Optimum temperatures for colour development in apples

by
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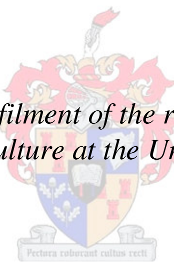

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Declaration

By submitting this thesis/dissertation electronically, I declare that the entirety of the work contained therein is my own, original work, and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

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SUMMARY

Peel colour is an important quality factor in the production of bi-coloured apple fruit. Most markets set minimum requirements for red colour coverage. Fruit that do not meet these requirements are downgraded and has a major impact on the profitability of apple production in South Africa. South African apple production areas are amongst the warmest in the world. Since anthocyanin accumulation requires induction at low temperature and synthesis require mild temperatures, experiments were conducted to investigate optimum day and night temperatures for red colour development throughout fruit development for red and bi-coloured apple cultivars grown in South Africa. We found that redder strains of bi-coloured apple cultivars did not appear to owe their enhanced pigmentation to higher temperature optima for anthocyanin synthesis. The optimum day temperatures for red colour development in the different cultivars seemed to differ between seasons, but not between production areas. In general, red colour in the cultivars evaluated developed maximally between 17 ºC and 25 ºC. The optimum day temperature for red colour development remained constant throughout fruit development for most cultivars, but increased roughly from 14 ºC to 22 ºC in ‘Cripps’ Pink’ between January and April. The extent of red colour development increased during fruit development in all the cultivars assessed. We were unable to determine optimum induction temperatures for red colour development. ‘Royal Gala’ from Ceres seemed to benefit from induction at 4 ºC while red colour in ‘Fuji’ decreased with decreasing temperature.

To explain the presence of anthocyanins in immature apple fruit, we tested the hypothesis that anthocyanins protect the peel from photoinhibition and photooxidative damage during conditions of increased light stress. First we established that the rate of colour change in response to a passing cold front appears to be sufficient to provide photoprotection during a cold snap. Also in agreement with the hypothesis, ‘Cripps Pink’ peel incurred significantly more photoinhibition at low temperature (16 ºC) compared to mild (24 and 32 ºC) and high (40 ºC) temperature under high irradiance with visible light. Recovery rate was temperature-dependent, being the slowest at low temperature and increasing with temperature. The photoapparatus in ‘Cripps Pink’ peel appears to be particularly sensitive to light stress at low temperature throughout the season, with significant photoinhibition occurring even at moderate temperature (24 ºC). The sensitivity of the apple peel to photoinhibition increased

throughout the season at lower irradiance levels, but remained the same at higher irradiance. In our final experiment, fruit were exposed to high irradiance at low and mild temperature before exposure to high temperature in combination with high irradiance. This was done to test the hypothesis that photoinhibition incurred during cold snaps predisposes peel to photothermal damage when temperature increases again after the cold snap. Unfortunately, due to the severity of the stress incurred in response to high temperature treatment, the results were inconclusive.

OPSOMMING

Vrugkleur is ‘n belangrike kwaliteitsfaktor in die produksie van tweekleurappels. Die meeste markte stel minimum vereistes vir rooi kleurbedekking. Vrugte wat nie aan hierdie vereistes voldoen nie, word afgegradeer. Suid-Afrika se appel produksie areas word beskou as van die warmste ter wêreld. Antosianien akkumulasie benodig induksie by lae temperature gevolg deur sintese in lig by matige temperature. Gevolglik het swak rooi kleurontwikkeling onder plaaslike toestande ‘n groot impak op die winsgewendheid van appelproduksie in Suid-Afrika. Eksperimente is uitgevoer om die optimum dag- en nagtemperature vir rooi kleurontwikkeling tydens vrugontwikkeling vir die rooi en tweekleur appel kultivars wat in Suid-Afrika geproduseer word te bepaal. Ons het gevind dat die verhoogde pigmentasie van rooier seleksies van tweekleurappel kultivars nie aan ‘n hoër temperatuur optimum vir antosianiensintese toegeskryf kan word nie. Die optimum dag temperature vir rooi kleurontwikkeling vir die onderskeie kultivars verskil klaarblyklik tussen seisoene, maar nie tussen produksie areas nie. Oor die algemeen het kleurontwikkeling maksimaal plaasgevind tussen 17 ºC en 25 ºC. Die optimum dagtemperatuur vir rooi kleurontwikkeling het konstant gebly tydens vrugontwikkeling, buiten vir ‘Cripps’ Pink’ waar dit toegeneem het van ongeveer 14 ºC tot 22 ºC vanaf Januarie tot April. Die mate van rooi kleurontwikkeling het in al die kultivars toegeneem deur die loop van vrugontwikkeling . Ons kon nie daarin slaag om optimum induksie temperature vir rooi kleurontwikkeling vas te stel nie. Rooi kleurontwikkeling van ‘Royal Gala’ uit Ceres is moontlik bevorder deur induksie by 4 ºC, terwyl ‘Fuji’ se rooi kleur afgeneem het met ‘n verlaging in induksie temperatuur.

