UNIVERSITY OF NATAL

MEANS OF IMPROVING COLOUR EXPRESSION IN PEPPER FRUIT

By

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DECLARATION

I hereby certify that the research work reported in this dissertation is the result of my own investigation, except where acknowledged.

Signed: - ______________________

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I certify that the above statement is correct.

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ABSTRACT

Colour is a characteristic that makes fruit appealing to the consumer, hence, is a prime factor when (s)he chooses to buy certain horticultural commodities. If colour is unappealing, consumers tend to ignore other quality characteristics of the fruit. Although colour expression in *Capsicum annuum* L. is genetically influenced, it is also affected by environmental factors as well as crop management practices. In an attempt to identify which are the dominant red colour-imparting carotenoids in red pepper and how to influence their occurrence, extracts from crude and saponified mature-green, colour-break and red-ripe fruit were purified on TLC silica gel 60 GF_{254} plates. TLC R_f values of the identified intensive red bands from saponified red-ripe extract were compared to those of authentic capsorubin, capsanthin, 13-Z-capsanthin and 9-Z-capsanthin standards. Two dominant red carotenoids were isolated from red-ripe fruit, by separating either crude or saponified extract by TLC. The two bands were then subjected to reversed-phase HPLC analysis. Both bands were characterized by comparison of their absorption spectra to that of authentic capsanthin and capsorubin standards. Red colour-imparting compounds of the red pepper cultivar Capistrano surfaced at colour-break stage and their intensity increased with the ripening process. HPLC results show that there was structural modification of ketocarotenoid, mainly capsorubin.

Trials were carried out in a plastic tunnel and in a growth room to investigate the impact of various plant management practices (moisture regimes, macronutrient applications, thinning of reproductive structures, subjecting pepper plants to optimal growing temperature) on pepper fruit pigment concentration. This concentration was determined at the mature-green and red-ripe stage. Subjecting pepper plants to optimal growing temperature (23/18°C) from the vegetative stage onwards, providing them with recommended optimal amount of K fertilizer (25.2 Kg ha^{-1}) and supplying Mg at supra-optimal level (455 Kg ha^{-1}) as well as subjecting them to drought stress starting from 30 or 50 days after transplanting and water-logging conditions from 30 days after first petal fall onwards increased pepper fruit carotenoid concentration significantly. On the other hand, other levels of the above-mentioned factors (subjecting pepper plants to the optimum temperature later than the vegetative stage, supplying 'very low', 'low' or 'high' K or, 'very low', 'low' or 'medium' Mg concentrations as well as thinning of reproductive structures of pepper plants early in the fruit developmental stage and subjecting plants to drought from 30 days after first petal fall onwards or to water-logging from 50 days after transplanting or 30 days after first petal fall onwards) resulted in a significant decrease in the carotenoid concentration. Therefore, management practices can be explored to improve red colour formation in red-ripe *Capsicum* fruit.

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CHAPTER ONE

GENERAL INTRODUCTION

1.1 INTRODUCTION

Colour is an important quality factor with respect to consumer preference and the commercial value of almost every horticultural commodity. A certain combination of colour with taste and pungency can play an important role in improving popularity of fruit, such as pepper (Howard *et al.*, 1994). Furthermore, different pigments impart different colours to plant parts. The most abundant pigments are chlorophylls, which are responsible for green colour. The presence of water-soluble anthocyanins results in various shades of red or blue of many flowers and fruit (Markakis, 1982) and the lipid-soluble carotenoids impart yellow, orange and red colours to a large number of fruit (Gross, 1987). In addition to that, carotenoids serve as accessory light harvesting pigments in all photosynthetic membranes (Siefermann-Harms, 1987; Frank and Cogdell, 1993; 1996; Behera *et al.*, 2002) and protect the photosynthetic apparatus against destructive effects of excess light and O_2 (Siefermann-Harms, 1987; Frank and Cogdell, 1993; Niyogi, 1999). Moreover, carotenoids contribute to attractiveness of fruit, and provide additional nutritional value in the form of dietary antioxidants (Bosland and Votava, 1999; McGhie and Ainge, 2002).

A non-subjective measurement of colour is necessary, as perception varies tremendously between individuals. Furthermore, there may be other colour-perception abnormalities apart from the well-known “red-green blindness” (Voss, 1992). The visual colour of a fruit is determined by the wavelength reflected from the fruit and is influenced by a variety of factors such as environmental signals, primarily light (Bartley and Scolnik, 1995), chemical components of the fruit such as fat and water content (Little and McKinney, 1972), its texture and luminosity (Delwiche, 1987) as well as the subjective judgement of the viewer. Moreover, other factors like the size of the surface viewed, its background or "surround", the directions of illumination and viewing and the nature of the light source may have an influence on the perception of colour (Voss and Hale, 1998).

Communicating perception of colour entails not only the evaluation and description of colour by an individual, but also the visualisation of described colours. Careful colour evaluation is desirable for scientific, botanical and horticultural descriptions (Voss, 1992). However, visual colour evaluation is often flawed. Even though evaluation of colours using colour charts (e. g. Royal Horticultural Society colour charts or Outspan Citrus colour chart) is nondestructive it is subjective and depends on an individual's experience and ability to differentiate between colours. This discrepancy necessitates the use of colour-measuring devices, which enable more objective notation of specimen colours and result in minimal deviation from "normal" colour vision. Currently used analytical methods for colour determination in pepper fruit include, firstly, the most commonly used spectrophotometric analyses, which are essential where spectral characteristics (of the colour) are relevant to research objectives (Voss, 1992), such as the ASTA 20.1 method (*American Spice Trade Association*) (ASTA, 1985); secondly, tristimulus colorimetry, which is currently gaining preference over spectrophotometric methods (Conrad *et al.*, 1987). This method provides a more descriptive and precise colour measurement by determining the spectrum of transmitted radiation from near UV to far red (350-750 nm). Thirdly, high performance liquid chromatography (HPLC) combined with a UV-Vis rapid-scanning detector can be used to identify and quantify the chemical composition of pigments in plant tissue. However, the latter methods are costly and destructive.

1.2 MOTIVATION

Worldwide, natural food colouring agents are gaining preference over the currently used synthetic ones, as natural agents are perceived to be healthier and more nutritious than their synthetic counterparts. Some of these natural colourants are carotenoids found exclusively in pepper fruit (Hornero Méndez *et al.*, 2002). Marketability of pepper fruit will, hence, revolve more and more around colour and colour intensity of the fruit. Therefore, research on colour regulatory aspects in pepper fruit has great merit for improved consumer acceptance of this fruit. Hence, the aims of the current study were: Firstly, to determine changes in pigment concentration (total carotenoids and chlorophylls) during pepper fruit development and, secondly, to investigate at which stage of development certain environmental factors and horticultural management practices can affect total carotenoid and chlorophyll concentration of pepper fruit, with an intention to fast-forward colour change.

1.3 Botany and taxonomy of *Capsicum* species

Capsicum is a term given to a fruit producing genus that, together with other vegetable crops like tomato and eggplant, agronomic crops such as potato and tobacco as well as floricultural crops like petunia, is a member of the *Solanaceae* family (Bosland and Votava, 1999). *Capsicum* fruit are considered vegetables, although botanically they are berries (Bosland, 1992). They are not related to *Piper nigrum*, the source of black and white pepper (Bosland, 1992), nor are they related to Guinea pepper or grains paradise, *Aframomum meleguata* (Bosland and Votava. 1999). *Capsicum* pepper seems to be gaining more and more popularity worldwide, as a high-valued crop due to its relatively high content of antioxidant vitamins A, C and E (Bosland and Votava, 1999) compared to other fruit vegetables like eggplant or cucumber.

The *Capsicum* terminology is confusing. Pepper, chilli, chile, chili, paprika and *capsicum* are used interchangeably for plants of the genus *Capsicum* (Bosland, 1992). The term '*Capsicum*' will, therefore, in this study be used according to Bosland (1992) when discussing the taxonomy of the genus, while 'pepper' will be used for the non-pungent bell pepper type, which was the subject of investigation in this study.

All *Capsicum* species, except *Capsicum anomalum,* originated in America (Rubatzky and Yamaguchi, 1997). *C. anomalum* originated in Asia and is taxonomically distinct from the other *Capsicum* species; it is even questionable whether it belongs to the genus *Capsicum* (Bosland and Votava, 1999). There has also been some debate about the number of species in the genus *Capsicum* (Bosland and Votava, 1999). First references on the classification of *Capsicum* are found in 16th century botanical books. In 1699 Morris published *Plantarum Historia Universalis Oxoniensis* in which 33 'variants' of pepper were described. A year later Tourneforte named the genus *Capsicum* and listed 27 species. In 1753 Linneaus reduced the number of *Capsicum* species to two: *C. annuum* and *C. fructescens.* Later, in 1767, the same author added two more species, *C. baccatum* and *C. grossum.* Towards the end of the 18th century Ruiz and Pavon (1790) and Willdenow (1798) described further species, *C. pubescens* and *C. pendulum*, respectively.

By the end of 19th century, more than 90 species were listed within the genus *Capsicum.* Similar to Linneaus, Irish (1898) recognised only two species, C. *annuum* and *C. frutescens.* Controversy about the number of *Capsicum* species continued until 1953 when Heiser and Smith re-categorised the genus into four species, *C. annuum, C. frutescens, C. baccatum* and *C. pubescens.* Four years later, Smith and Heiser (1957) determined that *C. chinense* was also a *Capsicum* species, which resulted in the current amount of five domesticated *Capsicum* species.

The genus *capsicum* has been classified into five complexes; each of these comprises related wild and domesticated species (Fig. 1.1) (Bosland and Votava, 1999). New forms of domesticated *Capsicum* have evolved by continued human manipulation; some of these forms have lost the ability to reproduce without artificial pollination.

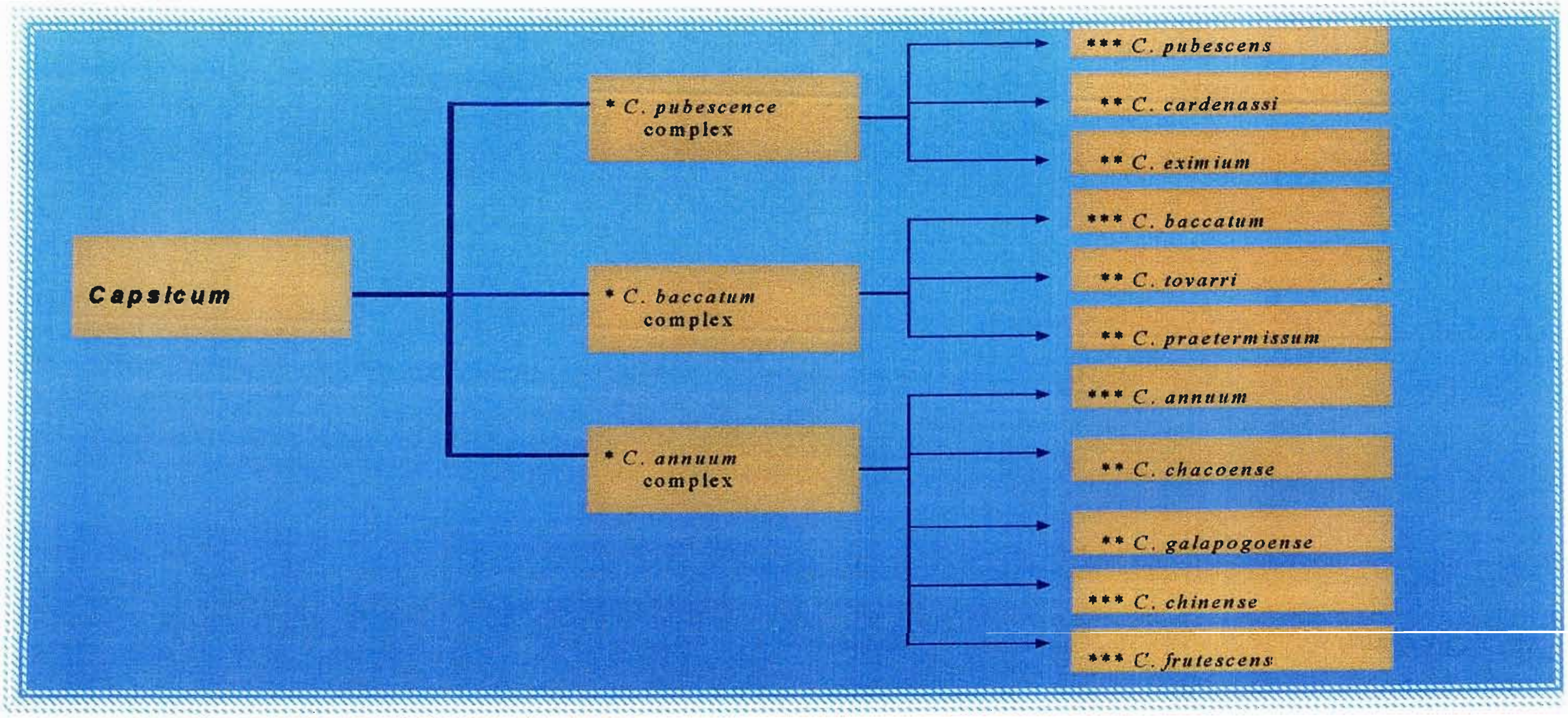

Figure 1.1 Classification of the genus *Capsicum*. * Species complexes. ** Wild *Capsicum* species. *** Domesticated *Capsicum* species (after Bosland and Votava, 1999).

1.4 CAROTENOID BIOSYNTHESIS

An understanding of the biosynthesis and interrelationship of carotenoids is deemed essential before any attempt at colour manipulation by cultural practices can be undertaken (Oberholster, 2001). Many authors (Bartley *et al.*, 1994; McGarvey and Croteau, 1995; Chappell, 1995; Cunningham and Gantt, 1998) investigated the carotenoid biosynthetic pathway, particularly its biochemical and molecular aspects. Recent information about the molecular biology of carotenoids is primarily derived from the biosynthetic pathway of carotenoids in ornamentals (mostly *Narcissus bulbocodium*) and fruit vegetables (mostly *Capsicum annuum*) (Bartley *et al.*, 1994).

Carotenoid biosynthesis can be divided into several stages, namely, formation of mevalonic acid (MVA), formation of geranylgeranyl pyrophosphate (GGPP), formation of phytoene, desaturation of phytoene, formation of lycopene, the first carotenoid in the MVA pathway. Further modifications result in various other carotenoids and apocarotenoids (Gross, 1987).

1.4.1 Formation of isopentenyl pyrophosphate

Mevalonic acid (MVA) is the precursor of the building block isopentenyl pyrophosphate (IPP). Cunningham and Gantt (1998) have shown that IPP is the central metabolite, building block precursor of all isoprenoid compounds. Formation of IPP, "the active isoprene unit" (McGarvey and Croteau, 1995), starts in all living organisms, from the fusion of three molecules of acetyl-CoA via acetoacetyl-CoA and β–hydroxy-β-methyl-glutaryl-CoA (HMG-CoA), through reduction of the latter to MVA (Gross, 1987; McGarvey and Croteau, 1995) (Fig. 1.2B). The mevalonate resulting from the aforementioned reduction step is sequentially phosphorylated by mevalonate kinase and phosphomevalonate kinase to form 5-pyrophospho-mevalonate. Formation of IPP is then catalysed by pyrophospho-mevalonate decarboxylase. Recent studies (Lichtenthaler *et al.*,

1997) show that an additional IPP (non-mevalonic) biosynthetic pathway (Fig. 1.2A) occurs in higher plants, eubacteria and green algae. This pathway allows an interpretation of results concerning the biosynthesis of chloroplast isoprenoids, which had been interpreted assuming compartmentation differences (Lichtenthaler *et al.*, 1997). In the non-mevalonic pathway glyceraldehyde 3-phosphate (GAP) and pyruvate are precursors of IPP. It seems MVA is a precursor of cytoplasmic IPP, while pyruvate and GAP, which have been reported to be precursors of plastidic prenyllipids, are in fact precursors of the alternative GAP/ pyruvate pathway of IPP formation (Lichtenthaler *et al.*, 1997).

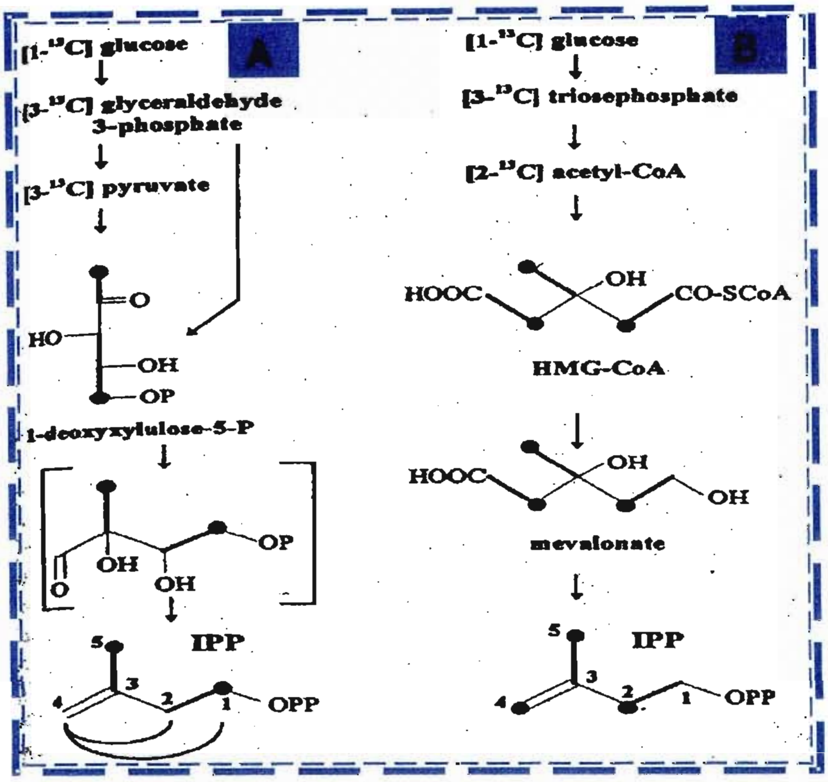

Figure 1.2 Isopentenyl pyrophosphate pathways. **A**: non-mevalonic (GAP/pyruvate) pathway. **B:** classical acetate/mevalonate pathway (Lichtenthaler *et al.*, 1997).

IPP is the basic building block that is added to prenyl pyrophosphates to form longer chains. Owing to the fact that IPP is insufficiently reactive to undergo ionization to initiate condensation to higher terpenoids, it is first isomerised to allylic ester dimethylallyl pyrophosphate (DMAPP) by IPP isomerase (McGarvey and Croteau, 1995). Prenyl transferase catalyses the generation of higher terpenoids by forming multistep reactions beginning with DMAPP and IPP to form higher isoprenologs, generally with all *trans* (E) geometry (McGarvey and Croteau, 1995).

1.4.2 Desaturation of phytoene and cyclization of lycopene

A series of desaturation reactions results in the conversion of the colourless lipophillic compound, phytoene, into colourful carotenoids by creating the conjugated double bonds that form a chromophore. The enzyme phytoene synthase (PSY) catalyses the two-step conversion of two molecules of GGPP into prephytoene pyrophosphate (PPPP) and then into phytoene (Dogbo *et al.*, 1988).

The cyclization of lycopene is the first important branch point in the carotenoid biosynthetic pathway in higher plants (Deli *et al.*, 2001). Two types of ionone rings are formed; the *β*-ring as an end group of both sides of the *β*-carotene molecule and the ε-ring at one side of an α-carotene molecule in addition to a β-ring on the opposite side. The mechanism of both types of cyclization involves a proton attack at either the C_2 or the C_4 position of the lycopene molecule. The resulting carbonium ion intermediate is stabilized by the loss of a proton from either C_2 or C_4 to yield a *β*- or ε-ring, respectively. The formation of these two rings is under different genetic control. The formation of *β*-carotene (an example of two *β*-rings) is catalyzed by lycopene *β*-cyclase whereas, in the case of α-carotene, lycopene *β*-cyclase and lycopene ε-cyclase are involved (Sandmann, 1994). In unripe peppers, both of these enzymes as well as the carotenoids *β*-carotene and lutein predominate. Physiological maturation of fruit results in the reduction of ε-cyclase activity and disappearance of lutein (Deli *et al.*, 2001).

1.4.3 Synthesis of Xanthophylls

Xanthophylls are hydroxy-, epoxy-, furanoxy- and oxy-derivatives of the carotenes formed in the late stages of the carotenoid pathway (Bartley and Scolnik, 1995; Deli *et al.*, 2001). Formation of xanthophylls occurs after cyclization when corresponding carotenes are oxidised (Gross, 1987; Deli *et al.*, 2001). The first xanthophylls are formed from cyclic carotenoids such as β-carotene by introduction of hydroxy-groups in position C_3 and C_3' of the ionone rings, followed by epoxidation at the 5, 6 and 5', 6' positions. Bartley and Scolnik (1995) reported that xanthophyll epoxides undergo light-dependent epoxidation and de-epoxidation cycles by interconversion of zeaxanthin and violaxanthin. Blue arrows illustrate that in Figure 1.3.

Interconversion of the xanthophylls violaxanthin, antheraxanthin and zeaxanthin, is due to the "xanthophyll cycle" (Haldimann, 1996), which involves de-epoxidation of violaxanthin in the light and epoxidation of the formed zeaxanthin in the dark, via antheraxanthin by two enzymes, a de-epoxidase and an epoxidase (Gross, 1987). Studies on fruit carotenoids of *Capsicum annuum* L. showed that, in red fruit, capsanthin and capsorubin were formed from antheraxanthin and violaxanthin (Fig. 1.3), presumably by pinacolic arrangement of the 3-hydroxy-5,6-epoxide end group (Camara, 1980; 1985). Bouvier *et al.* (1994), in confirmation of this, isolated and characterised capsanthin-capsorubin synthase, the enzyme responsible for the conversion of the 5,6 epoxy-carotenoids, antheraxanthin and violaxanthin, into the keto-xanthophylls capsanthin and capsorubin, respectively (Davies *et al.*, 1970). These two compounds are reported to be the predominant carotenoids of pepper fruit (Candela *et al.*, 1984), resulting in the red colour of ripe pepper fruit. This conversion tracks the transformation of chloroplasts to chromoplasts (Camara *et al.*, 1995).

