvested fruit do not produce ethylene & fruit ripened on the plant produce very low levels of ethylene
- There is a positive correlation between ethylene treatment, increased chlorophyll degradation & increased CCS gene expression. We conclude that in green-harvested fruit, chlorophyll masks colour development while capsanthin & capsorubin synthesis is minimal.
- We are now investigating chlorophyllase gene expression & levels of lycopene/carotene.

This research was presented at Plant and Animal Genome XV, 2007. Pham Thang is supported by a Vietnamese Government Scholarship.

Ripening behaviour of Pepper fruit (*Capsicum annum L.*) on and off the plant

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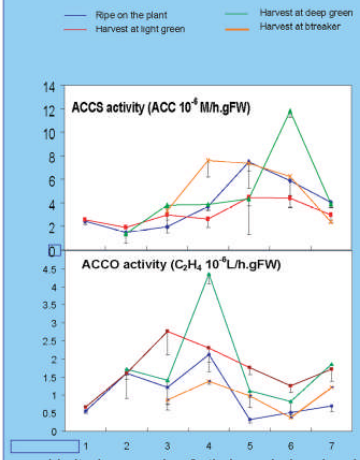
Introduction+ Aims

- Paprika peppers are widely used for spice powder production due to their rich red colour and pungency, however fruit do not ripen off the plant.
- We wanted to know
  - o Are they climacteric?
  - o What role does ethylene play during ripening?

Methodology

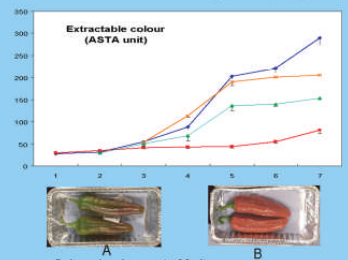
- *Capsicum Annum L.* (cv. Papri Queen SPS 2591) plants were grown in a glasshouse at the University of Adelaide, Waite Campus.
- Fruit were harvested at three different ripeness stages or allowed to ripen on the plant.
- Several parameters including internal carbon dioxide (CO_2) and ethylene (C_2H_4) concentration, extractable colour, soluble solid content, 1-amino-1-carboxylic acid (ACC) oxidase and synthase activity were determined during ripening.
- Seven sampling times are based on visual colour changes of fruit ripen on the plant:
  1. Light green
  2. Deep green
  3. Breaker
  4. Breaker red
  5. Light red
  6. Deep red
  7. Deep red and partially dried

Results

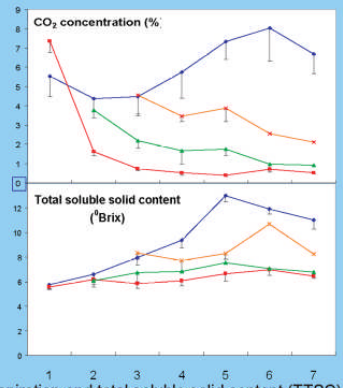


Limited or no role of ethylene during ripening

- o Internal ethylene concentration generally was nil or extremely low.
- o There were no significant differences in ethylene-forming enzyme activities between ripening on and off the plant as well as no clear correlation with the changes in ethylene production



Colour development of fruit:
A - Ripen off the plant
(harvest at the light green stage)
B - Ripen on the plant



Respiration and total soluble solid content (TTSC)

- o Ripen on the plant: climacteric-like pattern with regards to CO2 concentration and TSS content both peaked when fruit turned totally red.
- o Harvested at deep green or breaker stages: respiration remains at the same levels during ripening.
- o Harvest at light green stage: respiration decreased to very low levels and TTS content appeared unchanged.

Conclusions

Fruit that:
- o Ripen on the plant is climacteric
- o Ripen off the plant in non-climacteric

We are currently investigated whether:
- o Sugar supply is required for colour development?
- o Other phytohormones involve in ripening of paprika fruit.