Ten einde die teenwoordigheid van antosianien in onvolwasse appelvruggies te verduidelik, het ons die hipotese getoets dat antosianien die vrugskil beskerm teen fotoinhibisie en foto-oksidatiewe beskadiging gedurende tydperke van verhoogde ligstres. Eerstens het ons bevestig dat die tempo van kleurontwikkeling in reaksie op ‘n koue front waarskynlik vinnig genoeg is om fotobeskerming te verleen. Vervolgens is gevind dat ‘Cripps’ Pink’ vrugskil aansienlik meer fotoinhibisie ervaar het by lae temperatuur (16 ºC) in vergelyking met matige (24 ºC en 32 ºC) en hoë (40 ºC) temperatuur onder hoë irradiasie met sigbare lig. Die hersteltempo was temperatuur-afhanklik; dit was die stadigste by lae temperatuur en het toegeneem met ‘n toename in temperatuur. Die foto-apparaat in ‘Cripps’ Pink’ vrugskil blyk

besonder sensitief te wees vir ligstres by lae temperatuur regdeur die groeiseisoen met aansienlike fotoinhibisie by selfs matige temperatuur (24 ºC). Die sensitiwiteit van die vrugskil vir fotoinhibisie het toegeneem deur die groeiseisoen by laer ligvlakke, maar het dieselfde gebly by hoër vlakke van irradiasie. Laastens is vrugte blootgestel aan hoë irradiasie by lae en matige temperatuur voordat dit vervolgens blootgestel is aan hoë temperatuur in kombinasie met hoë irradiasie. Dit was om die hipotese te toets dat fotoinhibisie wat opgedoen word gedurende 'n onverwagte koue periode, die skil meer vatbaar maak vir foto-termiese skade sodra die temperatuur weer styg na die koue periode verby is. Ongelukkig het die hoë temperatuur stres al die behandelings tot so 'n mate geaffekteer dat dit onmoontlik was om enige gevolgtrekkings vanuit ons resultate te maak.

DEDICATION

Ek dra hierdie tesis op aan hul wat voetspore langs my neergelê het in sand, in klip en in die dieptes van my wese. Jul sal in my gedagtes wees met elke byt aan 'n blos-gekleurde, Gouws-groen appel.

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GENERAL INTRODUCTION

Light and temperature are the major factors that determine the extent of red colour development in apple fruit (Lancaster, 1992; Reay & Lancaster, 2001; Saure 1990). Anthocyanins in ripening apples are apparently induced at low temperatures (<10 ºC) (Curry, 1997) and synthesis takes place under high irradiation at mild temperatures (20 ºC to 27 ºC) in detached, mature apples (Curry, 1997; Reay, 1999; Saure, 1990 citing Nauman, 1964). Considering the importance of low night and mild day temperatures for anthocyanin synthesis in apple peel (Curry, 1997; Reay, 1999), it is not surprising that the high temperatures experienced in the Western Cape province give rise to poor red colour development (Wand et al., 2002, 2005). Different cultivars may differ in their optimum temperatures for anthocyanin synthesis (Curry, 1997). Knowing the optimum day and night temperatures for colour development for different cultivars may allow for more informed decisions with regard to cultivar choice in different production areas. Considering the above, we set out to determine the optimum day- and night-time temperatures for anthocyanin accumulation for red and bi-coloured apple cultivars grown in South Africa. Redder strains of some of these cultivars were also evaluated to determine whether their enhanced anthocyanin synthesis is due to a shift in the optimum temperature for anthocyanin synthesis. Fruit were harvested from two different production areas to assess whether growing conditions may influence the temperature requirements for anthocyanin synthesis.