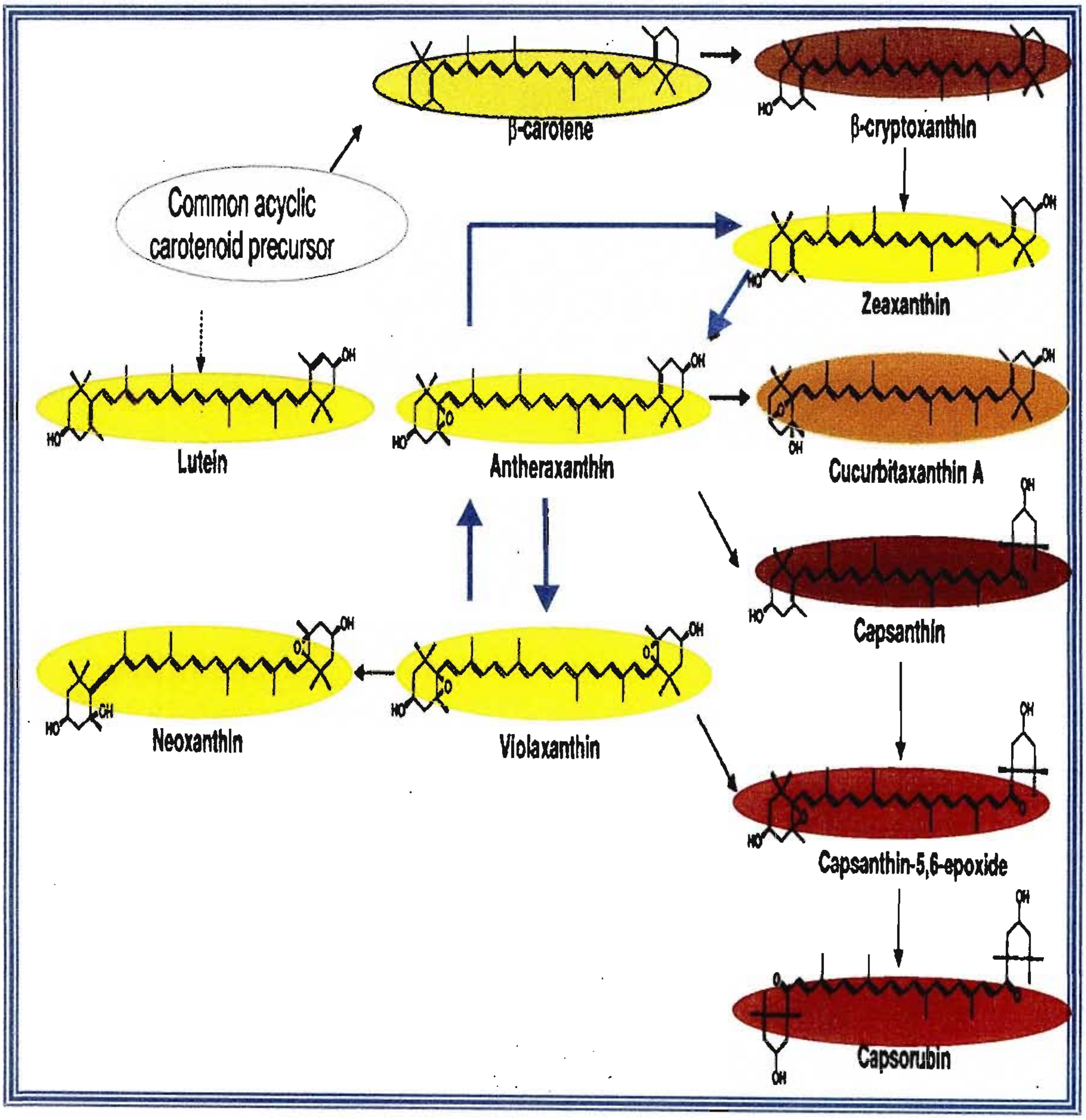

Figure 1.3 A modified carotenoid biosynthetic pathway in *Capsicum annuum* fruit (after Hornero-Méndez *et al.*, 2002).

1.4.4 Unique carotenoids of peppers

Carotenoids of pepper have been the subject of study by several authors (Gomez-Landron de Guevara and Pardo-Gonzalez, 1996). The complexity of the mechanisms involved in the biosynthesis of unique carotenoids such as capsanthin, capsorubin and cryptocapsin and the spectacular changes carotenoids undergo during ripening, have resulted in research interest in pepper carotenoids over the past 30 years (Fisher and Kocis, 1987; Almena *et al.*, 1990, 1991; Minguiz and Hornero-Méndez, 1993, 1994; Biacs and Daood, 1994). Gross (1987) reported that the genus *Capsicum*, regardless of pungency level, contains unique ketocarotenoids with a keto-group at the central chain and a cyclopentanol ring at one or both ends (Fig. 1.4).

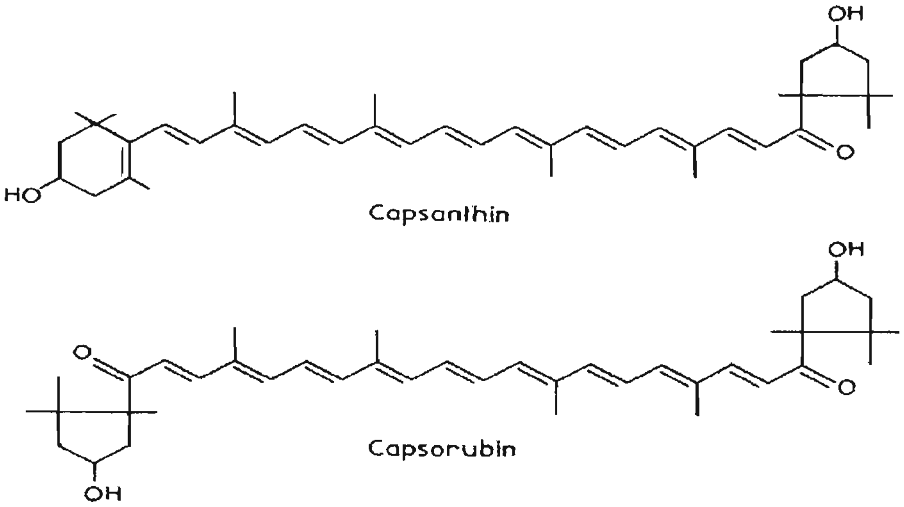

Figure 1.4 Unique carotenoids found in *Capsicum* species (Gross, 1987).

Separation and quantification of carotenoids in peppers using paper chromatography began as early as 1956 (Cholnoky *et al.*, 1956). Buckle and Rahman (1979) analysed pepper carotenoids by column chromatography. Many researchers (Minguiz-Mosquera and Garrido-Fernandez, 1983) utilized thin layer chromatography (mainly with silica gel as stationary phase) for the separation of pepper carotenoids. The results of the investigation by Phillip and Chen (1988) indicate that polar phases have a disadvantage of potentiating isomerization and transformation during separation. This happens, for example, with violaxanthin, which is converted to 5,8-epoxide derivatives.

1.5 COLOUR DEVELOPMENT

Colour expression is used as an indicator of maturity (Newman *et al.*, 1989) in all colourful fruit. There are various stages of fruit ripening, a regulated developmental process, which starts after seed maturation (Gillaspy *et al.*, 1993). In all coloured fruit chloroplasts differentiate into non-photosynthetic chromoplasts with concommitant degradation of chlorophylls and starch in a process accompanied by extensive structural reorganization (Camara *et al.*, 1989).

1.5.1 Chloroplast- chromoplast transformation

Czygan (1980) defined a true chromoplast as a non-photosynthetic, carotenoid-bearing plastid, which performs anabolic processes, particularly carotenoid biosynthesis.

Chromoplasts can be classified into four types on the basis of the characteristics of the carotenoid-containing substructure (Thomson and Whatley, 1980; Marano *et al.*, 1993; Vishnevetsky *et al.*, 1999). Those substructures can be: (a) globular:- containing many carotenoid-containing lipid droplets in the chromoplast stroma; (b) tubulous:- characterized by carotenoid-carrying internal elements (fibrils) which are more or less circular in section; (c) membranous:- containing a set of concentric membranes as their most prominent internal structure and (d) crystallous:- containing crystals.

Globular chromoplasts are the most common type (Gross, 1987) and the evolutionary oldest. They are lens-shaped or spheroidal and contain carotenoid-carrying lipid droplets termed plastoglobules. Globular chromoplasts occur in peppers (Kirk and Juniper, 1967) oranges (Thomson, 1966; Thomson *et al.*, 1967) muskmelon, pummelo, kumquat flavedo (Huyskens *et al.*, 1985), pears and plums (Sitte *et al.*, 1980). However, recent studies (Pozueta-Romero *et al.*, 1997) indicate that bell pepper chromoplasts are of the fibrillar (tubulous) type at the ripening stage, although these types are not common in some cultivars such as 'Albino' and 'Golden Summer'. In general, bell pepper chloroplasts undergo highly organised changes to chromoplasts (Spurr, 1970; Magne, 1971).

Evidence from comparative biochemical and structural studies indicates that the shape of chromoplasts is governed by the lipid-to-protein ratio. A high proportion of lipids induces the formation of globular structures, whereas a high proportion of proteins induces the formation of membranous or threadlike structures (Deruère *et al.*, 1994b). Thus, the proteins associated with carotenoid lipids may govern fibril formation in chromoplasts. Support of this theory is provided by the description of a 30-kD protein (fibrillin) from fibrillar chromoplasts of *Kerria japonica* (Wuttke, 1976) and *Tropaeolum majus* (Winkenbach *et al.*, 1976; Emter *et al.*, 1990). A biochemical model for fibrillar structures was proposed for *Tropaeolum majus* (Winkenbach *et al.*, 1976) and *Palisota barteri* (Knoth *et al.*, 1986) in which this 30-kD protein is buried in the polar sheath of the chromoplast and interacts with the core composed mainly of carotenoids. However, according to Deruère *et al.* (1994b) it has never been determined whether the 30-kD protein or other antigenically related proteins are present in chromoplast fibrils of other species.

During the ripening process of pepper fruit, specific membrane vesicles and fibrils appear in the developing chromoplasts (Camara and Brangeon, 1981). Deruère *et al.* (1994b) further observed that, upon ripening of pepper fruit, the thylakoid membranes become progressively disorganised and is replaced by a new membrane network. This is accompanied by a massive accumulation of plastoglobules that eventually elongate into fibrillar structures. Similar behaviour of thylakoid membranes in fruit have already been observed in *Capsicum annuum*

(Simpson *et al.*, 1977a), *Kerria japonica* (Steffen and Walter, 1955), *Palisota barteri* (Knoth *et al.*, 1986) and in sepals of *Nuphar lutea* (Gronegress, 1974) and *Strelitzia juncea* (Simpson *et al.*, 1975), in which globules elongate into fibrils during the initial period of chromoplast differentiation.

In coloured pepper varieties the appearance of chromoplast red and yellow carotenoids is accompanied by the simultaneous loss of chlorophyll. Deruère *et al.* (1994b) proposed that during the chloroplast to chromoplast transition, chromoplasts obtain their new internal membrane components from degrading thylakoid membranes. A year later Rafia (1995) confirmed this proposal and added that fibrils, tubules and peripheral membranes develop and shape the characteristic fibrillar chromoplasts. This is inconsistent with observations in the bell pepper colour mutant, 'Sweet Chocolate', which derives its unusual dark pigmentation from carotenoids that develop in the presence of chlorophyll. Therefore, Deruère *et al.* (1994b) concluded that the thylakoids do not degrade in 'sweet chocolate' but remain well preserved despite carotenoid accumulation and fibril formation. It is, therefore, likely that fibrils are synthesized *de novo* (Laborde and Spurr, 1973; Deruère *et al.*, 1994b).

Investigations by Newman *et al.* (1989) and Cervantes-Cervantes *et al.* (1990) revealed that bell pepper fruit contain three specialised proteins important in carotenoid biosynthesis; a carotenoid-binding protein (Chr A) (Oren-Shamir *et al.*, 1993; Bouvier *et al.*, 1994; Houlne *et al.*, 1994), a chromoplast-specific protein (Chr B) (Newman *et al.*, 1989) and fibrillin, a chromoplast-specific protein responsible for the linear arrangement of lipochromes into fibrils (Deruere *et al.*, 1994b). According to Pozueta-Romero *et al.* (1997) the presence of PAP protein in chloroplast plastoglobules and chromoplasts extracts confirms the hypothesis that plastoglobules and fibrils are ontogenetically related. The above-mentioned point coupled with the plastid transformation that occurs concomitantly with carotenoid accumulation indicates that PAP expression precedes fibril formation. Pozueta-Romero *et al.* (1997) reported that PAP is a gene that, although expressed in all plant tissues, is developmentally controlled and mainly expressed in fruit at the later stages of ripening. The same authors indicated that PAP accumulates in the fibrils during bell pepper chromoplast differentiation and is designated as a plastidial, lipid-associated protein. Ultimately, they inferred that in bell pepper Chr A, Chr B and fibrillin are encoded by the same single gene.

Structural and biochemical data indicate that carotenoids are not randomly dispersed or freely accessible within the chromoplasts of pepper fruits but are sequestered into fibril structures by fibrillin. Carotenoids are aligned along the longitudinal axis of chromoplasts. Therefore, Deruere *et al.* (1994b) developed a new model, differing from previous ones by Knoth *et al.* (1986) and Emter *et al.* (1990). According to this new model the major components of fibrils are their bicyclic carotenoids, which occupy the fibril core (Fig. 1.5). The core is surrounded by polar galactolipids and phospholipids, which orientate their hydrophobic tails towards the carotenoids and their polar head groups outwards towards the surface, which is composed of fibrillin. This model implies that the precursors of carotenes, such as phytoene, phytofluenes or ξ-carotene (Camara and Monéger, 1982), once incorporated into fibrils, remain constituents of the fibrils. Different to the previous model they no longer act as intermediates of the biosynthetic pathway. This model also postulates that fibrillin interacts directly with the polar lipids surrounding the core and thus act as a shield in addition to its role in governing the shape of the fibrils. Hansmann and Sitte (1982) proposed a model of a plastoglobule structure, with PAP protein as part of the interface between the stroma and lipophilic core of globules.

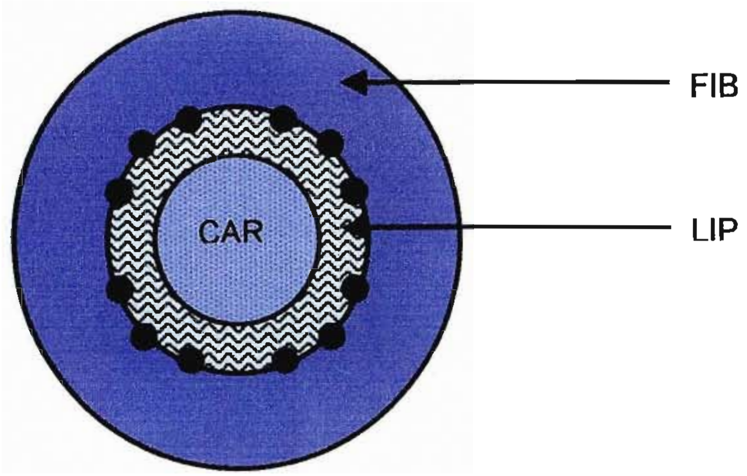

Figure 1.5 Model of chromoplast fibril assembly (not drawn to scale). The core contains carotenoids (**CAR**) that interact with the acyl residues of the more polar galactolipids and phospholipids (**LIP**), whose polar head groups (dark circles) interact with fibrillin molecules (**FIB**), which are directly in contact with the plastid stroma (re-drawn after Deruère *et al.*, 1994b).

1.5.2 Environmental and plant impact on carotenoid concentration in fruit

Previous investigations into the regulation of carotenoid biosynthesis in pepper fruit have mostly generalised the effects of certain environmental factors. Goodwin and Goad (1970) and Goodwin (1980) claimed that providing optimal growing conditions could optimise carotenoid accumulation in pepper fruit but that there are, however, cultivar differences. Various cultural practices, such as pruning or thinning, might affect carotenoid accumulation due to the presence or absence of building blocks and the manipulation of sink-source relationships. Furthermore, nutrition, moisture regime as well as growing temperatures might impose stress on the plant, which might influence resource allocation. Information on the impact of thinning of pepper flower/fruit/fruitlet on carotenoid accumulation in the remaining fruit seems to be lacking.

1.5.2.1 Moisture stress

Bell pepper is one of the most susceptible horticultural plants to drought stress (Alvino *et al.*, 1994). Doorenbos and Kassam (1986) observed that optimum moisture is required during the entire growth period to obtain high yields. Water stress and other stresses are reported to positively impact on the senescence of whole plants or leaves (Young and Britton, 1990; Alvino *et al.*, 1999), and the onset of senescence in harvested pepper fruit (Lurie *et al.*, 1986). The presence of zeaxanthin in plant tissues is frequently associated with stress conditions. In support of this point low amount of zeaxanthin (2-3% of total carotenoids) have been reported in drought-stressed senescent barley leaves that have been maintained in the light (Young and Britton, 1990).

1.5.2.2 Temperature effect

The day/night temperature regime affects flowering, fruit set and fruit development of bell pepper (Rylski and Spigelman, 1982; Polowick and Sawhaney, 1985; Bakker, 1989). Other solanaceous fruit vegetable crops, such as tomato, also show an effect of temperature on colour expresssion. Koskitalo and Ormrod (1972) observed that tomato fruit exposed to a 26/18°C (day/night) regime had a higher pigment content with lycopene accounting for 90% of the carotenoid than those subjected to sub- and supra-optimal temperatures. Furthermore, Noordegraaf and Wells (1995) observed that sub-optimal air temperature had a negative impact on fruit colour development as it increased time to ripening in tomato.

1.5.2.3 Nutritional effects

The chlorophyll and carotenoid concentration of fruit correlates strongly with colour, quality and nutritional parameters of horticultural commodities such as green and red peppers (Anchondo and Wall, 2001). The nutritional status of the soil has a clear impact on colour development of fruit. Nitrogen deficiency, for example, promotes less intense green colour in pepper (Miller, 1960). Furthermore, the form of nitrogen has been found to affect the nutrient composition, growth and development of plants. Ammonium nitrogen (NH_4-N) applied in the form of ammonium nitrate can promote colour intensity in citrus fruit compared to nitrate nitrogen (NO_3- N) (Collado *et al.*, 1996). It also has an inhibitory effect on Ca, Mg and K absorption by pepper (Zornaza *et al.*, 1987), tomato (Geraldson, 1957), and corn (Blair *et al.*, 1970; Clark, 1970). On the contrary, NH_4-N can act as a catalyst in P absorption (Wilcox *et al.*, 1977; Ikeda and Osawa, 1983).

Information on the effect of K fertilization on colour development on solanaceous crops is scarce. Gross (1987) and Trudel and Ozbun (1970) reported that an increase in K supplied to tomatoes increases carotenoid accumulation in the fruit. Phosphorus has been reported to have a promotive effect on tomato fruit colour (Woodrow and Rowan, 1979). Finally, as Mg is a component of chlorophyll its deficiency can result in chlorosis. However, there seems to be no published information on the impact of Mg on colour development in pepper fruit.

1.5.2.4 Fruit load

Thinning and its impact on colour development in peppers has not been investigated. The impact of fruit load on fruit growth, biomass allocation and firmness has been investigated for other crops such as cucumber (Marcelis, 1993) and apple (Johnson, 1992,1994). Johnson (1995) reported that thinning resulted in increased degreening of 'Cox Orange Pippin' apples. The increase in K concentrations of the fruit coincided with colour development in apple fruit (Palmer *et al.*, 1991; Johnson, 1992, 1994; Fallahi and Simsons, 1993; Volz *et al.*, 1993). Although pigments responsible for fruit colour in apple are anthocyanins and not carotenoids it has been reported that thinning can affect nutrient content in apple fruit (Johnson, 1995). Furthermore, as the carotenoid concentration of tomato fruit is correlated to its mineral concentration, particularly the K concentration (Gross, 1987), thinning might affect colour expression in peppers.

CHAPTER TWO

GENERAL MATERIALS AND METHODS

2.1 Chemicals

Butylated hydroxy toluene (BHT) was purchased from Sigma Chemical Co. (St Louis, Mo, USA), while triethylamine (TEA) was purchased from Merck (Darmstadt, Germany). Potassium hydroxide (KOH) was purchased from BDH Laboratory Suppliers (Poole, England).

2.2 Solvents

High Performance Liquid Chromatography (HPLC) grade solvents (ethyl acetate, methanol, acetonitrile) were purchased from Burdick and Jackson (Allied Signal Inc., Muskegon, MI, USA). The analytical grade solvents (ethanol, methanol, acetone, N, N-dimethylformamide, petroleum ether, chloroform) were obtained from BDH Laboratory Suppliers (Poole, England). Diethylamine was bought from Sigma Chemical Co. (St. Louis, Mo, USA). Diethyl ether was purchased from Merck (Darmstadt, Germany).

2.3 Thin layer chromatography (media)

Thin layer chromatography plates of silica gel, type 60 GF_{254} (20 x 20 cm, 0.7 mm thickness), were obtained from Merck (Darmstadt, Germany).

2.4 High performance liquid chromatography (HPLC) system

The HPLC system composed of a 5 μm Vydac 201 TP_{54} C_{18} (250 x 4.6 mm i.d.) column (VYDAC, Hespera, CA, USA), a Spectra System P2000 pump, UV3000 rapid scanning detector, and PC1000 software (Thermo Separations Products, Fremont, CA, USA).

2.5 Pigment extraction

As pigments are easily destroyed by photo-bleaching (Lichtenthaler, 1987), extraction, purification and concentrating procedures were performed on ice and under dim daylight (electric lamps were switched off).

2.6 Comparative extraction efficiency of organic solvents for spectrophotometric carotenoid and chlorophyll determination

Pepper fruit are usually harvested at horticultural maturity stage, that is when they are mature-green, and physiological maturity stage, that is when they are either red-ripe or yellow-ripe depending on the cultivar and the purpose for which they are harvested. In this study, mature-green and red-ripe pepper fruit were harvested and chopped into small pieces, shock frozen in liquid nitrogen and ground to a powder using a mortar and pestle. One gram of this powder was extracted, in triplicate, with the following organic solvents: 80% acetone (v/v), 95% ethanol (v/v), 100% methanol, 100% N, N-dimethylformamide (DMF), 100% chloroform and 100% acetone. Each sample was homogenized in 10 mL solvent, to which BHT (100 mg L^{-1}) was added, with an ULTRA TURRAX® T25 (Janke and Kunkel IKA®-Labortechnik, Berlin, Germany), and centrifuged in a SC-158T (Yih Der Instruments CO., Taiwan) centrifuge. The acetone extraction was carried out according to Lichtenthaler (1987). The 80% acetone extract was re-extracted with 10 mL 100% acetone to remove the less polar pigments. The total amount of pigments was calculated using Lichtenthaler`s formulae for 80% and 100% acetone (Table 2.1.1) and the values were added. Further aliquots of samples were extracted with the solvents listed in Table 2.1.1. The absorbance was measured with a BECKMAN DU ® Series 65 spectrophotometer (Beckman, USA) at the wavelengths shown in equations in Table 2.1.1. The calculations for the determination of concentrations of chlorophyll a (C_a), chlorophyll b (C_b), total chlorophyll (C_{a+b}), and total carotenoids (C_{x+c}) were performed using equations presented in Table 2.1.1 (Lichtenthaler, 1987; Porra *et al.* 1989; Wellburn, 1994). Solvent extraction efficiency expressed in percentage was calculated on the mean value of total chlorophylls/ carotenoids at mature-green and red-ripe stages.

2.7 Spectrophotometric determination of pigment concentration in *Capsicum* fruit

Bell pepper fruit were harvested at two maturity stages (mature-green and red-ripe), except from the temperature regime experiment where they were harvested at three maturity stages. The first harvest was at the mature green stage (stage 1), the second at the red-ripe stage (stage 2) and the third at the overripe red stage (stage 3) when fruit were soft to touch. Fruit were processed as described in Section 2.1.6. As the extraction with 80% acetone (v/v) followed by 100% acetone yielded the highest extraction efficiency (Table 2.1.2), this method was utilized for all pigment extractions. The absorbances were measured according to Lichtenthaler (1987) with a SECOMAN ANTHELIE Advanced Spectrophotometer (Topac Inc. St. Hingham. USA) at the wavelengths shown for 80% and 100% acetone in equations presented in Table 2.1.1.

Table 2.1.1 Equations for determination of concentrations of chlorophyll a (C_a) chlorophyll *b* (C_b), total chlorophyll (C_{a+b}), and total carotenoids (C_{x+c}) in *Capsicum* extracts for various organic solvents

Solvents	Equations
Acetone, 100%	$C_a = 11.24\ A_{661.6}{}^{x} - 2.04A_{644.8}$
	$C_b = 20.13A_{644.8} - 4.19A_{661.6}$
	$C_{a+b} = 7.05A_{661.6} + 18.09A_{644.8}$
	$C_{x+c} = (1000A_{470} - 1.90\ C_a - 63.1\ C_b)/214$
Acetone, 80% (v/v)	$C_a = 12.25A_{663.2} - 2.79A_{646.8}$
	$C_b = 21.50A_{646.8} - 5.10A_{663.2}$
	$C_{a+b} = 7.15A_{663.2} + 18.71A_{646.8}$
	$C_{x+c} = (1000A_{470} - 1.82\ C_a - 85.02C_b)/198$
Ethanol, 95% (v/v)	$C_a = 13.36A_{664.2} - 5.19A_{648.6}$
	$C_b = 27.43A_{648.6} - 8.12A_{664.2}$
	$C_{a+b} = 5.24A_{664.2} + 22.24A_{648.6}$
	$C_{x+c} = (1000A_{470} - 2.13\ C_a - 97.64C_b)/209$
Chloroform, 100%	$C_a = 11.47A_{665.6} - 2A_{647.6}$
	$C_b = 21.85A_{647.6} - 4.53A_{665.6}$
	$C_{x+c} = (1000A_{480} - 1.33C_a - 23.93C_b)/202$
Dimethyl formamide, 100%	$C_a = 12A_{664.2} - 3.11A_{648.6}$
	$C_b = 20.78A_{648.6} - 4.88A_{664.2}$
	$C_{x+c} = (1000A_{480} - 1.12\ C_a - 34.07C_b)/245$
Methanol, 100%	$C_a = 16.72A_{665.2} - 9.16A_{652.4}$
	$C_b = 34.09A_{652.4} - 15.28A_{665.2}$
	$C_{a+b} = 1.44A_{665.2} + 24.93A_{652.4}$
	$C_{x+c} = (1000A_{470} - 1.63C_a - 104.96C_b)/221$

[x] Numbers following the letter '*A*' represent the wavelength (nm) at which absorbance is determined. The pigment concentration obtained by inserting the measured absorbance values are micrograms per milliliter plant extract solution. Equations for 80%, 100% acetone, 100% methanol and 95% ethanol as described by Lichtenthaler (1987), for 100% chloroform and 100% dimethyl formamide by Wellburn (1994); calculations of C_a and C_b for dimethyl formamide after Porra *et al.* (1989).

Table 2.1.2 Amount of chlorophyll and carotenoid extracted from fresh pepper tissue with different solvents. Absorbances were measured spectrophotometrically and pigment concentration calculated using equations described by Lichtenthaler (1987), Wellburn (1994) and Porra *et al.* (1989)

Solvents	**Total chlorophyll (µg g^{-1} fresh tissue mass)**				**Total carotenoids (µg g^{-1} fresh tissue mass)**			
	MG	**%**	**RR**	**%**	**MG**	**%**	**RR**	**%**
95% Ethanol	42.47^{d3}	93.60^{d}	7.60^{f}	52.1^{f}	10.70^{d}	93.0^{e}	81.47^{c}	123.3^{c}
80% Acetone	58.53^{c}	128.90^{c}	14.03^{d}	96.1^{d}	11.27^{cd}	98.0^{d}	117.27^{b}	177.4^{b}
100% Acetone. (after 80% Acetone)^{1}	14.63^{g}	32.20^{g}	5.97^{g}	40.9^{g}	7.97^{e}	69.3^{f}	15.23^{g}	23.0^{g}
Cumulative: 80% +100% Acetone.^{2}	73.16^{a}	161.20^{a}	20.00^{c}	137.0^{c}	19.23^{a}	167.2^{a}	132.50^{a}	200.5^{a}
100% Acetone	40.07^{e}	88.30^{e}	31.33^{a}	214.6^{a}	11.87^{c}	103.2^{c}	73.83^{d}	111.6^{d}
100% Meth.	39.83^{e}	87.70^{e}	9.13^{e}	62.5^{e}	11.33^{cd}	98.5^{d}	60.40^{e}	91.4^{e}
100% Chlor.	30.27^{f}	66.70^{f}	25.10^{b}	171.9^{b}	6.53^{f}	56.83^{g}	36.03^{f}	54.5^{f}
100% DMF	64.20^{b}	141.40^{b}	4.33^{h}	29.6^{h}	13.15^{b}	114.3^{b}	12.20^{h}	18.5^{h}
Mean	45.4	-	14.60	-	11.5	-	66.10	-
LSD$_{(0.05)}$	1.5	4.11	1.5	4.11	0.66	4.24	0.66	4.24
F.probability								
Solvents	***	***	***	***	***	***	***	***
Maturity	***	ns	***	ns	***	ns	***	ns
Maturity x Solvents	***	***	***	***	***	***	***	***

[1]Pigment extraction with 100% acetone from the same sample after extraction with 80% acetone. ***: significant at P< 0.001, ns:- not significant, [2] Cumulative : sum of extraction with 80% and re-extraction with 100% acetone. [3]Different letters within the same column indicate significant difference at P= 0.05. **LSD**: least significant difference between solvents, **MG**: mature-green, **RR**: red-ripe, **Meth**: methanol, **Chlor**: chloroform, **DMF**: N, N dimethylformamide.

2.8 Statistical analysis

The data obtained were subjected to analysis of variance (ANOVA). These statistical analyses were carried out with Genstat 4.0 Release 5.1. and Microsoft Excel was utilised to construct graphs.

CHAPTER THREE

ISOLATION AND IDENTIFICATION OF CAROTENOIDS IN PEPPER FRUIT

3.1 CAROTENOID COMPOSITION OF RED PEPPER FRUIT DURING RIPENING: ISOLATION AND IDENTIFICATION OF RED COLOUR-IMPARTING KETOCAROTENOIDS

3.1.1 INTRODUCTION

The formation of red colour is one of various visible changes during the maturation process of pepper fruit, hence the external fruit colour is considered as a maturity index. In most cases the colour of fruit is a result of the dominant pigments (Gross, 1987). For example, red colour of *Capsicum annuum* fruit is directly related to the predominant genus-specific red carotenoids capsanthin and capsorubin (Mínguez-Mosquera *et al.*, 1984) whereas yellow cultivars are characterized by the yellow 5,6-epoxy-carotenoids (neoxanthin and violaxanthin), which are biosynthetic precursors of ketocarotenoids, that is a keto-group is present at the central chain and a cyclopentanol ring at one or both ends. Likewise, lycopene dominates in red tomato fruit (Türk *et al.*, 1994), while 9-Z-violaxanthin and *β*-citraurin predominate in *Citrus sinensis* 'Navel' and Valencia' flavedo (Oberholster, 2001). In many fruit, ripening involves differentiation of chloroplast carotenoids into chromoplast carotenoids.

Red pepper, however, has a high concentration of carotenoids and is, hence, used to extract pigments serving as commercial colourants (Gregory *et al.*, 1987) - such as paprika (Fisher and Kocis, 1987). In general carotenoids are required for human epithelial cellular differentiation (Byers and Berry, 1992) and have been found to have antioxidant (Di Mascio *et al.* 1989) and anticarcinogenic (Nishino

1998) properties. For the pepper industry, however, colour is the major quality factor determining the commercial value of the fruit (Gómez *et al.*, 1998).

A recent study by Hornero-Méndez and Mínguez-Mosquera (2000) indicates that fruit colour in *Capsicum annuum* is genetically influenced, with the gene for red colour dominant over that for yellow colour. During ripening of some fruit, such as citrus, all chloroplast pigments decrease except violaxanthin, which rises to a level of 50% of total carotenoids (Gross, 1987). At the onset of colour-break in citrus, the synthesis of chromoplast carotenoids such as β-citraurin and cryptoxanthin begins (Gross, 1987). On the other hand, chloroplast carotenoids, such as lutein and zeaxanthin, decrease gradually in ripening red pepper (Péter *et al.*, 1989), while chromoplast carotenoids, mainly the unique pigments capsanthin and capsorubin, are synthesised (Valadon and Mummery, 1977). This process coincides with the development of achlorophyllous membranes. Further maturation of red *Capsicum* fruit results in a massive biogenesis and accumulation of the dominant colour–imparting ketocarotenoids capsanthin and capsorubin (Camara and Brangeon, 1981). Therefore, the objectives of this study were, therefore, firstly, to purify and isolate red colour-imparting carotenoids from red-ripe pepper fruit by Thin Layer Chromatography (TLC) and, secondly, to identify these pigments via their spectral characteristics and via their retention time using reversed-phase High Performance Liquid Chromatography (rHPLC).

3.1.2 MATERIALS AND METHODS

3.1.2.1 PLANT MATERIAL

Pepper fruit (*Capsicum annuum* L.) cv. Capistrano, at three different maturity stages were used in this study. Fruit were harvested at each of the three consecutive ripening stages according to the external skin colour (Fig. 3.1.1): mature-green (the stage when fruit are horticulturally mature approximately 40 days after first petal fall; colour-break (a ripening stage during which the skin colour of fruit is brownish due to an accumulation of a combination of green and red pigments), and red-ripe (fully red fruit).

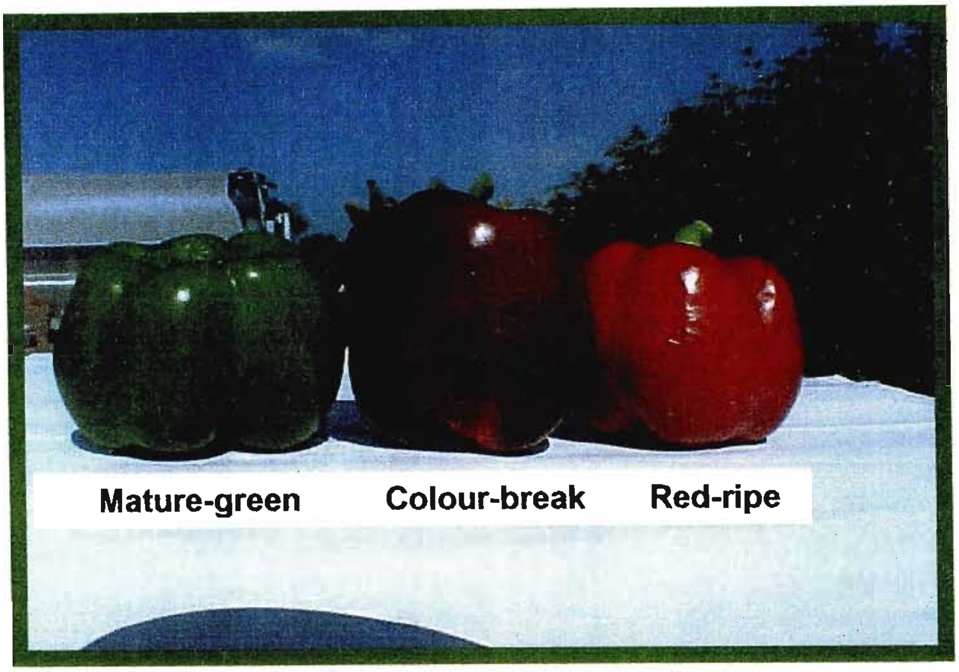

Figure 3.1.1 Three ripening stages of bell pepper fruit.

3.1.2.2 Pigment extraction

Extraction, purification and concentrating procedures were performed as desribed in Section 2.1.5.

3.1.2.3 Saponification

Fresh pepper tissue was saponified to disintegrate chlorophylls which might mask carotenoids and for hydrolysis of carotenoid acyl esters (Britton and Young, 1993). Saponification does not affect the basic carotenoid structure (Gross, 1987). This process was carried out according to Mínquez-Mosguera and Hornero-Méndez (1993) with some changes given hereafter. Thirty mL diethyl ether were added to the extract in a separation funnel, the solution shaken, and then left to settle for 15 minutes. The saponified extract was suspended in 2 mL methanol/ acetonitrile (9:1, v/v): ethyl acetate (50:50, v/v) and stored under nitrogen in the refrigerator until analysis (Oberholster, 2001).

3.1.2.4 Thin layer chromatography (TLC)

Saponified carotenoid pigments were separated on TLC silica gel 60 GF_{254} plates. The solvent system used was light petroleum ether (bp. 60-80°C): acetone: diethylamine (10:4:1, v/v/v). Three hundred µl of concentrated saponified red-ripe pepper fruit were loaded onto the TLC plates and left to develop to 15 cm in a closed TLC tank at temperature less than 10°C in darkness (Molnâr and Szabolcs, 1980). Thereafter the plates were left to dry. R_f values of each band were determined and compared with those reported by Mínguera-Mosquera and Hornero-Méndez (1993) (Table 3.1.1). In order to confirm the R_f values the authentic capsorubin, capsanthin, 13-Z-capsanthin and 9-Z-capsanthin standards as well as the extract of red-ripe fruit were loaded onto the TLC plate and the retention times and colours compared. The purified pigments (bands) were scraped off the plate and taken into solution with 100% acetone. These extracts were evaporated to dryness in a rotary evaporator at less than 30°C. Concentrated, purified, dry bands were re-suspended in 2 mL methanol/acetonitrile (9:1, v/v): ethyl acetate (50:50, v/v) and stored under nitrogen in the refrigerator until analyzed by HPLC (Oberholster, 2001).

3.1.2.5 High performance liquid chromatography (HPLC)

Extracts were filtered using 0.2 µm syringe filters (National Scientific, Kansas City, USA). Five microliters of the filtered extract were loaded onto a 50 µm Vydac C_{18} HPLC column (Western Analytical Products Inc., Murrieta, California, USA). Samples were eluted off the column isocratically at 26°C using methanol/acetonitrile (9:1, v/v), containing 0.1% (w/v) butylated hydroxy toluene

(BHT) and 0.05% (v/v) triethylamine (TEA) as the mobile phase at a rate of 1 mL min^{-1}. Compounds of interest were detected at 460 nm by a UV3000 rapid-scanning detector (Thermo Separations Products, Fremont, CA, USA) over a spectral region of 370-550 nm. Identification of compounds was achieved using a software package (PC 1000), which allows for comparison of absorption spectra of unknown compounds to authentic carotenoid standards. The two unknown bands were characterized by comparison of their absorption spectra to that of authentic carotenoid standards obtained from P. Molnár, Pécs University, Hungary. The two dominant red colour bands, obtained by separation of saponified extracts, were analysed by reversed-phase HPLC. The elution time of compounds was determined as well as the absorption maxima between 370 and 550 nm of the compounds (Table 2.1.4).

Table 3.1.1 Characteristics of carotenoids in saponified extract from red-ripe pepper fruit in comparison of pigments identified by Mínguez-Mosquera and Hornero-Méndez (1993) to unknown pigments. Solvent system: light petroleum ether (bp. 65-95°C)-acetone-diethylamine (10:4:1)

Pigment	Colour in the chromatogram	R_f value		
		A^1	B^2	C^3
β-carotene	Yellow-Orange	0.96	0.96	-
β-cryptoxanthin	Yellow	0.57	0.65	-
Cryptocapsin	Pale Red	0.51	0.58	-
Capsolutein	Yellow	0.47	0.56	-
Zeaxanthin	Yellow-Orange	0.44	0.44	-
Antheraxanthin	Yellow	0.42	0.41	-
Capsanthin	Intense Red	0.39	0.39^A	0.39
Violaxanthin	Yellow	0.34	0.33	-
Capsorubin	Red-Brown	0.31	0.31^B	0.31

A^1: R_f values used for identification of unknown pigments (after Mínguez-Mosquera and Hornero-Méndez, 1993). B^2: R_f values of unknown saponified pigments. ^{A} and ^{B} are the top and bottom bands (respectively) scrapped off the TLC plate for HPLC analysis. C^3: R_f values of authentic carotenoid standards obtained from P. Molnár, Pécs University, Hungary.

3.1.3 RESULTS

3.1.3.1 Separation and tentative identification of red colour-imparting carotenoids by Thin Layer Chromatography (TLC)

An increase in colour intensity of two bands ('A' and 'B') as well as the disappearance of green bands (chlorophyll) was observed with increasing fruit maturity (Fig. 3.1.2). Furthermore, yellow and orange bands were present, which prevailed at colour-break and increased in intensity as the fruit ripened (Fig. 3.1.2). The R_f values of these two bands from the saponified extract of red-ripe fruit corresponded well with those of the known ketocarotenoids capsorubin and capsanthin (Table 3.1.1) as well as those of authentic capsorubin and capsanthin standards (0.31 and 0.39 respectively).

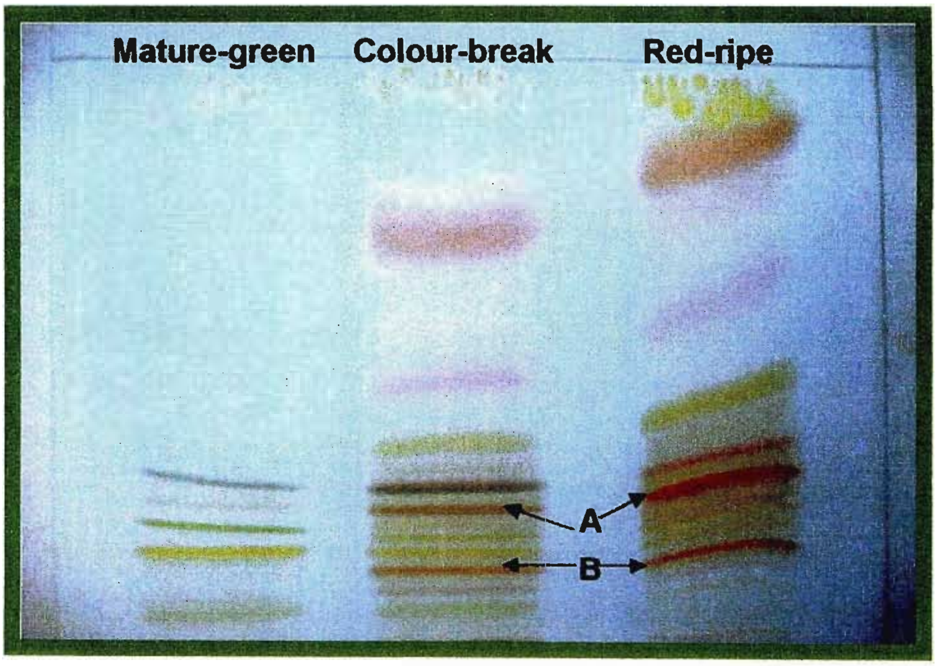

Figure 3.1.2 TLC separation of crude bell pepper pigments extracted with 80% acetone followed by 100% acetone indicating two predominant red colour-imparting bands 'A' and 'B'. TLC system utilised was light petroleum ether (bp. 60-80°C): acetone: diethylamine (10:4:1, v/v/v). Fruits were at three ripening stages as illustrated in Figure 3.1.1.

3.1.3.2 Carotenoid identification by reversed-phase High Performance Liquid Chromatography (rHPLC)

The rHPLC absorption spectra show that both band ‘A’ and ‘B’ (Fig. 3.1.2) are identical (have broad absorption spectra) (Figure 3.1.3A and B).

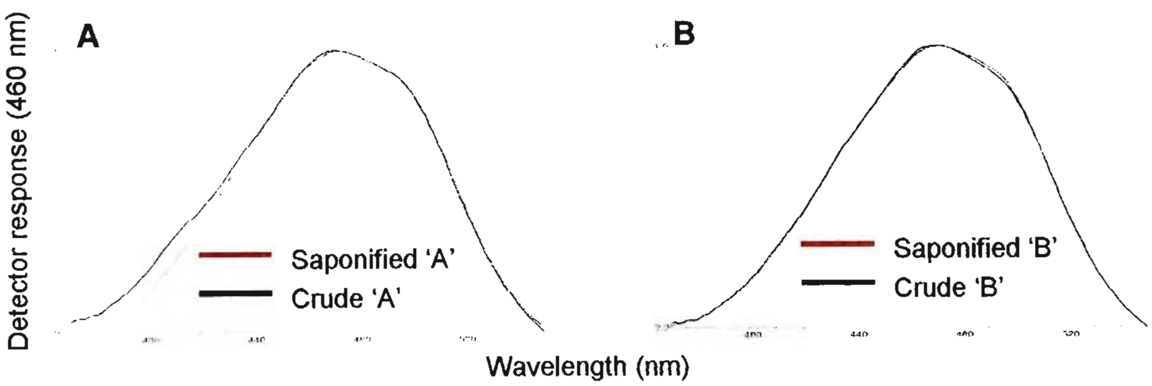

Figure 3.1.3A HPLC absorption spectra, monitored at 460 nm, of saponified and crude band ‘A’.
Figure 3.1.3B HPLC absorption spectra, monitored at 460 nm, of saponified and crude band ‘B’.

The comparison of the spectral characteristics of authentic capsanthin and capsorubin standards with those of the unknown band ‘A’ and ‘B’ show a similarity between ‘A’ and the authentic capsanthin standard and however, there is little a difference between ‘A’ and authentic capsorubin standard (Fig. 3.1.4A). Similarly, the spectral characteristics of saponified ‘B’ are different from that of authentic capsorubin and are similar to that of authentic capsanthin standard (Fig. 3.1.4B).

Saponification of band ‘A’, prior to analysis by rHPLC, yielded one peak less than that of crude band ‘A’ (Fig. 3.1.6B and D) while the retention times of the remaining peaks are slightly reduced (too insignificant, however, to change the positions of the peaks) (Table 3.1.2). The remaining peaks still co-chromatographed with authentic standard of capsanthin (Fig. 3.1.6D). Similarly, the retention times of band ‘B’ (crude extract = 2.941 minutes and saponified extract = 2.936 minutes) did not change due to saponification (Fig. 3.1.5; 3.1.7A and B). Furthermore, the absorption spectra of both crude and saponified band 'A' as well as 'B' are identical (Fig. 3.1.5).