Little is known about anthocyanin accumulation during early fruit growth in apple due to its economical non-significance and only speculated biological significance. Immature apples of at least some cultivars seem to accumulate anthocyanins at lower temperatures than mature fruit (Faragher, 1983). Anthocyanin synthesis in plants generally coincides with periods of high excitation pressure and increased potential for photo-oxidative damage (Steyn et al., 2002). The same appears to be true for apple fruit (Steyn et al., 2009). Chlorophyllous tissues that receives more light energy than can be used in photochemistry undergo a decrease in quantum efficiency of photosynthesis, better known as photoinhibition (Adams et al., 2008; Long et al., 1994). As a response chloroplasts generate Reactive Oxygen Species (ROS) that, when in superabundance, may potentially destroy thylakoid membranes, damage DNA

and denaturate proteins associated with photosynthetic electron transport (Alscher et al., 1997). ROS production increases in response to stresses such as low temperature (Prassad et al., 1994; Prassad, 1996) and have been implicated in photoinhibition (Hull et al., 1997) and cellular damage (Wise, 1995). We argued that anthocyanins in immature apple fruit protect apple peel from photoinhibition and photooxidative damage during conditions of increased light stress, which occur during sudden cold snaps. Subsequently, we set out to determine whether anthocyanins can accumulate fast enough to provide photoprotection during cold snaps. We also considered whether protection of fruit peel against photoinhibition during cold snaps lowers the risk of subsequent high light and high temperature- induced damage to fruit peel when temperatures increase again after the cold snap. Lastly, we determined whether the sensitivity of fruit peel to photoinhibition increases during fruit development, thereby explaining why anthocyanins apparently accumulate at lower temperatures in immature apples.

The biosynthesis of anthocyanins have been widely studied and has been the theme of various reviews (e.g. Heller and Forkman, 1988; Lancaster, 1992; Macheix et al., 1990; Davies, 2009). Also, numerous literature studies on anthocyanin synthesis have been conducted in the Department of Horticultural Science at Stellenbosch University (Marais, 2000; Reynolds, 2001; Schmeisser, 2002; Steyn 2003; Viljoen and Huysamer, 2005). Rather than repeating these reviews, we decided to focus the literature study of this thesis on the use of suspension cultures to study the regulation of anthocyanin synthesis. Unlike apples, (Marais et al., 2001; Steyn et al., 2005), pears do not synthesize anthocyanins after removal from the tree making it difficult to study the regulation of anthocyanin synthesis in pears. Under laboratory conditions, suspension cultures could be subjected to different environmental conditions (Kakegawa et al., 1987), potentially allowing the determination of optimum day- and night-time temperatures for anthocyanin accumulation.

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<u>LITERATURE REVIEW</u>: USING CELL SUSPENSION CULTURES TO STUDY ANTHOCYANIN SYNTHESIS

1. Introduction

Apple fruit have the ability to synthesize anthocyanins from the tree, thus allowing researchers to study the temperature and light regulation of red colour development under controlled conditions in the laboratory (Curry, 1997). Unlike apples, pears do not synthesize anthocyanins after removal from the tree, i.e. after harvest (Marais et al., 2001b; Steyn et al., 2005). This makes it difficult to study the regulation of anthocyanin synthesis in pear. However, plant cells of the fruit skin retain the ability to divide and synthesize anthocyanins (and other phenolics) as part of secondary metabolism - seeing that secondary metabolite synthesis is believed to be related to cell growth (Nakamura et al., 1998) and differentiation (Kakegawa et al., 1995). This concept of organogenesis can thus be used in laboratory conditions to potentially grow cells from a piece of fruit peel (by using callus culture suspension cultures) and subject them to many different environmental conditions, including temperature and light (Kakegawa et al., 1987), in order to study the regulation of anthocyanin synthesis.

2. Cell Suspension Cultures

Cell suspension cultures consist of cells that rapidly divide within a liquid medium (Evans et al., 2003). The term normally refers to dispersed single cells as well as some cell aggregates, seeing that a cell suspension culture consisting entirely of only single cells is rarely achieved (Evans et al., 2003). The cells within the medium proliferate and complete a growth cycle while suspended in the liquid medium. Cell suspension cultures, initiated from callus cultures, grow more rapidly than a callus culture and are more suitable for experimental manipulations. In order to establish and maintain a fine cell suspension culture, consisting of many dispersed single cell and some small cell aggregates, it is compulsory to select and subculture for several generations (Evans et al., 2003).

2.1 History

Making use of cell suspension techniques to study biological processes is a relatively new concept compared to other tissue culture work. Methods for generating plant callus tissue from several sources were only really well established in the 1950's, more than 130 years after the Cell Theory, suggesting totipotency of cells (Gautheret, 1983). Callus formation in various species and the process of wound healing were first described in 1853. In 1878, more detailed reports followed on the process of callus development (Gautheret, 1983). In 1902, the first but unsuccessful attempt at tissue culture was made by Gottlieb Haberlandt, which later led to root cultures, embryo cultures and the first true callus culture (Thorpe, 2007).

During the 1950's, the study of human cancers became a very popular field of research. Since it was understood that plant callus development shared similarities to mammalian cancer development, research laboratories intensified their studies on plant callus and suspension cultures. These studies on "plant cancers" were well rewarded with generous grants (Trigiano et al., 2005). The prediction was made in the early 1950's that a somatic plant cell could undergo embryogenesis. This idea was proved valid by Steward et al. (1958) and Reinert and Stewart (1958) who showed that somatic cells of carrots would differentiate into embryos when cultured within a proper nutrient medium. It led to the vision of great applications in propagation and genetic engineering. Today, somatic embryogenesis, or nonzygotic embryogenesis, has been demonstrated in most higher plant species (Trigiano et al., 2005). Murashige and Skoog (1962) developed a medium for rapid growth and bioassays with tobacco tissue cultures. This well established MS-medium is still being used as basic agar and liquid medium for callus growth as well as suspension growth.

2.2 Callus Development

A callus can be defined as an amorphous mass of unorganized (thin-walled) parenchyma cells (Evans et al., 2003). Callus formation can be seen at the cut surface of a wounded plant and is therefore thought to be a natural response by the plant to protect itself. In a culture, a callus can be initiated by simply placing a piece of plant tissue (called explant) on a solid culture media under aseptic conditions. The callus will then be induced and formed from proliferating cells. During initiation, the differentiated and specialized cells of the explant are

basically rejuvenated to an undifferentiated state. By simply applying the right growth medium, a callus can be initiated from a variety of tissues, depending on the species (Evans et al., 2003). In some species, rapid cell division is more easily induced. The presence of plant growth factors (hormones) in the medium enhances callus formation and proliferation (Gamborg and Phillips, 1995). Examples of such hormones are auxins and cytokinins that promote cell division and elongation.

Callus varies in appearance and physical features depending mostly on the parent tissue, growth conditions and the age of the callus (Evans et al., 2003). Callus may be white, green or coloured due to the absence or presence of chlorophyll and anthocyanin. In general, two types of callus can be defined. Type 1 callus is non-friable, regenerates somatic embryos and organs and frequently produces leaf-like structures. Type 2 callus is friable, undifferentiated and regenerates only somatic embryos (Evans et al., 2003). The growth of the callus can be monitored in several different ways including fresh weight measurements, dry weight measurements and by *in vitro* estimation of the callus diameter. The first two techniques are only really useful for optimizing the growth medium and/or conditions, seeing that it will result in the death of the culture (Evans et al., 2003).

2.3 Suspension Development

The time it takes to establish a cell suspension culture varies among species and the medium used for culturing will also play a significant role. Dicots more easily generate a suspension culture (Evans et al., 2003). High callus friability is an important factor for successful suspension initiation because of the need for easy fragmentation during agitation. Established cultures are sub-cultured every 1-3 weeks (when in early stationary growth phase) depending on the growth of the culture (Evans et al., 2003). The initiation of a suspension culture will usually entail the agitation of a healthy and vigorously *in vitro*-grown callus fragment in a liquid medium and on an orbital shaker. This breaks the callus into small masses of cells and single cells. The colour of the callus can give a good indication of the state of the callus. A light colour (white/cream) is generally indicative of a healthy callus whereas a dark brown callus most likely contains many dead cells (Evans et al., 2003). Evans Blue dye can be used to determine the number of healthy cells (staining of cells). Of course the most reliable method of determining viability and vigour of a callus is the culture growth rate (Evans et al., 2003).