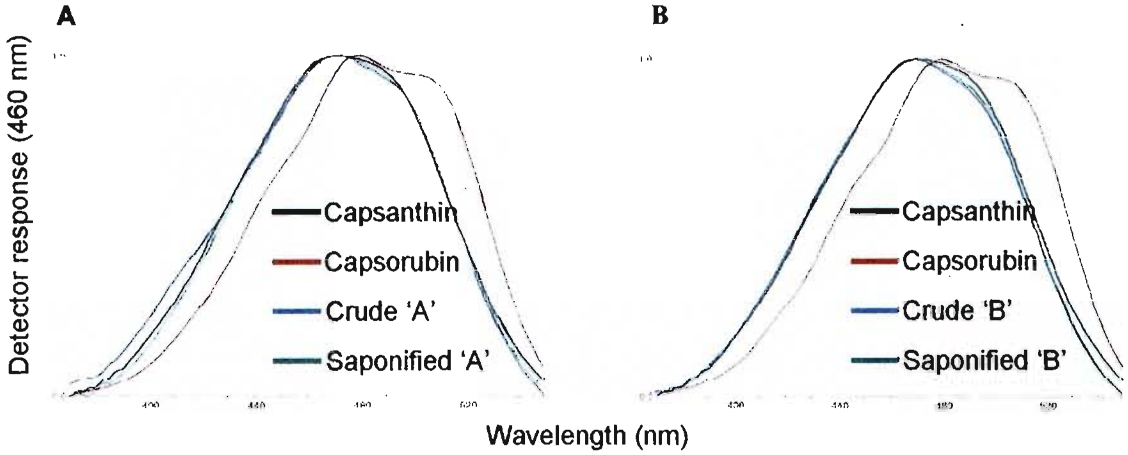

Figure 3.1.4A HPLC absorption spectra, monitored at 460 nm, of authentic capsanthin and capsorubin standard as well as the saponified and crude band 'A'. **Figure 3.1.4B** HPLC absorption spectra, monitored at 460 nm, of authentic capsorubin and capsanthin standard as well as the saponified and crude band 'B'.

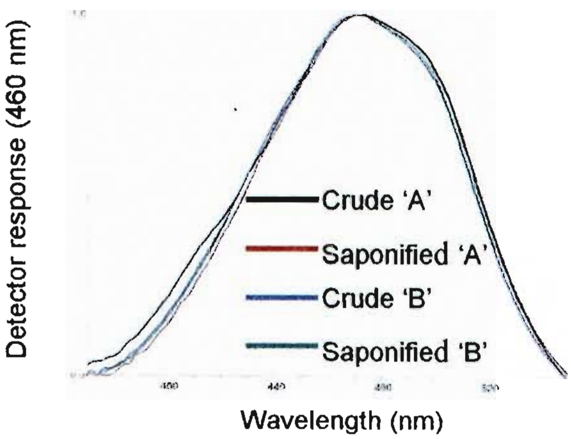

Figure 3.1.5 HPLC absorption spectra of crude band 'A' and 'B' as well as saponified band 'A' and 'B' scraped off TLC plate of red pepper.

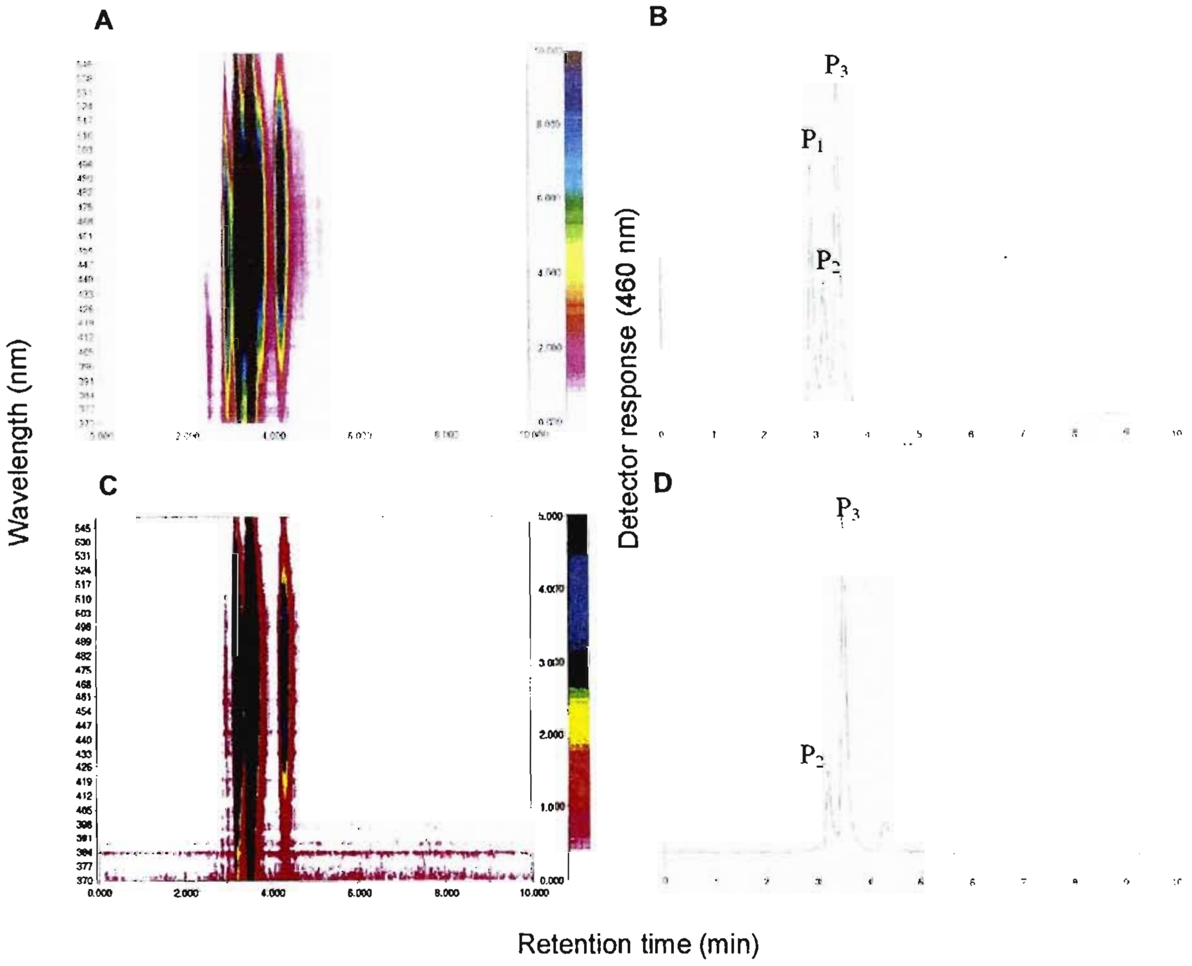

Figure 3.1.6 HPLC isoplots (left) and chromatograms (right), monitored at 460 nm, of crude (A and B) and saponified (C and D) band 'A' (illustrated in Fig. 3.1.2) scraped off TLC plate. 'P' stands for peak.

Table 3.1.2 Retention times of peaks from crude and saponified band 'A' extracted from red-ripe pepper fruit, eluted isocratically with methanol/acetonitrile (9:1, v/v) at 1 mL min^{-1} by the rHPLC system described in Section 3.1.2.5

Peak	**Retention time (min)**	
	Crude extract	**Saponified extract**
P_1	2.948	-
P_2	3.204	3.196
P_3	3.500	3.497

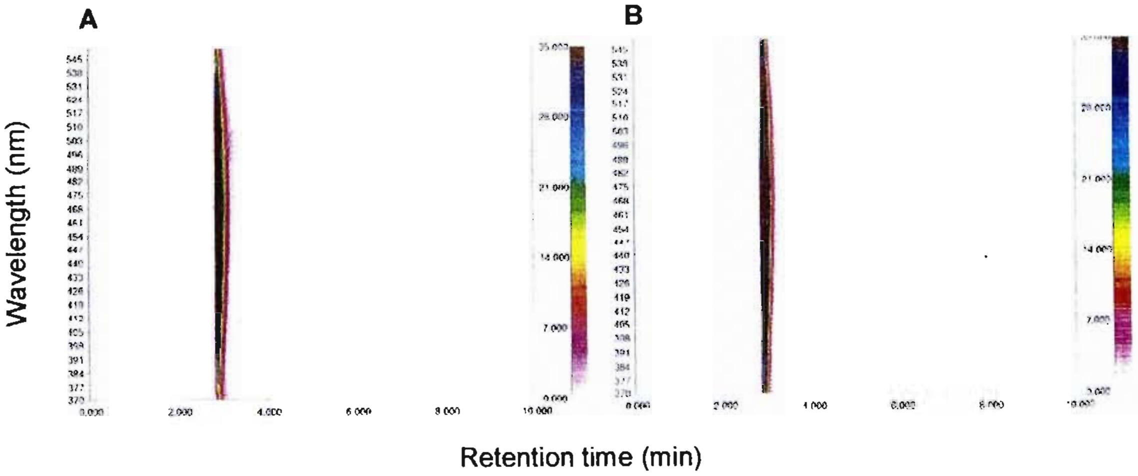

Figure 3.1.7A and B HPLC isoplots, monitored at 460 nm, of crude (left) and saponified (right) band 'B' (illustrated in Fig. 3.1.2) scraped off TLC plate of red pepper.

3.1.4 DISCUSSION

The change in skin colour of pepper fruit during the ripening process from mature-green through colour-break to red-ripe, as illustrated in Figure 3.1.1, ran parallel to the appearance and accumulation of red carotenoids and was inversely related to the presence of green pigments in pepper fruit (Fig. 3.1.2). This suggests that the increasing intensity of the red colour during fruit ripening was, indeed, due to the increasing concentration of certain dominant red pigments. These findings are in agreement with those of Lancaster *et al.* (1997) who showed that pigments, such as chlorophylls, carotenoids and phenolics, are the sole biochemical determinants of skin colour of fruit and vegetables. The presented results also accord with those of McGhie and Ainge (2002), who reported that green fruit do contain yellow and orange carotenoids but in a concentration too insignificant to influence the fruit colour brought about by chlorophylls. However, this suggests that these carotenoids were biosynthesised and accumulated in chloroplasts (Britton and Young, 1993). Furthermore, the carotenoid pattern differed between mature-green and red-ripe fruit; neither of the dominant carotenoids that had been tentatively identified as capsanthin and capsorubin respectively (Fig. 3.1.3A and B), could be identified in mature-green fruit (Fig. 3.1.2).

During the ripening of red *Capsicum* fruit, a massive synthesis of certain ketocarotenoids (mainly capsanthin and capsorubin) (Camara *et al.*, 1995), (structures of which are illustrated in Section 1.4, Fig. 1.4), takes place through the sequential action of several enzymes. Hornero-Méndez *et al.* (2002) affirmed that the intense and characteristic red colour of *Capsicum* fruit is solely due to the ketocarotenoids specific to this genus. Furthermore, the same authors further pointed out that pepper fruit contain C_{40} isoprenoids with nine conjugated double bonds in the central polyenic chain, with different end groups (β, ε, k, 3-hydroxy-5,6-epoxide), which change the chromophore properties of each pigment, resulting in either red or yellow isochromic fractions. Earlier reports by Oren-Shamir *et al.* (1993) and Lefevre *et al.* (1998) had shown that the formation of ketocarotenoids is regulated by four genes, *y*, c_1 and c_2-, which control carotenoid accumulation, and by *cl*- which controls the chlorophyll content in the chromoplast. Entschel and Karrel (1960) reported that ketocarotenoids are formed by a pinacolic rearrangement of the 3-hydroxy-5,6-epoxide end group such that capsanthin is formed from zeaxanthin via antheraxanthin whereas capsorubin is derived from violaxanthin via capsanthin-5,6-epoxide (Section 1.4, Fig. 1.4). These precursors (antheraxanthin, zeaxanthin and violaxanthin) of ketocarotenoids are in turn formed sequencially from β-carotene and β-cryptoxanthin leading to an increase in the red isochromic pigment fraction (Hornero-Méndez and Mínguez-Mosquera, 2000).

The two bands began to appear at the colour-break stage, suggesting a possible role of these compounds in visual fruit colour development from green to brown and later to red. The preliminary results showed a clear correspondense of the R_f values of band 'A' and 'B' with those of the known capsanthin and capsorubin (Mínguez-Mosquera and Hornero-Méndez, 1993, Table 3.1.1) as well as those of authentic capsanthin and capsorubin standards. Therefore, these bands could be tentatively identified as capsanthin and capsorubin. The amount of these carotenoids increased proportionally from colour-break to the red-ripe stage, bringing about an intensification of red colour with ripening. This suggests an important aspect of both bands on visual colour change of *Capsicum* fruit and, hence, their concentration could be used as an indication of ripening stage. This coincided with the reduction in intensity of green bands (chlorophyll), which marked its importance in the final red colour of fruit (Oberholster, 2003). Therefore, these results confirm earlier reports by Davies *et al.* (1970) as well as Hornero-Méndez *et al.* (2002) that red colour in *Capsicum* is due to carotenoid pigments that are synthesised massively during ripening and that the red colour of *Capsicum* fruit is due to the reddish carotenoids capsanthin and capsorubin.

Some researchers (Baranyai *et al.*, 1982; Gross, 1987; Mínguez-Mosquera and Hornero-Méndez, 1993) have reported that most of the pigments of red pepper are present in an esterified form and, therefore, are liposoluble. As occurs in senescent leaves, the synthesis and/or transformation of carotenoid pigments associated with ripening in pepper fruit involve esterification. The first carotenoid esters begin to form at colour-break. Esterification results in increased fruit colour due to the accumulation of xanthophylls in plastoglobules. Deruère *et al.* (1994b) further demonstrated the greater efficiency of esterified xanthophylls in the differentiation of *Capsicum* chromoplasts and their participation in the organization of fibrillar structures responsible for accumulation of carotenoids in the ripe fruit. Esterification also makes xanthophylls more lipophilic and more readily integratable into the lipid-rich plastoglobules. On the other hand, in mature-green fruit all xanthophyll carotenoids are unesterified (Hornero-Méndez and Mínguez-Mosquera, 2000).

Although preliminary results led to the tentative conclusion, based on TLC R_f values, that the pigments 'A' and 'B' are capsanthin and capsorubin respectively rHPLC analysis, however, a most sensitive, accurate and reproducible method for carotenoid identification (Britton and Young, 1993), confirmed identification of only band 'A' as capsanthin. The identity was confirmed by overlaying the absorption spectra of the authentic standards with those of the tentatively identified (on the basis of R_f values) carotenoids. The absorption spectra of both crude and saponified band 'A' and 'B' co-chromatographed well with those of authentic capsanthin standard indicating >99% similarity (Fig. 3.1.4A). In contrast, the results depict that band 'B' was not capsorubin. The absorption spectra of both crude as well as saponified 'B' and authentic capsorubin standard did not overlap very well indicating only less than 83% similarity (Fig. 3.1.4B). However, a significantly high (>98%) similarity among the absorption spectra of both band 'A' and 'B' (crude and saponified) (Fig. 3.1.5) suggests that saponification did not affect their structure. A similar report, although on *Citrus sinensis* 'Navel' and 'Valencia' flavedo, has been given by Oberholster (2001) and can be accounted for by an assumption that esterification did not alter the visual properties, and therefore, perceived colour of carotenoids (Oberholster, 2001). A slight, though insignificant change in retention times of both bands even after saponification might mean that the compounds were not esterified at some stage.

As mentioned earlier, identification of 'B' as capsorubin was later rejected on the basis of rHPLC results. It is not clear why band 'B' had an absorption spectrum only 85% similar to that of authentic capsorubin standard. However, it could be attributed to the influence of a carbonyl group - possibly an in-ring carbonyl group - (due to the hypsochromic shift of a spectral band of <10 nm, Fig. 3.1.4B) in the compound, which results in the loss of a fine structure of a pigment. The spectrum of such a compound is characterized by only one broad absorption maxima (Gross, 1987). The opposite effect can possibly be obtained upon reduction of the carbonyl group, which will result in a typical fine structure of a carotenoid. In support of this, Gross (1987) further indicated that the reduction of carbonyl group of the common apocarotenoid citraurin could result in the formation of a fine structured (with visible shoulders) carotenoid citraurol. Another possibility is that carotenoid 'B' is a product of degradation of a ketocarotenoid, which is characterised by a loss of certain fragments of carbon skeleton from one or two ends of the parent pigment, either during extraction or purification by TLC, resulting in structural modification. The latter postulation is also supported by a report by Britton and Young (1993), who indicated that the formation of artifacts such as apocarotenoids and epoxides is possible in the presence of oxygen rich environments. However, it is questionable whether this could have materialized considering the stringent precautions that have been taken (described in Sections 2.1.5, 2.1.6, and 3.1.2.4) during handling, processing and purification of the extract to avoid the presence of an oxidizing environment. Alternatively, it might be that the formation of certain complexes of the pigment with proteins resulted in a change in Rf value, which would not result in an alteration of the absorption spectrum.

3.1.5 CONCLUSIONS AND FUTURE AREAS OF STUDY

This study confirms that, apart from chlorophylls, yellow and orange carotenoids are biosynthesised and accumulated in green pepper fruit. In addition to that, it is demonstrated that red colour-imparting carotenoids surface at the onset of ripening and play an important role in breaking the green fruit colour of *Capsicum*. Furthermore, the brown colour of *Capsicum* at the colour-break stage and the red colour at maturity are due to an increase in the amount of the dominant red carotenoids capsanthin and possibly capsorubin, and are, therefore, correctly called the only 'red colour-imparting' carotenoids of the red pepper cultivar Capistrano. In addition to that more knowledge is needed to focus on the ratio of ketocarotenoids to epoxy-xanthophylls and how manipulation of environmental factors and/or plant management practices can affect pepper fruit carotenoid concentration as well as the proportion of yellow to red isochromic fractions of pepper fruit. Furthermore, any further study can focus on means of regulating the concentration of these two colour-imparting compounds for paprika production.

MANIPULATION OF PIGMENTS IN PEPPER FRUIT

3.2 INFLUENCE OF CERTAIN TEMPERATURE REGIMES ON COLOUR DEVELOPMENT OF PEPPER FRUIT

3.2.1 INTRODUCTION

Climatic conditions can influence fruit quality (Jackson and Lombard, 1993) by affecting the physiological processes of plants. Krajewski (1996) indicated that, although several factors are involved in colour development, temperature seems to be of paramount importance. In accordance with Krajewski (1996), Somogyi *et al.* (2000) reported that the impact of temperature on pigment accumulation in *Capsicum annuum* is three-fold greater than that of any other climatic factor. Additionally, temperature has an effect on flowering, fruit set and fruit development of sweet peppers (Polowick and Sawhaney, 1985; Bakker, 1989).

Together with size, colour is a prime fruit quality determinant of the market value of any horticultural commodity (Türk *et al.*, 1994). Colour in fruit and other plant parts is imparted by colour pigments such as chlorophylls, carotenoids (Mínguez-Mosquera and Garrido-Fernandez, 1983) and flavonoids (Markakis, 1982). Noordegraaf and Wells (1995) reported that under low air temperature physiological processes in plants are negatively affected, which may result in poor fruit colour and slow ripening while high temperatures may accelerate fruit ripening and, therefore, possibly colour expression.

Wells (1967) showed that an optimal day/night temperature for pepper is 25/18°C. Moreover, Somogyi *et al.* (2000) indicated that this optimal temperature regime (25/18°C) two to three weeks prior to physiological maturity (red-ripe stage) might increase carotenoid accumulation in pepper fruit. Contrary to that, Bakker (1989) reported that pepper grows well under 23/18°C (day/night) temperature regime. In temperate regions, plants experience seasonal temperature changes to below optimum values during the production season. It was, therefore, hypothesised that seasonal temperature changes (from summer to fall) might have a considerable influence on pigment concentration in pepper fruit as compared to more tropical conditions. *Capsicum* pepper is a perennial crop in the tropics even though it can be grown in temperate areas as an annual crop (Bosland, 1992). Consequently, the objectives of this investigation were to: firstly, investigate the effect of the 'optimal' 23/18°C (day/night) (Bakker, 1989) temperature cycle, on carotenoid accumulation in peppers, and, secondly, to determine whether the transfer of pepper plants from relatively high and fluctuating temperatures (plastic tunnel) at various developmental stages to a stable 23/18°C day/night regime (growth room) affects fruit carotenoid content.

3.2.2 PLANT MATERIAL AND ENVIRONMENTAL CONDITION

Bell pepper (*Capsicum annuum* L. cv. Capistrano) seedlings were germinated in speedling® trays in a plastic tunnel (PT) at the University of Natal and transplanted when they had six true leaves into 25 cm-diameter plastic pots containing a mixture of composted pine bark (GROMOR, Camperdown, RSA) and sand (2:1 v/v). The moisture content of the growing medium was measured daily and maintained at container capacity throughout the growth period with the help of 15 cm-depth tensiometers (Irrometer®, Riverside, California, USA). Seedlings were grown and transferred from the PT to the growth-room (GR) at various stages of development. During the experiment in 2001/2002 summer season the average daily temperature in the PT was determined and it ranged from 20°C (min) to 30°C (max), the average light intensity was 500 Wm^{-2} (≈ 90 $\mu mol\ m^{-2}\ s^{-1}$ [PAR]). The average sunshine hours per day were six (South African Weather Service, 2002). The conditions in the GR were set to 23/18°C (day/night) 60% RH, 14/10 hr photoperiod (day/night) and light intensity of 335 $\mu mol\ m^{-2}\ s^{-1}$ (PAR). Fluorescent tubes (L58W/77; 02250IM (OSRAM, Berlin, Germany) and 100W incandescent (Top bulb, Indiana Boulevand, Chicago, USA) light bulbs were used to provide light. Light intensity was measured with a Campbells Scientific Quantium Sensor (LI-COR, Lincoln, NE).

Table 3.2.1 Duration pepper plants were subjected to a constant 23/18°C (day/night) temperature regime in the growth room

Developmental stage at exposure to optimal temperature	Days of exposure to optimal temperature regime		
	Evaluation time		
	49 DAPF	56 DAPF	63 DAPF
Control (never)	0	0	0
Vegetative	>49	>56	>63
20 DAPF	29	36	43
30 DAPF	19	26	33
40 DAPF	9	16	23

DAPF : number of days after first petal fall. Plants subjected to 23/18°C starting at the vegetative stage took 25 days (from exposure time) to reach first petal fall (approximately 50 to 60 days from transplanting day). Therefore this value can be added to values of vegetative stage per evaluation time in order to find total number of days plants stayed in the growth room.

Plants were grown in the PT and transferred at four different developmental stages, namely, vegetative = 14 days after transplanting, 20, 30 and 40 days after first petal fall [DAPF] to the GR where they were kept for various durations (Table 3.2.1) till the last harvest (over-ripe) while control plants stayed in the PT until the last harvest. At 49, 56, 63 DAPF the number of fruit which had reached colour-break (a combination of chlorophyll and carotenoid concentration and is normally brownish) and red-ripe fruit on each plant was counted. Each treatment was replicated six times and each individual plant was considered a replicate.

Pigment extraction, spectrophotometric determination of pigment concentrations and statistical analyses of data in this study and the subsequent ones were performed as described in Sections 2.1.5, 2.1.7 and 2.1.11, respectively.

The same number (two per plant) of pepper fruit were initially harvested from all plants when mature-green (stage 1) and red-ripe (stage 2 = approximately 15 days post the mature-green stage) (Fig. 3.2.1). The carotenoid concentration in red-ripe fruit from plants grown under constant 23/18°C from the vegetative stage onwards was significantly lower than in fruit from plants grown in the PT up to physiological maturity. The effect of optimal temperature on visual colour development and carotenoid accumulation was assessed further by leaving the remaining fruit on the plant, after 'stage 2' harvest, till over-ripe (stage 3= approximately 20 days post the mature-green stage). Hornero-Méndez *et al.* (2002) have demonstrated that keeping fruit till the over-ripe stage is a way of determining the maximal carotenogenic potential of fruit.

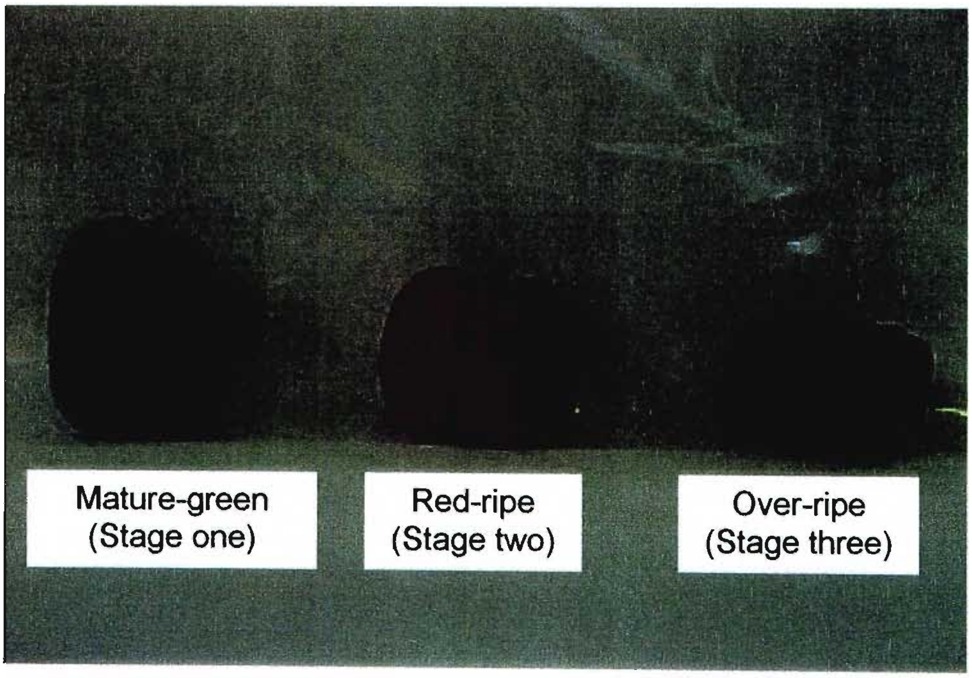

Figure 3.2.1 Ripening stages of pepper fruit.

3.2.3 RESULTS

A remarkable increase in carotenoid concentration (45% of carotenoid concentration of red-ripe fruit) was determined in fruit at the over-ripe stage from plants exposed to 23/18°C at the vegetative stage while the increase in carotenoid concentration of fruit from plants exposed to the 23/18°C regime 20, 30 and 40 DAPF was not significant (Table 3.2.2). At both ripening stages (2 and 3) carotenoid concentration decreased with each delay in transfer of pepper plants to the optimal temperature in the GR. The lowest fruit carotenoid concentration was noted from plants transferred 40 DAPF to GR conditions and the highest at the red-ripe stage was from the control plants while those transferred at the vegetative stage yielded the highest at the over-ripe stage. However, the difference in carotenoid concentration of over-ripe fruit from plants transferred 20 to 40 DAPF was not statistically significant.

Table 3.2.2 Fruit carotenoid concentration at the red-ripe and over-ripe stage

Transfer stage	**Carotenoid concentration of pepper fruit (µg/g fresh tissue mass)**	
	Red-ripe	**Over-ripe**
Control (never)	193.0^{a}	199.0^{b}
Vegetative	160.0^{b}	231.6^{a}
20 DAPF	161.1^{b}	164.0^{c}
30 DAPF	159.3^{b}	162.4^{c}
40 DAPF	150.2^{b}	153.4^{c}

Values followed by letters denote significant differences at five percent. $LSD_{0.05}$= 17.09.

For the purpose of a clear presentation only the pigments of mature-green and over-ripe fruit will be discussed.

3.2.3.1 Visual colour change as affected by transfer to optimal temperatures at various stages of development

The transfer of pepper plants at various developmental stages to optimal growing temperatures had a significant influence ($P< 0.001$) on the visual colour change of fruit. The number of fruit reaching colour-break from control plants and those transferred at either the vegetative stage, 20 or 30 DAPF to 23/18°C was significantly lower when evaluated 49 and 56 DAPF. On the later evaluation date, 63 DAPF, however, only control plants and those transferred to optimal temperature at the vegetative stage had a lower number of red-ripe fruit compared to other treatments.

Figure 3.2.2 indicates that delaying the transfer of pepper plants (to 40 DAPF) to the optimal temperature regime, even though the duration of exposure to optimal

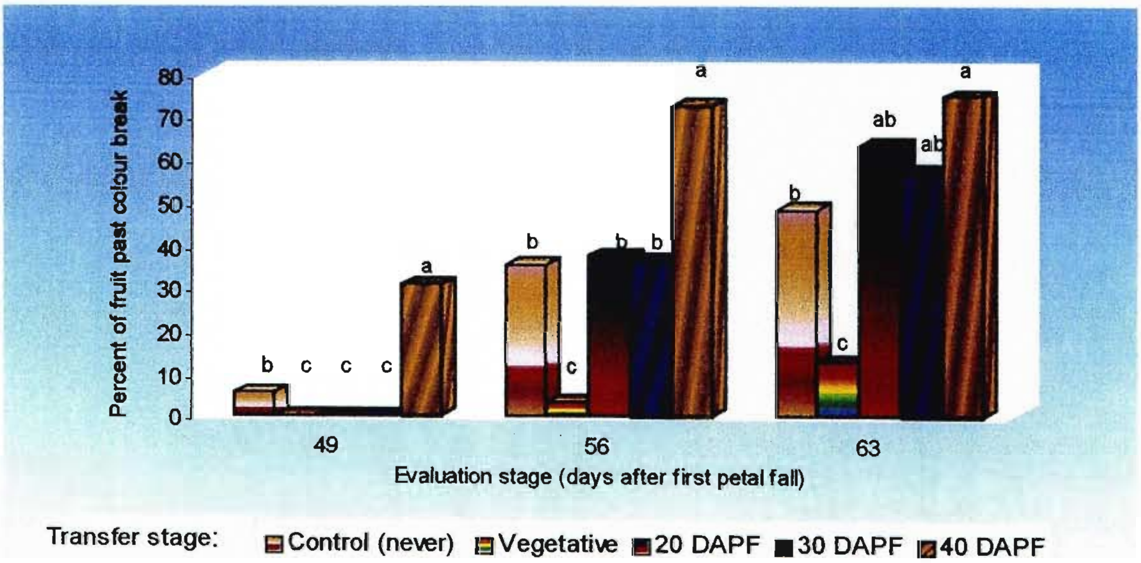

Figure 3.2.2 Effect of transfer of pepper plants to optimal growing temperature on fruit colour change. Plants were transferred from the PT to the GR at the vegetative stage, 20, 30 and 40 days after first petal fall (DAPF). Control plants were kept in the PT from seedling to fruit physiological maturity. Bars per a given period (days) with different letters indicate statistical significant difference. $LSD_{0.05}$= 19.95.

conditions was shorter (Table 3.2.1), resulted in a significantly higher percentage of fruit reaching colour-break earlier when evaluated 49 to 63 DAPF compared to control and other treatments.

3.2.3.2 Chlorophyll accumulation in pepper fruit as affected by exposure to optimal temperatures at various stages of development

Subjecting pepper to a 23/18°C temperature regime at various developmental stages had a significant impact ($P< 0.001$) on chlorophyll concentration in pepper fruit. Exposure of plants to the optimal temperature regime from the vegetative stage onwards resulted in a higher chlorophyll concentration of mature-green fruit (Fig. 3.2.3), than when plants were subjected to these conditions either starting from 20, 30 or 40 DAPF. At the over-ripe stage, however, the chlorophyll concentration had decreased to about one quarter of the mature-green fruit and no significant differences in chlorophyll concentration among the treatments were detected.

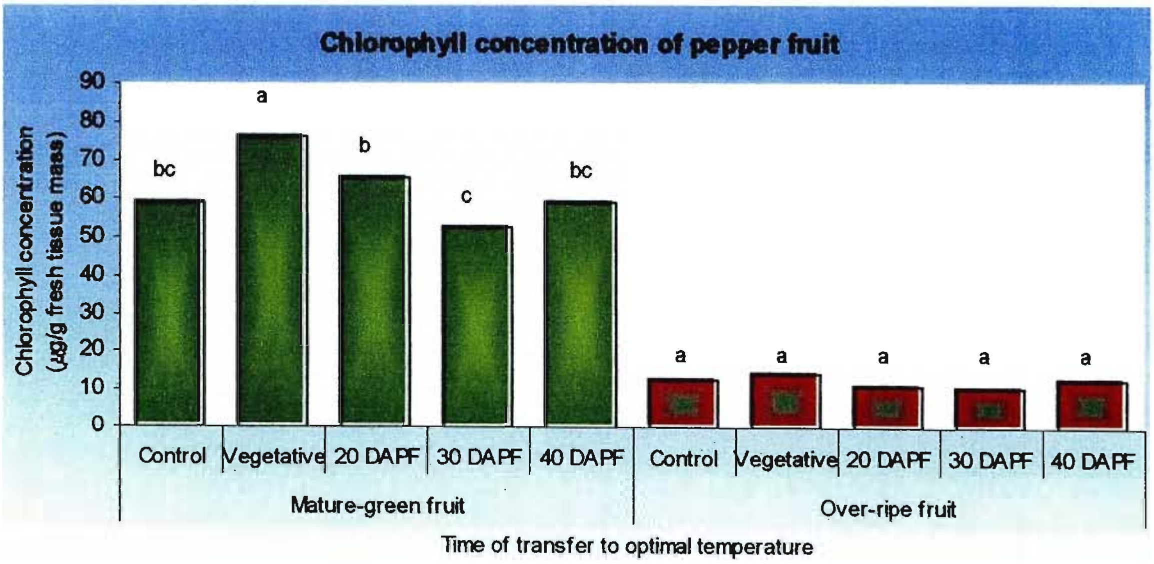

Figure 3.2.3 Effect of transfer at various stages of development of pepper plants to optimal temperature in relation to fruit chlorophyll concentration. Fruit were harvested at either the mature-green or the over-ripe stage. Bars per maturity stage sharing similar letters indicate insignificant difference. $LSD_{0.05}$= 17.05.

3.2.3.3 Carotenoid concentration in pepper fruit as influenced by transferring plants to optimal growing temperatures at various developmental stages

The carotenoid concentration of mature-green fruit among the various treatments was not different while the accumulation of carotenoids in the over-ripe fruit seemed to be affected by the time of transfer to optimal conditions. Subjecting plants to 23/18°C already at the vegetative stage resulted in the highest fruit carotenoid concentration (Fig. 3.2.4) relative to those kept under a temperature range of a wider amplitude (control). Transferring pepper plants to optimal temperature conditions at the late fruit developmental stages, 20 DAPF or later, resulted in a reduced carotenoid concentration to a level below that of the control. Total carotenoid concentration increased as fruit developed from mature-green to over-ripe, a trend opposite to that of total chlorophyll content.

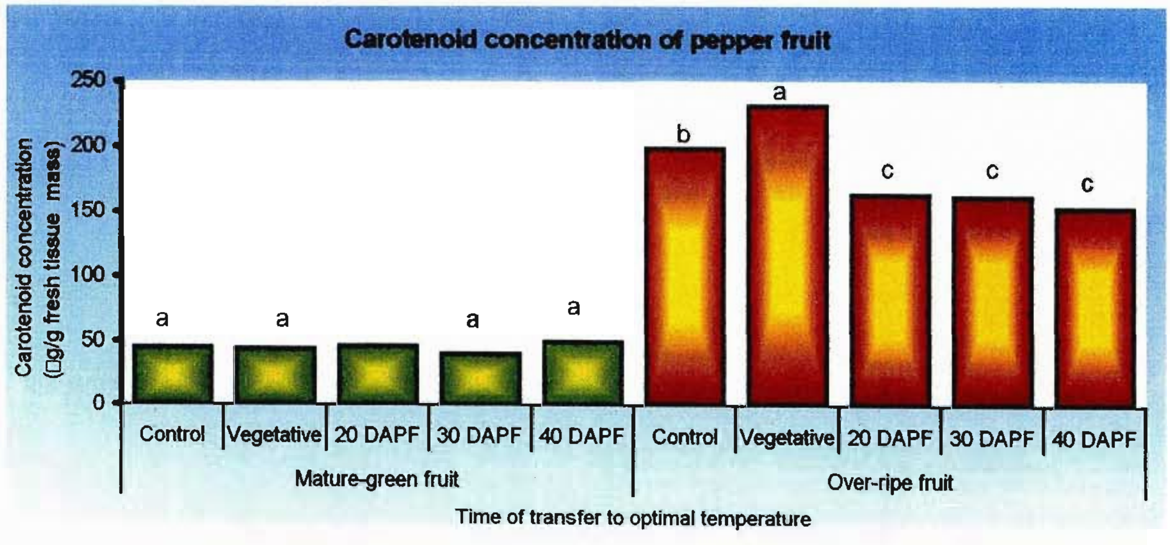

Figure 3.2.4 Effect of transfer of pepper plants to optimal temperature on carotenoid concentration. Fruit were harvested at either the mature-green or the over-ripe stage. Bars per maturity stage sharing the same letters indicate statistically insignificant difference. $LSD_{0.05}$= 7.79.

3.2.4 DISCUSSION

The outcome of this study accords with reports by Hurd and Graves (1985) in that transferring plants from high and fluctuating temperatures to relatively lower and constant temperatures at an early developmental stage (vegetative stage) resulted in prolonged time to maturation (Fig. 3.2.2). Hence, transferring pepper plants from relatively warm but highly fluctuating temperatures (ranging from 20 to 30°C) to relatively cool and stable optimal (23/18°C) temperatures resulted in a prolonged time to colour change of pepper fruit. This (delayed maturity) may be linked to a lack of substrates for carotenoid biosynthesis or a delayed channeling of isoprenoid pathway metabolites into fruit carotenoid biosynthesis under non-optimal conditions. This shows that fruit ripening might have provided the opportunity to accumulate more carotenoids in the fruit than relatively shorter ripening times (transfer 20, 30 or 40 DAPF). Furthermore, Figure 3.2.2 indicates that the transfer of plants to optimal growing temperatures 40 DAPF could reduce the time to maturity of pepper fruit, possibly due to stress developing as a result of a sudden change of temperature. The decrease in aerial temperature to optimal temperature (23/18°C) at that stage may fast-forward physiological processes (chloroplast-chromoplast transformation), hence fast visual fruit colour change. These data confirm Somongyi's (2000) results that an optimal growing temperature, two to three weeks before the ripening stage which would coincide with the 40 DAPF treatment in our experiment (Fig. 3.2.2), may impact positively on colour change in pepper fruit.

The enhanced rate of physiological maturity of fruit on plants transferred to 23/18°C 40 DAPF compared to fruit on plants transferred either earlier or not at all (Fig. 3.2.2) may be attributed to the enhancement of the action of gene encoding ripening related proteins, whose expression occurs prior to, or concomitant with, colour-break stage (Briggs *et al.*, 1986; Schuch *et al.*, 1989). This would, in our experiments, mean, that the exposure of pepper plants to optimal temperature at the horticultural maturity stage (mature-green) might speed up chlorophyll degradation and/or carotenoid synthesis in fruit and hence fast-forward colour change. This emphasizes that optimal growing temperatures are a means to influence the pigment concentration in pepper fruit. A sole effect on chlorophyll degradation would contrast with results of Noordegraaf and Wells (1995) who found that high temperatures accelerate degreening of pepper fruit. Furthermore, the response to exposure to a certain temperature regime may be varying with cultivar and species.

Exposing pepper plants to 23/18°C from the vegetative stage onwards resulted in the highest chlorophyll concentration of mature-green fruit. This may be due to more chloroplast organelles in fruit tissues. The same treatments resulted in the highest carotenoid concentration of over-ripe fruit, most likely due to an increased ability to synthesize carotenoids compared to shorter exposure periods to this optimal temperature regime (Fig. 3.2.4). Alternatively, the presumed high number of chloroplasts in mature-green fruit from the same treatment might have been transformed into chromoplasts, thus resulting in more carotenoid accumulation sites for a significantly higher carotenoid concentration in over-ripe fruit. As the visual colour change of pepper fruit, from green to red or yellow, is correlated to accumulation of carotenoids in chromoplasts within fruit, the carotenoid concentration is indicative of the internal quality of the fruit, because it shows how much carotenoids have been synthesized by the fruit (Madrid *et al.*, 1999).

The conclusion can, therefore, be drawn that exposure of pepper plants to optimal temperature condition (23/18°C) may fast-forward fruit carotenoid formation. The timing of transfer to optimal temperature is therefore more important than the duration of time exposed to optimal temperature regime. Similarly, Adams *et al.* (2001) and de Koning (1994) expressed the view that the sensitivity of tomato fruit to temperature, in relation to parameters such as fruit development and dry matter distribution, increases in mature-green fruit. Furthermore, de Koning (1994) indicated that, although tomato fruit sensitivity to temperature is dependent on the developmental stage, the middle stage is less sensitive. Similarly, transfer of pepper plants to optimal growing temperature 20, 30 and 40 DAPF neither affected chlorophyll concentration of mature-green nor carotenoid concentration of over-ripe fruit positively (Fig. 3.2.3 and 3.2.4). Figure 3.1.2 additionally reveals that the exposure of plants to GR conditions starting at the vegetative stage resulted in a delay of fruit colour change compared to later exposure to the optimal temperature regime. De Koning (1994) concluded that in tomato, relatively high temperatures post anthesis, during cell division, drastically hastens the ripening process. The late (40 DAPF) transfer to optimal temperature might have the highest stress effect on fruit resulting in fast-forwarding of physiological processes such as chloroplast degradation.

The present study affirms that colour change of pepper fruit was a result of a change in pigment spectrum, which at the mature-green stage comprised, predominantly, chlorophylls and to a lesser extent, carotenoids. As the fruit matured the chlorophyll concentration decreased and carotenoid concentration increased. Similarly, Brady (1987) showed in tomato, that there is an inverse relationship between chlorophyll concentration and ripening.

Contrary to chlorophyll, a continuous increase of the total carotenoid concentration was noted in fruit from all treatments, from the mature-green to over-ripe stage. The same trend has been previously observed in fruit of yellow gooseberry (Gross, 1982), 'Shamouti' orange (Rotstein *et al.*, 1972) and 'Dancy' tangerine (Gross, 1982b). Exposing pepper plants to 23/18°C from the vegetative stage onwards increased the ability of the plants to synthesise carotenoids. This accords with the work of Britton and Hornero-Méndez (1997) who reported that in most cases carotenoid concentration in fruit and vegetables will be greater under conditions that favour growth. This might imply that a 23/18°C regime did not have a stress effect on plants, which could cause a fast chlorophyll degradation and consequently fast degreening of fruit. On the other hand, if the exposure to optimal temperature was delayed to 20, 30 or 40 DAPF the ability of the fruit to accumulate carotenoids was reduced. The later two points might suggest that the visual skin colour change of a fruit might not necessarily reflect the pigment concentration of a fruit, even though there was a correlation between the skin colour and pigment concentration. Alternatively, it might suggest that exposing plants longer to a 23/18°C regime, even though it seemed it was an ideal temperature for optimal carotenoid biosynthesis (Fig. 3.2.4) did not impact positively on chlorophyllase, which is involved in the destruction of chlorophyll during fruit ripening (Ihl *et al.*, 1999), hence a delay in degreening of fruit (Fig. 3.2.2).

3.2.5 CONCLUSION AND FUTURE AREAS OF RESEARCH

In conclusion, the exposure of pepper plants to optimal growing temperatures affect pigment accumulation and this effect depend on the developmental stage of the fruit at the time of exposure. Transfer of plants to the optimal growing temperatures very late (40 DAPF) in fruit development may enhance maturity of pepper fruit. Furthermore, optimal growing temperatures from the vegetative stage onwards result in the highest accumulation of carotenoids in pepper fruit. Transferring plants from a high and fluctuating to an optimum temperature level, at the very late stage of development (40 DAPF), accelerate degreening of pepper fruit (Fig. 3.2.2). These results support the hypothesis of this study that seasonal temperature changes might impact positively on the visual colour change of pepper fruit. Therefore, fruit maturity can be enhanced when plants are exposed to optimal environmental conditions at or shortly after horticultural maturity. In mature-green fruit there was no sign that the carotenoid concentration of fruit was increased significantly for photoprotective purposes, as there was no significant difference in carotenoid concentration of mature-green fruit from all treatments. However, transfer from high to optimal growing temperatures or irradiance in the GR might have affected colour development. Further study is, therefore, needed to determine if these results are essentially attributed to a change in growing temperature or whether the illumination to which fruit are exposed is an important factor involved. Finally, in order to determine the exact developmental stage most sensitive to a change in temperature, plants should be subjected to the optimal temperature regime at any phenological stages for equal durations, starting at either the vegetative, first flowering or first fruit set stage.

In a nutshell this research indicates that the optimal growing temperature is needed for the highest carotenoid accumulation in pepper fruit, hence the best fruit quality. In addition to that it shows that supra-optimal temperatures may negatively affect carotenoid concentration in fruit.

3.3 INFLUENCE OF P, K, AND MG ON FRUIT PIGMENT CONCENTRATION OF *CAPSICUM ANNUUM* L. cv. *Capistrano*

3.3.1 INTRODUCTION

Nutrient availability can have an influential role in pigmentation of plants (Goodwin and Goad, 1970). The impact of mineral nutrition on pigment accumulation has been investigated in various plant species. It is generally agreed that high nitrogen fertilisation increases chlorophyll concentration in fruit such as pepper (Miller, 1960), citrus (Jones and Embleton, 1959; Huff, 1983), and apricot (Kotze and de Villiers, 1991). Information on the effect of other macronutrients such as P, K and Mg on colour development in fruit, and particularly pepper fruit, is scarce. However, as mentioned in chapter one (Section 1.4.2.3), high K levels can have either a promotive effect on colour development, as in tomato and apricot, or an inhibitory one, as in citrus. Owing to the fact that Mg is a component of chlorophyll, its deficiency results in poor green colour of vegetative plant parts (Marschner, 1995). But does Mg have an influence on colour development in pepper fruit? This should be investigated. Existing evidence indicates that an insufficient Mg content in plants such as rape may result in a decrease in plastid carotenoids (Baszynski *et al.*, 1980). The objective of this study was, therefore, to determine the effect of different levels of P, K, and Mg in the growing medium on chlorophyll and carotenoid accumulation in pepper fruit.

3.3.2 PLANT MATERIAL AND GROWTH CONDITIONS

The experiment was laid out as a randomised complete block design with the three nutrients (P, K and Mg) as variables and each nutrient supplied at four levels. The experiment comprised of four replicates and two plants per treatment level per replicate. 'Capistrano' bell pepper seedlings were established in speedling® trays filled with composted pine bark (GROMOR, Camperdown, RSA) in a plastic tunnel (PT). The moisture content of the growing medium was monitored and maintained at container capacity with the help of 15 cm-depth tensiometers (Irrometer ®, Riverside, California, USA). During the experiment the average temperature in the PT ranged from 20°C (min) to 30°C (max). The seedlings were transplanted when they had six true leaves into 25 cm-diameter plastic pots filled with a mixture of sand and vermiculite (2:1, v/v). The experiment was conducted from Spring 2001 to Summer 2002. The plants were irrigated four times a day with tap water by means of an automated sprinkler system.

N and Ca were supplied at medium (optimum) level (18.2 and 516 Kg ha^{-1} respectively) according to Manson *et al.* (2000) while P, K, and Mg were supplied at the following levels: 'very low' (no further application), 'low', 'medium' and 'high' (actual amount of P, K, and Mg applied in the medium as in Table 3.3.1). The 'medium' level was used as control, as it was based on the recommended level of the specific nutrient according to FERTREC system (Manson *et al.*, 2000).

Table 3.3.1 Amount of nutrients applied to the medium to achieve certain nutrient levels

Medium nutrient content	**Multiple of Recommended rate**	**Nutrient applied**					
		Phosphorus[1]		**Potassium**[1]		**Magnesium**[1]	
		kg ha^{-1}	**N:P ratio**	**kg ha^{-1}**	**N:K ratio**	**kg ha^{-1}**	**N:Mg ratio**
'Very low'	0	0.0	1:0	0.0	1:0	0.0	1:0
'Low'	1	3.6	1:0.2	5.0	1:0.3	18.2	1:1
'Medium'[2] (Recommended)	5	18.0	1:1	25.2	1:1.4	91.0	1:5
'High'	25	90.0	1:1.5	126.0	1:6.9	455.0	1:25

[1] Sources of elements: P as $(NH_4)_2$ PO_4 , K as K Cl and Mg as Mg O. The amount of nitrogen added with the P application was taken into consideration when adding the nitrogen (at 'medium' level) source. The recommended amount of nitrogen applied to the medium and also used for calculating the ratios was 18.2 Kg ha^{-1}. [2] 'Medium' was used as control as it is equivalent to the recommended soil level (Manson, 2000).

With the exception of the element varied, the other two were supplied at the 'medium' level. All plants were fertigated with a complete N: P: K solution (1450, 1080 and 1510 mg/L respectively) twice every week from transplanting to first flower opening after which the actual application of various levels was initiated. Urea was used as a nitrogen source. Ammonium phosphate was used as a source of N and P while potassium chloride was a source of K. According to soil analyses recommendation (Manson *et al.,* 2000) no additional micronutrient (Zn, B, Fe, Cu, Mn and Mo) or lime was required. The treatment application was initiated at the flowering stage, a stage when the nutrient uptake by pepper plants increases (Hector and Mills, 1991). Fruit were harvested at the mature-green as well as the red-ripe stage.

3.3.3 RESULTS

3.3.3.1 Effects of P, K and Mg nutrients on total chlorophyll concentration of pepper fruit

The application of either P, K or Mg to the medium at a 'low' level resulted in a higher chlorophyll concentration of mature-green pepper compared to 'very low', 'medium' or 'high' levels of these nutrients (Fig. 3.3.1). Only 'medium' (control) level of P resulted in lower fruit chlorophyll concentration at the mature-green stage than the 'low' P, while 'medium' application of K and Mg resulted in the lowest chlorophyll concentration of mature-green fruit. The results furthermore show that K concentration at sub-optimal ('very low', 'low') level resulted in a higher fruit chlorophyll concentration than 'medium' (control) K application. Further addition of K did not influence the chlorophyll concentration positively. Mature-green fruit from plants supplied with 'low' or 'high' levels of Mg had a significantly higher chlorophyll concentration than fruit of plants supplied with 'medium' Mg level.

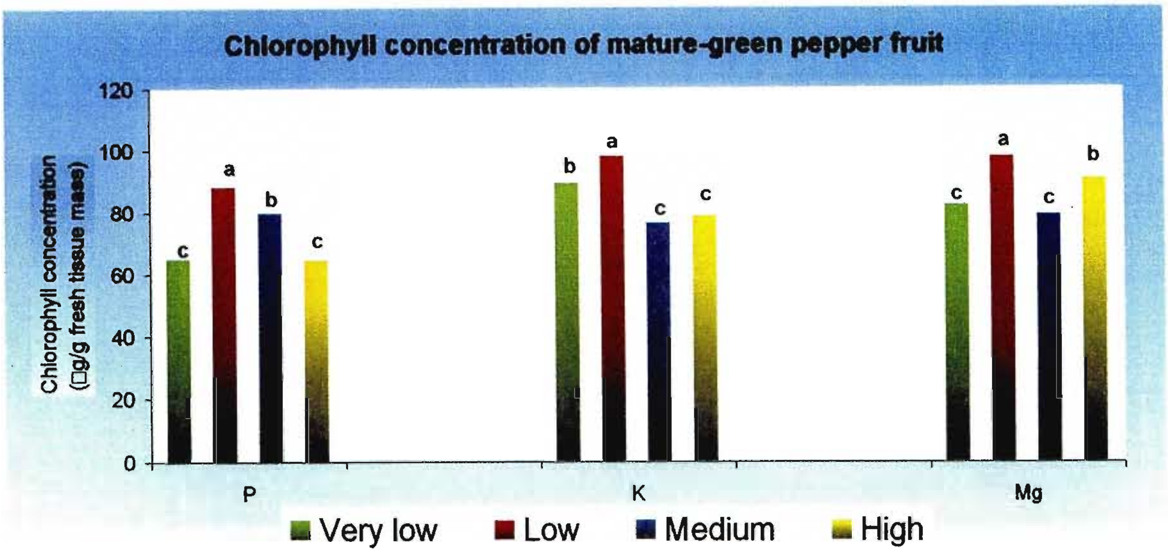

Figure 3.3.1 Chlorophyll concentration of mature-green pepper fruit as influenced by varying P, K, and Mg supply. Levels of nutrients applied as stated in Table 3.2.1. Data are the means of 12 fruit per treatment from four replicates (two plants per replicate). Bars, per nutrient, with different letters within the nutrient treatment indicate significant differences. $LSD_{0.05}$= 4.7.

The chlorophyll content of red-ripe fruit (Table 3.3.2) was very similar in the various treatments and decreased to less than one third of the concentration in mature-green fruit. With the exception of Mg supplied at 'very low' level, which resulted in a statistically lower chlorophyll concentration, the supply of K and P did not seem to affect the chlorophyll concentration of red-ripe fruit (Table 3.3.2).

In general, application of P, K and Mg at low levels (N: P, N: K, N: Mg ratios being 1.02, 1.03 and 1.1 respectively) resulted in the highest chlorophyll concentration at the mature-green stage in pepper fruit (Table 3.3.2).

Table 3.3.2 Chlorophyll concentration of red-ripe fruit as affected by varying P, K and Mg medium level

Nutrient	Chlorophyll concentration (μg/g fresh tissue mass)				
	'Very low'	'Low'	'Medium'	'High'	Main treatment effects
P	20.53^{a1}	20.17^{a}	19.78^{a}	22.14^{a}	n.s
K	18.48^{a}	18.96^{a}	17.25^{a}	17.29^{a}	n.s
Mg	14.33^{b}	19.26^{a}	16.75^{a}	19.12^{a}	$P< 0.05$

$LSD_{0.05}= 4.7$.

[1] Different letters within a row denote significant differences between fruit carotenoid concentration. Each value represents the mean (carotenoid concentration) from 12 fruit per treatment level

3.3.3.2 Effects of selected macronutrients (P, K and Mg) on concentration of total carotenoids in pepper fruit

The carotenoid concentration of mature-green fruit (Table 3.3.3) was, as anticipated, very much lower than of red-ripe fruit (Fig. 3.3.2). No significant differences in carotenoid concentration of mature-green fruit were found at the various P or Mg levels of mature-green fruit, however, 'very low' K resulted in a significantly higher carotenoid concentration than application to a 'medium' level (Table 3.3.3).

Table 3.3.3 Carotenoid concentration in mature-green fruit as affected by nutrient levels

Nutrient	**Carotenoid content (μg/g fresh tissue mass)**			
	'Very low'	**'Low'**	**'Medium'**	**'High'**
P	46.08^{a}	51.27^{a}	46.71^{a}	46.22^{a}
K	52.40^{a}	51.48^{ab}	45.07^{b}	46.47^{ab}
Mg	47.14^{a}	50.38^{a}	48.26^{a}	50.43^{a}

LSD = 5.6.

[1] Different letters within a row denote significant differences between fruit carotenoid concentration. Each value represents the mean (carotenoid concentration) of twelve fruit per treatment level. Levels of nutrients applied to growing medium are as presented in Table 3.3.1

The total carotenoid concentration of red-ripe pepper fruit was, however, affected by the nutrient levels with the exception of P, which did not have an effect on the carotenoid concentration (Fig. 3.3.2). An increase in K level from a 'very low' to either a 'low' or a 'medium' level resulted in a significant increase in the fruit carotenoid content, while a 'high' K application decreased the fruit carotenoid concentration to a level similar to the 'very low' K application. Progressively increasing the Mg level, however, was mirrored by similar increase in the fruit carotenoid concentration. Finally, none of the three macronutrients - or their levels - altered the relationship between chlorophyll and carotenoid concentration in both, mature-green and red-ripe pepper fruit.

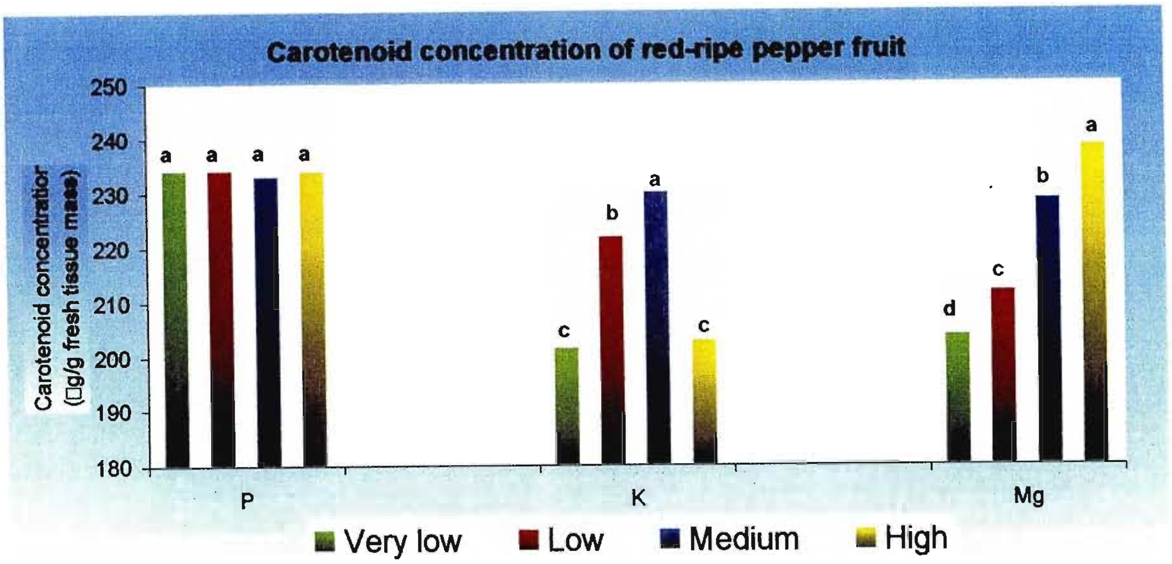

Figure 3.3.2 Carotenoid concentration of red-ripe pepper fruit as influenced by varying P, K, and Mg supply. Levels of nutrients supplied as stated in Table 3.2.1. Data are the means of 12 fruit per treatment from four replicates (two plants per replicate). Bars, per nutrient, with different letters within the nutrient treatment indicate significant differences. $LSD_{0.05}$= 5.6.

3.3.4 DISCUSSION

The comparison between Figure 3.3.1 and 3.3.2 demonstrates the relationship between the two pigment systems in pepper fruit. The relative concentration of chlorophylls and carotenoids at both ripening stages seemed to be inversely related. This finding is consistent with the results of Trudel and Ozbun (1970) who reported a similar trend in ripening tomato. Fruit from plants fertilized with 'very low' and 'low' K levels (N:K ratios = 1:0 and 1:0.3 respectively) had a higher chlorophyll concentration than fruit from control plants and 'high K' fertilized plants (N:K ratios = 1:1.4 and 1:6.9 respectively). This may be attributed to the effect of nitrogen under 'sub-optimal K' level. At 'very low' and 'low K' supply the response of pepper to 'optimal N' (low N:K ratio) was not limited by K content whereas at 'medium' and 'high K' level (high N:K ratios) the response to nitrogen in relation to chloroplast chlorophyll accumulation may be limited by K (Fig. 3.3.1). This confirms a report by Tisdale *et al.* (1990) who stated that an interaction between K and N is common in plants when other factors are at optimal level. This observation is inconsistent with the results of Trudel and Ozbun (1970) where 'high' K fertilization resulted in the highest chlorophyll concentration in mature-green tomato fruit. However, current results do accord with those of Trudel and Ozbun (1970) with respect to fertilizer levels having no significant impact on the chlorophyll concentration of fruit at the red-ripe stage.

At both maturity stages 'high' K fertilization (five times the recommended rate) did not outcompete the 'medium' (recommended) K application rate with respect to accumulation of both pigment types. In fact, there was a quadratic (an increase in carotenoid concentration of fruit with an increase in K fertilizer supply followed by a decrease in carotenoid concentration as K is supplied further) relationship ($P< 0.001$) between the K fertilizer level and the carotenoids accumulation in red-ripe pepper fruit. Supplying an 'optimal' K level (N:K ratio = 1:1.4) resulted in the highest accumulation of carotenoids, further addition of K (N:K ratio = 1:6.9) did not result in a further increase but a decline of the total carotenoid content (Fig. 3.3.2). This confirms earlier results by Goodwin and Goad (1970) and Britton and Hornero-Méndez (1997) who reported that plants grown under optimal conditions contain higher amounts of carotenoids in plants than if grown under either supra- or sub-optimal growing conditions. This phenomenon may be linked to the findings of various authors (Costes, 1962; Goodwin, 1967; Trudel and Ozbun, 1970; Bartley and Scolnik, 1995) that K is involved in channelling of the isoprenoid intermediates, such as GGPP, into carotenoid biosynthesis. It has been reported in several plant species that K activates acetic thiokinase and pyruvic kinase, two enzymes involved in carotenoid biosynthesis (Miller and Evans, 1957; Hiatt and Evans, 1960). Possibly, an optimal K level is conducive to the activities of the carotenoid biosynthetic pathway, which renders optimal K nutrition essential for carotenoid production and hence optimal pepper fruit colour development. Oversupplying K to levels above optimal, to possibly increase production or other fruit quality parameters seemed to negatively affect the quality parameter colour. These findings contradict Bussi and Amiot (1998), who showed that high K fertilization

(225 kg ha^{-1}) enhanced red colour in apricot fruit. A tentative postulation can, therefore, be made that excess K did not enhance carotenoid accumulation in pepper fruit. Alternatively, high concentration of K in the guard cells may result in stomatal closure, thus affecting the transport rate of photosynthates from source to sink through the phloem, hence little building blocks for carotenoid biosynthesis (Browling, 1987).

Supplying plants with 'low' P resulted in significantly higher chlorophyll concentration in mature-green peppers than the supply to a 'medium' level. 'Very low' and 'high' P levels showed a lower fruit chlorophyll concentration. In red-ripe fruit (Table 3.3.2), however, there was no significant difference in total chlorophyll concentration recorded among the four levels of P. Applying different levels of P did also not seem to have a significant effect on the carotenoid concentration of mature-green fruit (Table 3.3.3). This might suggest that there was already sufficient P in the medium. Application of 'low' and 'high' levels of Mg enhanced chlorophyll concentration significantly in mature-green fruit relative to those of 'control' plants. However, plants supplied with sub-optimal amounts of Mg had a lower fruit carotenoid concentration than control fruit. The low Mg might have resulted in less photosynthates hence, less carotenoid building blocks. This theory may be supported by Baszynski *et* al. (1980) who reported that Mg deficiency in plants my result in a decline in plastid carotenoid concentration. This might be due to the depression of Mg^+ by either K^+ and/or NH_4^+ in the growing medium (Baszynski *et al.*, 1980). Interestingly, high Mg fertilization did result in a significant increase in the chromoplast carotenoid concentration. This latter point may suggest that excess Mg might act as a stimulating stressor characterised by positive physiological response of plants to stress as judged by significantly higher carotenoids concentration in fruit relative to fruit from non-stressed ('medium' fertilized) pepper plants (Lichtenthaler, 1996). In addition to that, Demmig-Adams and Adams III (1992) reported that some environmental stress factors, in the presence of light, could affect the xanthophyll cycle pool. Among the three xanthophylls cycle elements zeaxanthin is reported to play an important role in the control of carotenoid biosynthesis in red pepper (Hornero-Méndez *et al.*, 2002).

Generally, an increase in carotenoid concentration during ripening, as a result of varying fertilizer levels, did not result in complete disappearance of chlorophyll in red-ripe fruit. Therefore, the chloroplast-chromoplast transformation in ripening fruit must be either not a complete conversion, or possibly reversible. However, it has also been reported that a distinctive feature of chromoplast development is the incorporation of a new set of proteins encoded in both nuclear and plastidic genomes which functions in the synthesis of carotenoids and their incorporation into fibrillar and globular structures (Camara *et al.*, 1995). On the basis of this an assumption can be made that chlorophyll synthesis took place throughout ripening, even though synthesis did not compensate for destruction.

3.3.5 CONCLUSIONS AND FUTURE AREAS OF STUDY

The chlorophyll concentration in fruit decreases with fruit ripening while total carotenoids increase during ripening. It is, therefore, logical to infer that fruit maturation is mirrored by a transformation of chloroplast to chromoplast, which is differentiation of plastids. A remarkable response to fertilizer application, expressed in terms of differences in carotenoid concentration, was observed at the physiological mature (red-ripe) stage. K and Mg play a role in the process of pigmentation during pepper fruit ripening. Different P fertilizer levels did not have a marked influence on carotenoid accumulation in red-ripe fruit. It can, therefore, be concluded that P did not play a direct role in carotenoid synthesis. Alternatively, the P available in the medium has been sufficient to fulfill its role in carotenoid synthesis.

To establish the exact role of K and Mg in pigment formation during ripening a thorough research on the impact of these nutrients on chloroplast-chromoplast transformation is needed, possibly by an anatomical study using electron microscopy. Secondly, a mineral analysis of K and Mg of fruit at the various stages of development should be carried out with the intent of correlating pigment concentration to mineral composition/ ratio of the fruit possibly including Ca in this ratio. Lastly, the effect of K and Mg on the proportion of xanthophyll carotenoids in pepper fruit of different maturity stages should be carried out.

3.4 EVALUATING THE EFFECT OF MOISTURE STRESS ON COLOUR DEVELOPMENT IN *CAPSICUM ANNUUM* L. cv. Capistrano

3.4.1 INTRODUCTION

Water is essential for crop growth, yield and quality. Recent studies have shown that water availability to plants is a prime factor for optimum yield (Doorenbos and Kanssam, 1986) and product quality (Gutezeit, 2001). Furthermore, stress-free conditions are more likely to allow physiological processes, such as pigment synthesis, to proceed normally than stressful conditions (Goodwin and Goad, 1970; Goodwin, 1980). However, varietal and species differences can also determine the response to certain growing conditions. A mildly stressful scenario, on the other hand, might have a positive impact on the physiological activity of a plant and does not have to result in damaging effects (Lichtenthaler, 1988). The same author indicated that a low concentration of a stress factor, such as a xenobiotic substance, could stimulate plant metabolism and plant growth, while at higher stressor concentration the opposite results may follow.

Pepper (*Capsicum annuum* L.) is among the horticultural plants most susceptible to water stress because of a wide transpiring leaf surface and elevated stomatal openings (Delfine *et al.,* 1994). Lately, several researchers have investigated the effect of water stress on pepper in relation to plant growth (Delfine *et al.*, 2001) and yield (Katerji *et al.*, 1993; Delfine *et al.*, 2001). However, information on the relationship between water stress and colour development in pepper seems to be missing. To what extend does water stress affect fruit colour in pepper? On the basis of Lichtenthaler`s (1988) assumption of an impact of moderately stressful conditions on physiological activity it is hypothesized that a certain degree of moisture stress stimulates carotenoid biosynthesis in plants. In an attempt to address the question how water stress can affect fruit carotenoid concentration and how this is related to the fruit chlorophyll concentration an experiment was designed, to firstly, investigate the effects of different moisture regimes on colour development, secondly, to determine the growth stage most sensitive to moisture stress and thirdly, to relate the fruit size to pepper fruit response to stress.

3.4.2 PLANT MATERIAL AND ENVIRONMENTAL CONDITIONS

'Capistrano' bell pepper seedlings were established in speedling ® trays filled with composted pine bark (GROMOR, Camperdown, RSA) and irrigated four times a day by an automated sprinkler system. The seedlings were transplanted into 25 cm-diameter plastic pots when they had six true leaves. The potting medium consisted of a sand-composted pine bark mixture (3:1, v/v). Plants were grown in a plastic tunnel during fall/winter 2001 (preliminary experiment) and spring/summer 2001/2002 (main experiment). Each experiment comprised four replicates, each consisting of either four (preliminary experiment) or two plants (main experiment) per moisture level (three levels) per exposure time (three stress period durations). The pots were arranged in a randomized complete block design. The moisture content of the growing medium was monitored and maintained at three regimes (drought = 6-10 KPa, water-logging = 0-2 KPa, and field capacity (which will be referred to as container capacity (Handreck and Black, 1984) = 2-5 KPa) (control) with the help of 15 cm-depth tensiometers (Irrometer®, Riverside, California, USA). Plants were considered to be water-logged at 0-2 KPa, as drainage of water following the application of excess water into the growth medium was restricted (Hausenbuiller, 1978). The first two moisture regimes were implemented simultaneously: either 30 days after first petal fall (DAPF) (preliminary and main experiment) or 30 or 50 days after transplanting (30 and 50 DAT), and 30 DAPF (main experiment) by either withholding the irrigation water (in the case of drought) or increasing the amount of irrigation water (in the case of water-logging condition) to maintain high level of water saturation point. On day one of the treatment water was applied till the desired moisture level was reached in the pots. The desired moisture level was maintained until final harvest (red-ripe stage). The third moisture regime, container capacity, was maintained throughout the growing period and served as control. The specific moisture level was, in all treatments, maintained until the end of the experiment. As no major differences were found between the results of the preliminary and the main experiment, only results of the latter will be presented and discussed.

The length of fruit was measured with digital callipers (Zodiac Electronic and Scientific Corporation, Ambala, India) immediately after harvest (early in the morning when the fruit were still turgid). It was measured from the proximal to the distal end. Three fruit were harvested simultaneously at the mature-green (about 40 DAPF) and the red-ripe stage (based on the visual colour of fruit).

3.4.3 RESULTS

3.4.3.1 Chlorophyll concentration in pepper fruit as affected by moisture stress

The variation of the amount of water administered to the plants, at different growth stages had a highly significant ($P< 0.01$) influence on chlorophyll concentration in pepper fruit 30 and 50 DAT, 30 DAPF (Fig. 3.4.1). A significantly lower concentration of chlorophyll was found in mature-green fruit (40 DAPF) of all drought stressed compared to non-stressed plants. Water stress applied 50 DAT reduced the chlorophyll concentration of fruit most drastically to approximately two thirds of fruit from plants grown at container capacity (Fig. 3.4.1). The earliest drought application, 30 DAT, also reduced the chlorophyll concentration significantly but not as drastically as 50 DAT. When plants were allowed to develop under container capacity until flowering and drought stress was only applied 30 DAPF the fruit chlorophyll concentration of those plants was still negatively affected compared to the control plants. On the other hand, water-logging plants 30 and 50 DAT resulted in a significantly (Fig. 3.4.1) higher chlorophyll concentration compared to container capacity plants. Moisture stress applied 30 DAPF resulted in mature-green fruit with a chlorophyll concentration equivalent to non-stressed plants.

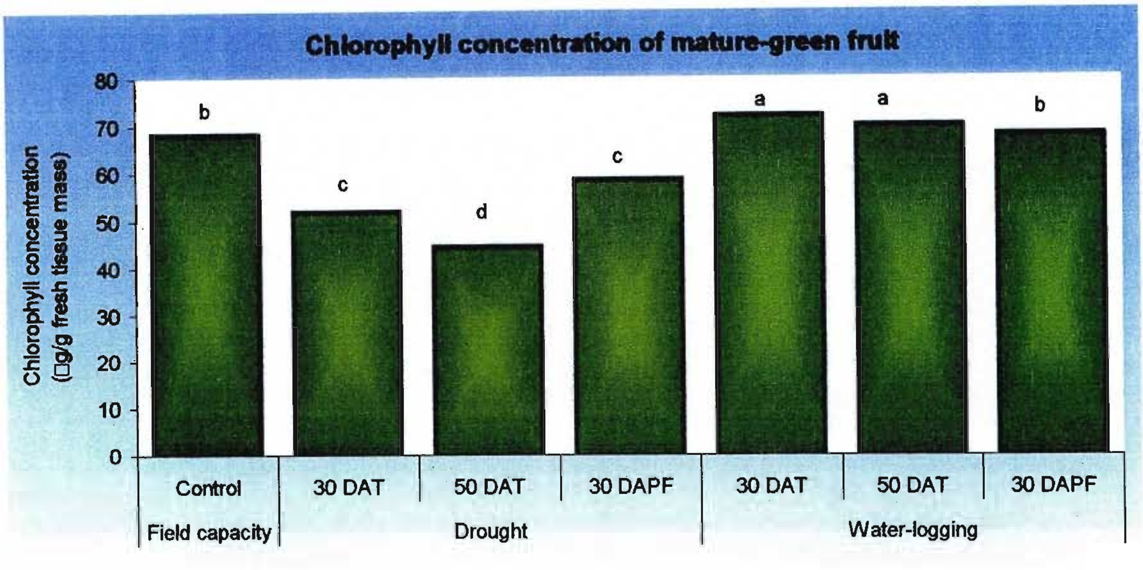

Figure 3.4.1 Chlorophyll concentration of mature-green pepper fruit exposed to various moisture regimes at different growth stages. Plants were subjected to container capacity (control), drought and water-logging conditions from 30 or 50 days after transplanting (DAT) or 30 days after first petal fall (DAPF) till harvest. Fruit were harvested at the mature-green stage. Bars with different letters indicate significant differences. $LSD_{0.05} = 1.5$.

However, later, at the red-ripe stage, when fruit chlorophyll concentration had decreased up to one third of the concentration in mature-green fruit, very little differences in chlorophyll concentration were found between treatments. Fruit from plants that were subjected to drought since an early stage of development, 30 DAT, had a significantly higher chlorophyll concentration than fruit from any other treatment, including the control (Fig. 3.4.2).

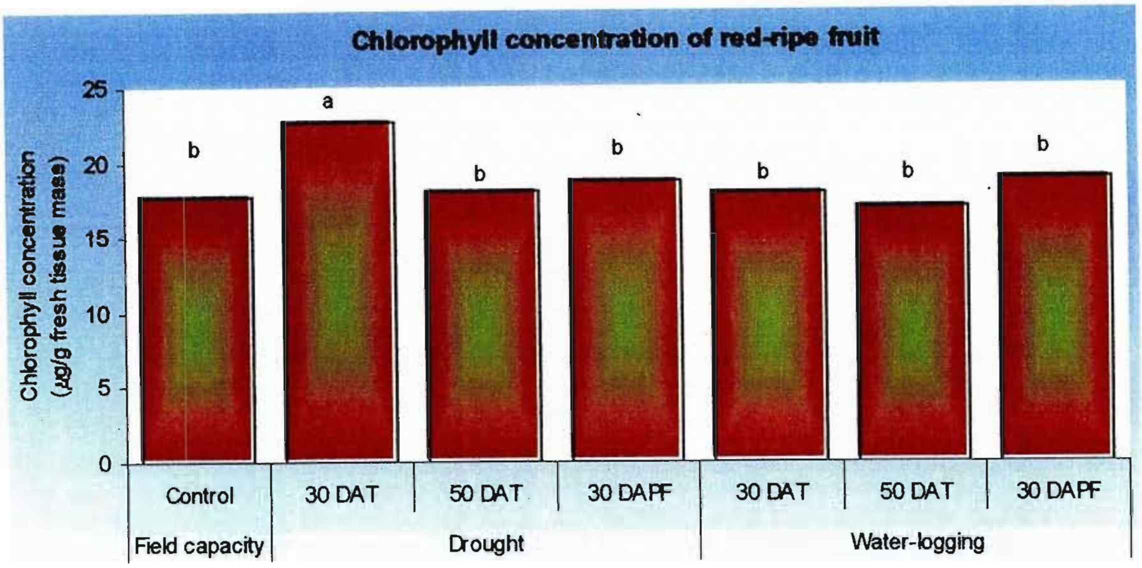

Figure 3.4.2 Chlorophyll concentration of red-ripe pepper fruit exposed to various moisture regimes at different growth stages. Plants were subjected to container capacity (control), drought and water- logging conditions from 30 or 50 days after transplanting (DAT) or 30 days after first petal fall (DAPF) to harvest. Fruit were harvested at the red-ripe stage. Bars with different letters indicate significant differences. $LSD_{0.05} = 1.5$.

3.4.3.2 Carotenoid accumulation in pepper fruit as affected by moisture stress

Similar to the response seen in fruit chlorophyll concentration, fruit carotenoid concentration was also affected ($P < 0.01$) by the moisture regime to which pepper plants were subjected. Mature-green fruit from plants that were subjected to drought stress either from 30 or 50 DAT or 30 DAPF onwards, as well as to water-logging from 30 DAT onwards had a significantly higher carotenoid concentration than non-stressed plants (Fig. 3.4.3). A later application of excess water (water-logging 50 DAT or 30 DAPF) did, however, not have any effect on the fruit carotenoid concentration at the mature-green stage.

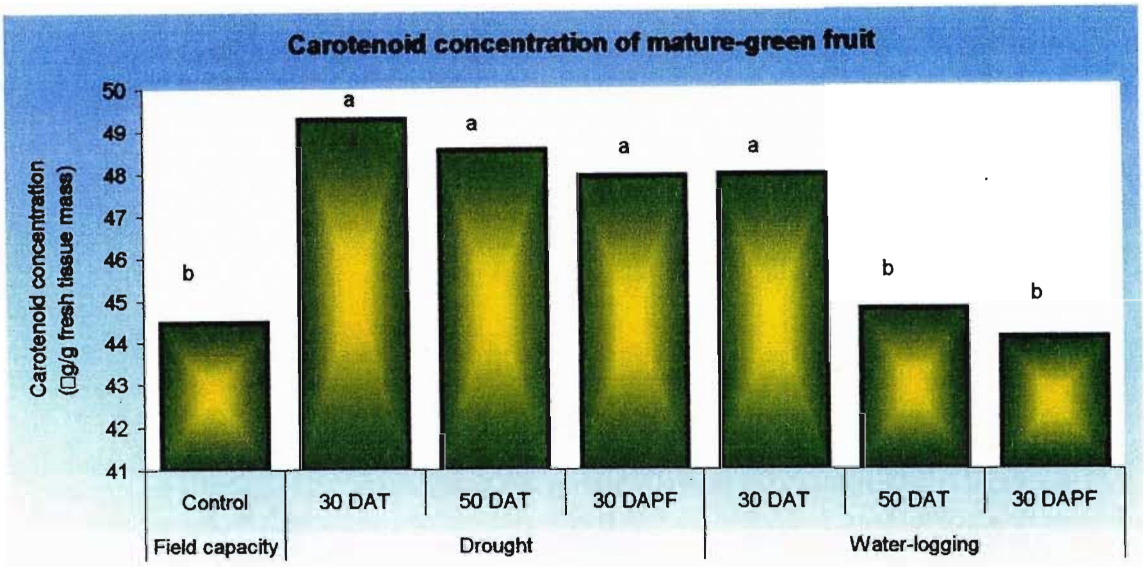

Figure 3.4.3 Carotenoid concentration of mature-green pepper fruit subjected to various moisture regimes at different growth stages. Plants were subjected to container capacity (control), drought and water-logging conditions from 30 or 50 days after transplanting (DAT) or 30 days after first petal fall (DAPF) onwards. Fruit were harvested at the mature-green stage. Bars with different letters indicate significant differences. $LSD_{0.05}$ = 1.8.

Similarly, subjecting pepper plants to water-logging conditions from 30 DAT and drought conditions from 30 or 50 DAT onwards resulted in a significantly higher fruit carotenoid concentration at the red-ripe stage compared to fruit from non-stressed plants. Fruit from plants subjected to drought stress 30 DAPF and to water-logging 50 DAT and 30 DAPF had a significantly lower concentration of carotenoids relative to fruit from plants grown at container capacity ($P< 0.01$). Nonetheless, in all treatments an increase in carotenoid concentration from mature-green to red-ripe fruit was found.

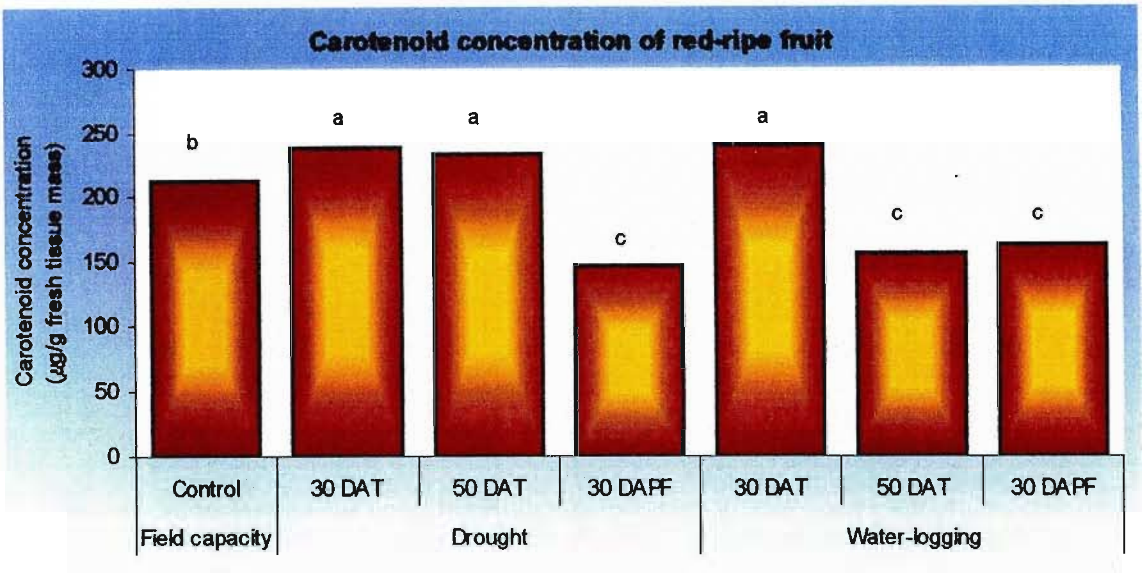

Figure 3.4.4 Carotenoid concentration of red-ripe pepper fruit subjected to various moisture regimes at different growth stages. Plants were subjected to container capacity (control), drought and water-logging conditions from 30 or 50 days after transplanting (DAT) or 30 days after first petal fall (DAPF) onwards till harvest. Fruit were harvested at the red-ripe stage. Bars with different letters indicate significant differences. $LSD_{0.05} = 1.8$.

3.4.3.3 Fruit size

Exposing pepper plants to various moisture conditions had a significant impact ($P < 0.001$) on fruit size as determined by fruit length (Fig. 3.4.5). Fruit from plants subjected to drought from 30 or 50 DAT onwards were significantly longer than fruit from the container capacity plants.

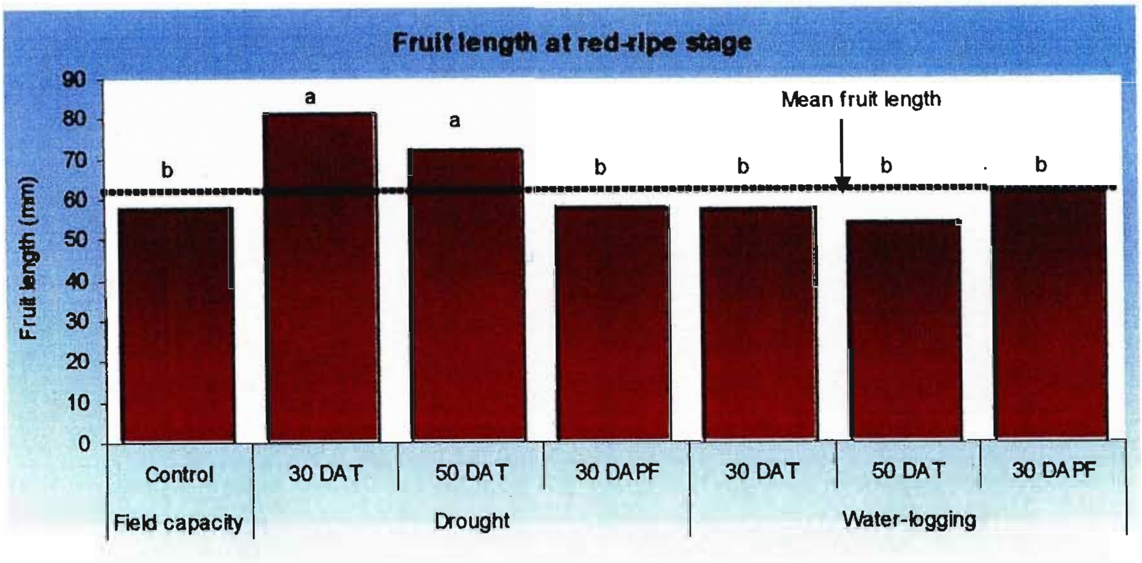

Figure 3.4.5 Length of the red-ripe pepper fruit. Plants were subjected to container capacity from transplanting, drought and water-logging conditions from 30 or 50 days after transplanting (DAT) or 30 days after first petal fall (DAPF) onwards until harvest. Fruit were harvested at the red-ripe stage. $LSD_{0.05} = 9.2$.

3.4.4 Discussion

The environmental conditions of temperate summers often cause drought stress in plants. Drought stress in peppers is almost inevitable if water deficit is not counteracted by irrigation (Delfine *et al.*, 2001). On the other hand, plants grown in poorly drained soils might experience excess stress of water-logging during rainy seasons and/or due to over-irrigation.

A mild drought stress (achieved by deficit irrigation) can be stimulative and beneficial to cell metabolism and physiological processes of a plant even under long stress conditions (Lichtenthaler, 1988). However, the effect is a "dose-dependent matter" (Lichtenthaler, 1996). This study aimed at testing the hypothesis that a certain degree of moisture stress stimulates pigment accumulation in pepper fruit, possibly as a "mild stress" response, hence, outcompete the effect of the optimum moisture level. This hypothesis is supported by studies of Bramley (1993) who reported that stressing plant tissues with carotenoid biosynthesis inhibitors (stressors) resulted in an increased carotenoid concentration compared to non-treated tissues. Exposing plants to drought stress (either 30 or 50 DAT, or 30 DAPF) resulted in a reduced chlorophyll concentration in mature-green fruit (Fig. 3.4.1) independent of time of first exposure to this stress condition. Water-logging, however, had the opposite effect (Fig. 3.4.1). Hence, either chlorophyll synthesis in mature-green pepper fruit was negatively affected by drought stress regardless of time of fruit exposure or chlorophyll breakdown was enhanced under these stress conditions. Both scenarios would have similar implications on photosynthetic capacity. This could explain the significantly higher carotenoid concentration in these fruit (Fig. 3.4.3), as chlorophyll molecules needed to be better protected by the radical scavenging carotenoids, which would result in shielding photosynthetic apparatus (Bramley, 2002). However, the carotenoid concentration at this stage of development was too low to impact on visual colour. Furthermore, mature-green pepper fruit must have been in an "alarm phase", as defined by Lichtenthaler (1996), which is characterized by a “low resistance minimum” (Fig. 3.4.6). Thus, as a stress coping mechanism, chloroplast carotenoid synthesis could have been stimulated.

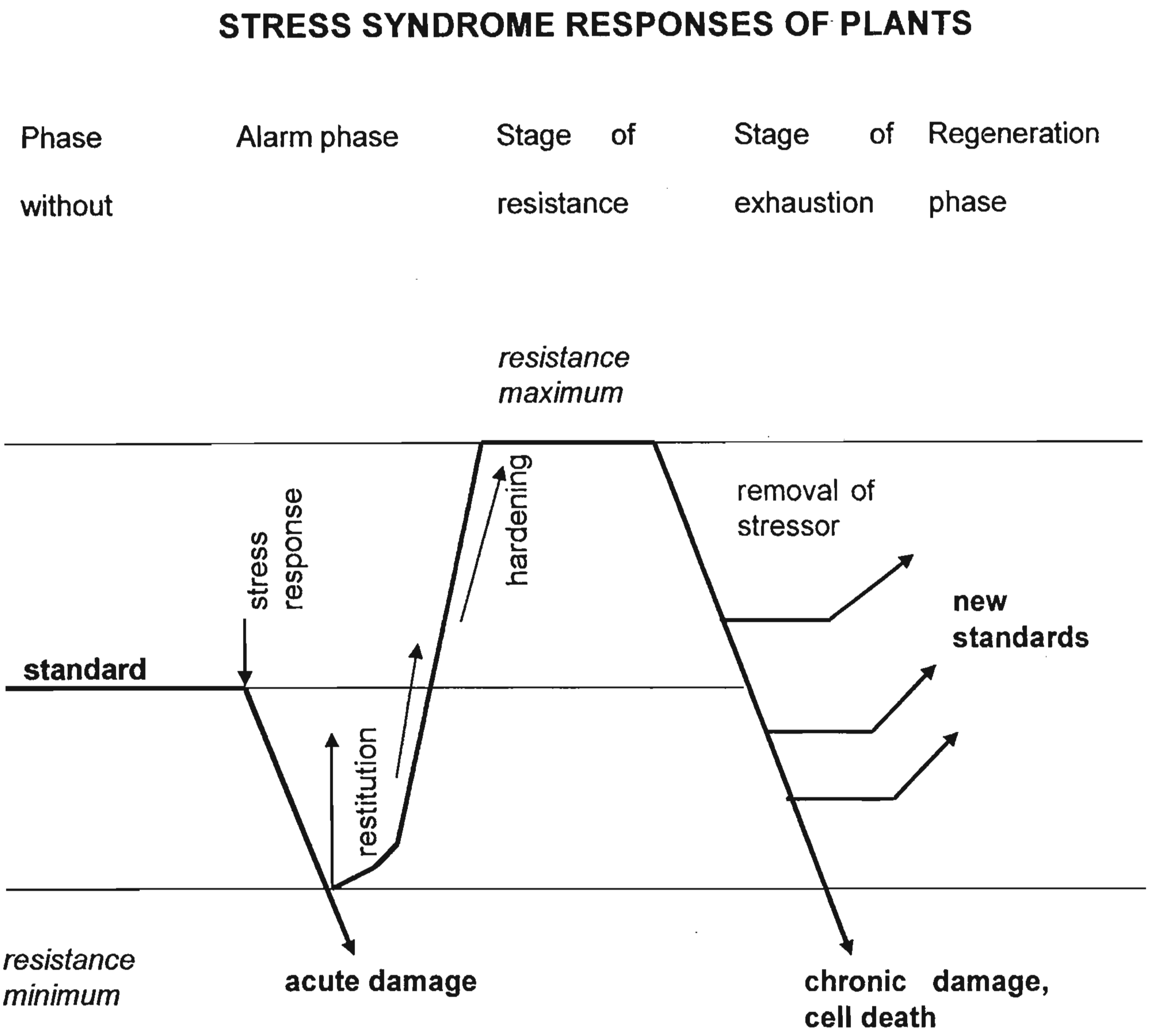

Figure 3.4.6 Sequences and responses of plants to stress. Plants at the resistance (maximum) stage will respond to and cope with stress. Removal of stressor(s) may result in development of new standards of physiology (depending on the time stress is removed as well as the duration and intensity of the stress (re-drawn from Lichtenthaler, 1996).

Drought applied at any of the three developmental stages and water-logging supplied early in the development (30 DAT) had a positive influence on chloroplast carotenoid concentration of mature-green fruit. This trend was maintained for chromoplast carotenoids (red-ripe fruit) with the exception of the late drought application. Moreover, plants subjected to drought stress early in the development (30 or 50 DAT) had longer fruit than those from the container capacity plants (Fig. 3.4.5). This phenomenon may be related to the stage of development at the time of treatment. If plants were under continuous drought stress (restitution phase) (Fig. 3.4.6) they could have developed a physiological adaptation as described by McKersie and Leshem (1994) characterized by a hardening process and the establishment of a new physiological standard (cellular acclimation as well as molecular organization within cells (Leopold, 1990)). This could have possibly resulted in morphological adaptation of fruit (a predominant form of stress response in plants (Leopold, 1990)).

Red-ripe fruit harvested from drought stressed plants (30 DAPF) and plants stressed by water-logging (50 DAT or 30 DAPF) had a significantly lower carotenoid concentration. Both forms of water stress, drought and water-logging, could have resulted in water deficit in plants either due to limited soil moisture (as in drought) or depletion of oxygen in the root zone and water intake might have been retarded or inhibited (as in water-logging) (Reid and Wample, 1985), thus causing water deficit stress. These results suggest that exposure of plants to drought (30 or 50 DAT) and water-logging (30 DAT) could have served as an "eu-stress". According to Lichtenthaler (1988) "eu-stress" is stimulative and has a positive impact on plant production. Water stress may affect plants differently depending on other prevailing environmental factors, such as temperature and light intensity (Delfine *et al.* 2001). Therefore, drought stress from 30 or 50 DAT and water-logging from 30 DAT onwards might have stimulated the development of stress coping (acclimation) mechanisms; in this case a significant increase in carotenoid synthesis in red-ripe fruit due to water stress from the early developmental stages. Hence, a bigger pool of carotenoids possibly, resulting in increased photo-protection (Lichtenthaler and Schindler, 1992; Demming-Adams and Adams III, 1993; Schindler and Lichtenthaler, 1994).

The carotenoid concentration of mature-green fruit from plants subjected to drought from 30 DAPF onwards was higher than that of control fruit. In later developmental stage (red-ripe), however, the carotenoid concentration of stressed fruit was lower than that of control fruit. Overall, however, the carotenoid concentration had increased from mature-green to red-ripe fruit.

3.4.5 CONCLUSION AND FUTURE AREAS OF STUDY

This study indicates that, in peppers, the production of carotenoids increases or decreases due to drought and water-logging stress depending on the developmental stage. This would, in mature-green fruit, result in a photo-protective function. However, the effect of increasing carotenoid concentration is sustained through to the red-ripe stage, if the stress is applied at an early fruit developmental stage. Colour expression in pepper was most sensitive to water stress 30 and 50 DAT. These findings may provide a clue to the means of influencing the appealing fruit colour through the imposition of water stress on pepper plants early in the developmental stage. An investigation into the response of the xanthophyll cycle carotenoids of pepper fruit to water stress would produce an insight into the importance of these carotenoids in colour expression of pepper under water deficit. Moreover, it is necessary to determine which carotenoids are predominant in fruit under stress and how this pattern differs from that under optimal growing conditions. Finally, it is important to consider the effect of water stress on post harvest quality as it might be improved under drought stress.

3.5 INFLUENCE OF THINNING OF REPRODUCTIVE STRUCTURES FROM PEPPER PLANTS (*Capsicum annuum* L. cv. Capistrano) ON FRUIT PIGMENT CONCENTRATION

3.5.1 INTRODUCTION

Climatic factors, cultural operations and the interaction among these factors can affect fruit quality (Jackson and Lombard, 1993). According to Naor *et al.* (2002) environmental and plant factors may have direct effects on the chemical composition of fruit. Smart (1985) reported that the management of plant canopy by thinning of vegetative or reproductive structures, or both, might affect the microclimate in the vicinity of fruit. Thinning is common practice in many horticultural commodities.

What effect does thinning have on the composition of the fruit? As demonstrated in Chapter 1 (Section 1.4.2.4) thinning of reproductive structures may affect the mineral content of fruit. Reports by Bravdo and Hepner (1987) and Jackson and Lombard (1993) indicate that an increase in fruit load on grape vines may result in delayed fruit colour expression. Based on this information it is hypothesised that thinning of reproductive structures of pepper plants impact positively on pigment accumulation in pepper fruit. The effect of thinning of flowers/fruits and timing of thinning in relation to colour expression in *Capsicum* fruit has, to date, not been investigated. The objectives of this study were, therefore, firstly, to investigate the effects of thinning of flowers and fruitlets on pepper fruit carotenoid concentration and, secondly, to determine whether timing of thinning affects fruit carotenoid concentration.

3.5.2 PLANT MATERIAL AND GROWTH CONDITIONS

The experiment was carried out in a plastic tunnel (PT) at the University of Natal, Pietermaritzburg. Bell pepper (*Capsicum annuum* L. cv. Capistrano) seedlings with six true leaves were transplanted into 25 cm-plastic pots containing composted pine bark (GROMOR, Camperdown, RSA) as a medium. The experiment was conducted from winter to summer 2002. The plants were provided with optimal moisture (container capacity) throughout the entire growth period with the help of 15 cm-depth tensiometers (Irrometer® Company Inc., Riverside, California, USA). Each plant was staked with a stick inserted into the pot. Plants were fertigated with Calmagro plus (wetable solution) comprising of: 137 g N/Kg; 128 g Ca/Kg; 34 g Mg/Kg; 170 mg Zn/Kg; 3075 mg B/Kg; 78 mg Mo/Kg) (Rozie Agencies C.C., Pietermaritzburg, RSA), at the rate of 2 kg/100 liters of water, fortnightly throughout the vegetative growth period up to first fruit set. The average daily aerial temperature in the PT ranged between 22 and 30°C during the reproductive growth period till harvest at physiological maturity (red-ripe stage). Thinning was carried out by hand and involved the removal of all reproductive structures (flowers and fruit) from every 2nd, 4th, 6th, 8th node of each branch once on the 11th, 19th, 27th and 35th day after first petal fall (DAPF). The control plants were not thinned. Each treatment was replicated ten times and each individual plant was considered a replicate.

3.5.3 RESULTS

3.5.3.1 Chlorophyll concentration of pepper fruit as influenced by thinning of reproductive structures

Reducing the fruit load of pepper plants by thinning flowers and fruitlets had no impact on the chlorophyll concentration of neither mature-green nor red-ripe fruit

(Fig. 3.5.1). Therefore, neither the timing of thinning nor any once over thinning affected the chlorophyll concentration of mature-green and red-ripe pepper fruit.

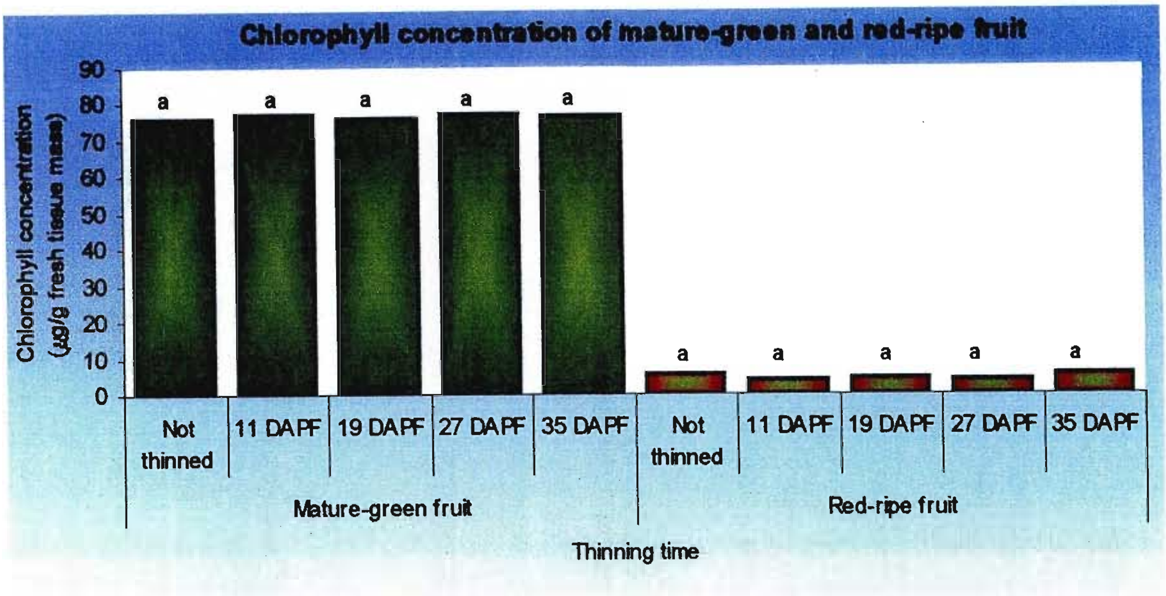

Figure 3.5.1 Effect of thinning of reproductive structures on chlorophyll concentration of mature-green and red-ripe pepper fruit. Fruit and flowers were removed once on the 11th, 19th, 27th and 35th DAPF. Control plants were not thinned. Fruit were harvested at the mature-green or red-ripe stage. Bars with different letters indicate significant difference. $LSD_{0.05}$= 5.

3.5.3.2 Carotenoid concentration of pepper fruit as affected by timing of thinning

There was no significant difference in carotenoid concentration of mature-green fruit between the treatments, irrespective of the timing of thinning. Thinning, however, influenced chromoplast carotenoid concentration. An early thinning (11 DAPF) of reproductive structures resulted in a significantly lower fruit carotenoid concentration while a later thinning (19, 27 or 35 DAPF) had no positive influence on fruit carotenoid concentration at the red-ripe stage compared to control plants (Fig. 3.5.2). However, a delay in thinning from 11 to 19 to 27 DAPF resulted in a linear increase in fruit carotenoid concentration.

3.5.3.3 Fruit number

Carotenoid concentration and fruit number per plant were inversely related (Fig. 3.5.2). The earliest thinning date resulted in the highest fruit number while later thinning showed no effect on fruit number.

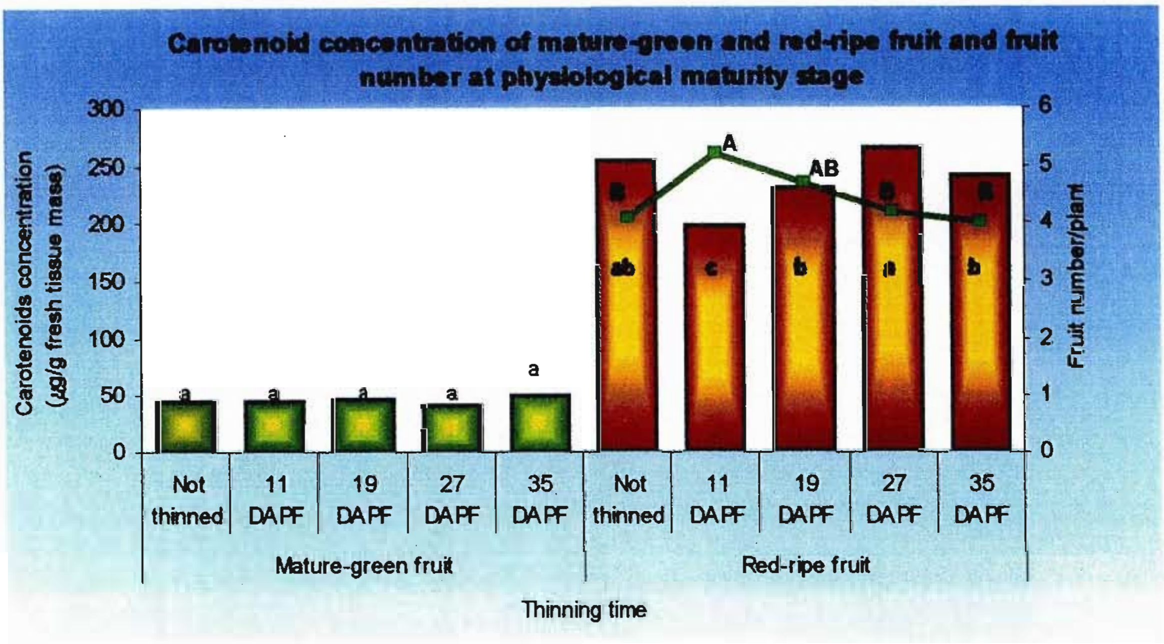

Figure 3.5.2 Effect of thinning of reproductive structures on carotenoid concentration of mature-green and red-ripe fruit (bars) as well as fruit number per plant (line). Fruit and flowers were removed once on the 11th, 19th, 27th and 35th DAPF. Control plants were not thinned. Fruit were harvested at the mature-green or red-ripe stage. Either upper or lower case letters per ripening stage indicate significant difference between the time of thinning. $LSD_{0.05}$= 15 and 0.8 for carotenoid concentration and fruit yield respectively.

3.5.4 DISCUSSION

Hall (1977) stated that, in pepper, fruit constitute the main sinks for assimilates during much of the fruit growth period. Therefore, inter-sink competition for assimilates and for possible carotenoid building blocks is likely. The results of this study indicate that thinning reproductive structures by flower removal and defruiting of pepper plants at different growth stages did neither affect total chlorophyll concentration of mature-green nor red-ripe fruit. This suggests firstly, a limited "inter-sink" competition for chlorophyll building blocks and secondly, no influence of the thinning treatments on chlorophyll degradation in ripening fruit. On the other hand, the early (11 DAPF) flower removal and defruiting resulted in a significant reduction in carotenoid concentration of red-ripe fruit. It is questionable whether the reduced ability of these fruit to accumulate carotenoids was simply due to relatively high fruit load or the timing of thinning. Statistical analysis of variance did show that the timing of thinning was related to pigment accumulation at red-ripe stage ($P< 0.001$).

The lack of positive influence of timing of thinning on carotenoid concentration of red-ripe fruit may be attributed to the naturally occurring loss of reproductive structures, through abscission. Furthermore, the sink competition between remaining fruit, newly set fruit and young leaves might have been insignificant. Stressful conditions, such as high temperature (Wien *et al.*, 1989a), as recorded in our experiment (in the PT), may have resulted in a reduced, but uniform, fruit number per plant of all treatments, except 11 DAPF. Hence, a uniform sink: source ratio through flower and/or fruit drop resulted, ensuring uniform allocation of assimilates and reserves to the remaining reproductive sinks and vegetative growing points (Naor *et al.*, 2002). High temperatures could have, furthermore, affected physiological processes in pepper plant, which may have resulted in abscission of reproductive structures in all treatments. High temperature stress may also affect hormone (auxin) transport out of the fruit via the pedicel and cause the formation of an abscission layer resulting in the abscission of reproductive structures (Wien *et al.*, 1993b). This may also be related to the poor fruit set, common in tomato under night temperatures warmer than 21°C (Moore and Thomas, 1952). This assumption is supported by results obtained by Erickson and Markhart (2001) who reported that poor fruit set in bell pepper is primarily due to flower abscission. Lastly, high temperatures might have imposed water stress on the plants, and, hence poor fruit set, low fruit load and fresh fruit weight per plant resulted. The average fresh fruit weight per plant from all the treatments was four times less than the average commercial fresh fruit weight (0.88 kg/plant) (Maynard and Hockmuth, 1997).

The higher fruit number from plants thinned 11 DAPF (Fig. 3.5.2) and the significantly lower fruit carotenoid concentration of these fruit (Fig. 3.5.2) indicated a correlation between fruit number and carotenoid concentration of fruit. A tentative conclusion can, therefore, be drawn that there is an inverse relationship between fruit load and carotenoid concentration of red-ripe fruit. This would entail that with a higher fruit number per plant the ability of a certain fruit to synthesise carotenoids decreased and *vice versa.* It can be interpreted as a result of reduction in sink strength, which is a product of sink size and sink activity (Sonnewald and Willmitzer, 1992). The reduction in sink strength may be due, possibly, to the lack of building blocks or energy, in plants thinned 11 DAPF, and, hence a low ability to synthesise carotenoids. Alternatively, the low carotenoid concentration in fruit from plants thinned 11 DAPF might be related to the variation of physiological maturity of these fruit rather than the number of fruit per plant. Furthermore, the pot size alone might have limited fruit carotenoid concentration, as there was, neither enough space for the root development nor, possibly, enough nourishment to meet the high demand for resources necessary for carotenoid biosynthesis. The relatively high fruit number recorded from the early-thinned plants (11 DAPF) was possibly due to the growth characteristic of pepper. Additional flushes of vegetative and reproductive growth were stimulated by removal of flowers and defruiting, particularly of the first fruit (Hall and Brady, 1977) resulting in an increase of sinks.

3.5.5 CONCLUSION AND FUTURE AREAS OF STUDY

According to the results of this study, thinning of reproductive structures at different stages of development of pepper fruit may become a tool to manipulate carotenoid concentration of red-ripe fruit. Further studies are required to evaluate the effects of thinning of reproductive pepper structures on the fruit maturity rate in order to alter physiological maturity and maximize pigment accumulation. Additionally, the fruit load should be kept constant in all treatments when evaluating the effect of timing of thinning on fruit pigment accumulation. At each thinning date fruit load could be reduced to certain levels in all treatments. In addition to the treatments implemented in this study, another series could be added by allowing new growth of reproductive structures at specific levels of new fruits.

Furthermore, an investigation into comparative effect of thinning of reproductive and/or vegetative structures at various developmental stages on fruit carotenoid concentration would provide an insight into the importance of these practices in colour expression of pepper fruit. However, it is considered that the following precautions could be opted for in order to reduce abscission of reproductive structures during the hot summer season; firstly, stress resistant cultivars (Aloni *et al.*, 1994; Wien *et al.,* 1989a) could be utilized; secondly, temperature could be moderated through mitigation of low light stress in the glass houses and frequent irrigation in the field (Nederhoff and van Uffelen, 1988), and, finally, an exogenous application of ethylene inhibitor (Wien and Zhang, 1991), particularly to hot pepper because some cultivars are reported to produce ethylene at colour-break (Gross *et al.*, 1986),

CHAPTER FOUR

GENERAL CONCLUSIONS AND RECOMMENDATIONS

The concentration of pigments in fruit tissue depends fundamentally on the species, the cultivar, the stage of ripeness of fruit and the environmental conditions fruit are exposed to. *Capsicum* is not an exception.

A number of inferences can be drawn from this study. First and foremost, this study confirms that tissues of green plants contain yellow and orange carotenoids to influence predominant colour brought about by chlorophylls. It further shows that the fruit developmental stage is a key determinant of pigment concentration of pepper fruit. As fruit ripen the chlorophyll concentration decline while that of chromoplast carotenoids, mainly red colour-imparting carotenoids capsanthin and capsorubin in red pepper cv. Capistrano, increase. Under optimal management/ environmental conditions (temperature [23/18°C; day/night], nutrients [P, K, Mg] and moisture [field (container) capacity]) the carotenoid concentration of red-ripe fruit increases to a level five times greater than that of mature-green fruit. However, this effect of optimal environmental conditions depends on the developmental stage of the fruit at the time of exposure. Otherwise, either supra- or sub-optimal environmental conditions can have a positive or negative impact on fruit pigment concentration depending on how high or low the condition is or the developmental stage at which plants begin to experience the condition. Stress factors play an important role in stimulating fruit carotenoid concentration. Stress can enhance fruit carotenoid concentration, but the effect depends on the stage of development and the duration of the stress. In fact, exposure of pepper plants to a mild drought stress from 30 or 50 DAT as well as water-logging from 30 DAT onwards increases carotenoid concentration of red-ripe pepper fruit to five-fold of that of mature-green fruit. On the other hand, delaying this exposure to either 30 DAPF (drought) or 50 DAT or 30 DAPF (water-logging) results in chromoplast carotenoid concentration three to four times higher than the chloroplast carotenoid concentration of mature-green fruit. It is, therefore, logical to infer that fruit acclimation to drought stress in the form of an increment in carotenoid concentration, which might possibly result in increased photo-protection, is an essential step in stress avoidance and is, therefore, a stress coping mechanism. In addition to that, a change in fruit morphology is a form of response to drought stress imposed early in the developmental stage. The supra-optimal water supply (water-logging) affects fruit carotenoid concentration positively when imposed from 30 DAT onwards.

The effect of various K amounts added to the growing medium on fruit carotenoid concentration is apparent at either horticultural or physiological maturity whereas for Mg it is only apparent at the latter stage. Similar to moisture stress, a supra-optimal Mg level results in the highest fruit carotenoid concentration and seems to act as a stimulant, which is not the case with K. Instead, the 'optimal' K level at N:K ratio of 1:1.4 is the ideal amount to produce fruit with an optimal carotenoid concentration.

Manipulation of sink strength (determined by the fruit number per plant) of pepper plants by timing the thinning of reproductive structures can not affect fruit colour expression positively, unless other factors, such as temperature, soil moisture content and humidity, which might have direct or indirect influence on sink strength, are controlled. A higher fruit number seems to be correlated to a lower carotenoid concentration, indicating that manipulation of sink strength at certain stages of development influence fruit pigment concentration, and could be used as a means to impact on fruit carotenoid concentration.

Further studies are, therefore, essential in order to attain an insight into the importance of the afore-discussed environmental factors and manipulation of reproductive structures in colour expression of pepper fruit. These studies should focus on the response of the xanthophyll cycle carotenoids (violaxanthin, antheraxanthin and zeaxanthin) of pepper fruit to water stress as this might provide an insight into the role of these carotenoids in colour expression of fruit. Furthermore, it is important to consider the effect of water stress on post-harvest quality. A mild drought stress (deficit irrigation) might improve this quality. Therefore, the effect of different durations of water deficit on plastid membrane properties could be investigated. To establish supportive information about the role of K and Mg in fruit pigmentation during the ripening process a thorough investigation into the impact of various levels/combinations of these nutrients on chloroplast to chromoplast transformation is needed, possibly by an anatomical study using electron microscopy. Another alternative could be to carry out mineral analysis of K and Mg of fruit at various stages of development with the intention of relating pigment concentration to mineral composition/ratio of the fruit possibly including Ca in this ratio. In order to determine the exact developmental stage most sensitive to a change in temperature with respect to colour development plants should be subjected to the optimal temperature regime for equal durations, starting at either the vegetative, first flowering or first fruit set stage. This could shed light on the impact of optimal temperature at the appropriate developmental stage with the intention to optimise fruit carotenoid concentration and, hence, improve fruit quality. Furthermore, manipulation of sink strength should aim at evaluating the effects of thinning at various fruit developmental stages not only in relation to pigment concentration but also to the rate of fruit ripening in order to reduce days to harvest and maximize pigment accumulation. Otherwise, stress resistant cultivars, moderate temperatures and application of ethylene inhibitors, should be opted for in order to reduce abscission of reproductive structures during the hot summer season. In addition, further study should be geared towards regulating the expression of major red colour-imparting carotenoids in *Capsicum annuum* L. possibly by manipulation of some environmental factors and cultural practices, discussed in this study, as a means of improving quality of natural colourants in pepper fruit. Finally, further studies should focus on the determination of the concentration of dominant colour-imparting carotenoids in pepper fruit as influenced by the discussed horticultural practices.

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