Suspensions can be initiated from either a friable callus, a non-friable callus or from a callus treated with call wall degrading enzymes. Because of the easy fragmentation of a friable callus during agitation in a liquid medium, these calluses are the most commonly used. Considering the importance of friability in successful initiation of suspension cultures, procedures should be used to ensure that a suitable friable callus is produced. Examples of such procedures include cycling of a callus (e.g. 7-day cycle for 2-3 weeks) and ratio of hormones in the growth medium (e.g. higher auxin to cytokinin ratio) (Evans et al., 2003). The ratio of callus tissue to liquid medium at initiation is also important. For every 100 ml liquid medium used, the addition of about 2 to 3 g friable callus is recommended. The cells will start to break off from the callus in the liquid medium and form a suspension. It is necessary to subculture on a regular basis to fresh media to establish an actively growing culture of the desired density (Evans et al., 2003; Gamborg and Phillips, 1995). Pectinase, which breaks down the middle lamella of the plant cell wall and separates plant cells, is sometimes used as an enzyme treatment to promote suspension of cells (Evans et al., 2003).

According to Gamborg and Phillips (1995), the basic steps for initiation and maintenance of a cell suspension culture may consist of the following: pieces of broken-up calli are transferred to an Erlenmeyer flask containing the liquid medium. The importance of keeping the work area sterile is emphasized and the Erlenmeyer flasks must be capped. It is advisable to prepare additional replicate flasks. Incubation follows on a gyratory shaker for 1 week after which sub-culturing must be done weekly. For the first few subcultures, a portion of the spent medium should be removed and replaced with fresh medium. When the cell mass has doubled, the culture must be split into two flask (containing an equal amount of fresh medium) followed by the repeating of the incubation cycle. Upon the generation of a stable suspension culture consisting of finely dispersed cell clusters and aggregates, a dilution ratio of 1:4 to 1:10 old culture to fresh medium should be possible on a 7 to 10 day basis to maintain the cell line. A mesh can be used in order to obtain a suspension consisting of only fine aggregates and cell clusters.

A growth curve for a certain established cell suspension can be constructed as follows (Gamborg and Philips, 1995): Combine all replica cultures into a single batch for uniform

inoculums and prepare replicate suspensions from batch culture. Determine the zero-time value for the growth curve by spinning some of the culture in a centrifuge tube and measuring the volume of packed cells. Re-suspend the culture and incubate on rotary shaker. Repeat the centrifuge process every 2-3 days in order to attain 8-12 sampling times. Then the mean packed cell volume can be calculated as well as standard deviation for each sampling time. By using these data, a curve can be plotted as the growth curve of the established cell suspension.

2.4 Mediums Commonly Used

Mediums used for callus and suspension cultures will typically consist of a carbon source, inorganic salts, vitamins and growth regulators (plant hormones). Other components such as organic nitrogen, organic acids and/or plant extracts for example, can be added for specific purposes. The Murashige and Skoog medium (MS medium) (Table 1) (Murashige and Skoog, 1962), the Linsmaier and Skoog medium (LS-medium) (Linsmaier and Skoog, 1965) and the B5 medium (Table 1) (Gamborg et al., 1968) are the most frequently and widely used salt compositions. LS medium has the same salts as MS medium, but contains thiamone at 6.4 mg L^{-1} and 100 mg L^{-1} inositol instead of glycine and MS vitamins. Na_2EDTA and $FeSO_4.7H_2O$ can be replaced with Ferric Na EDTA and Sequestrene 300 Fe respectively for MS medium (Gamborg and Philips, 1995). Over the past few years, a number of media has been developed for specific purposes (Gamborg and Philips, 1995), e.g., basal media for tissue culture of cereals (N6, NN, ER and L2 mediums), media for woody species (DKW and WPM mediums), media for embryogenic soybean (FN and LV mediums) and specialized vitamin and organic supplements (B5 supplements, Kao vitamins and Koa organic acids).

According to Gamborg and Phillips (1995), compounds used as growth regulators include the following: cytokinins, e.g., benzyladenine (BA), isopentyl adenine (2-iP), kinetin (KIN) and zeatin (ZEA), synthetic cytokinins e.g., thidiazuron (TDZ), auxins, eg., indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), 1-naphthaleneacetic acid (NAA), 2,4-dichlorophenoxyacetic acid (2,4-D), 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) and picloram (PIC), gibberellic acid (GA3), abscisic acid (ABA) and silver nitrate (AgNO3).

2.5 Fruit Cell Suspension

Over the past few years, research on anthocyanins has been conducted utilizing suspension cultures from a range of different horticultural commodities such as strawberry (*Fragaria ananassa*), *Centaurea cyanus, Aralia codata*, grape (*Vitis vinifera*), carrot (*Daucus carota*), poplar (*Poplus deltoides*) and also pear (*Pyrus communis*).

2.5.1 Fragaria ananassa (Strawberry)

Mori and Sakurai (1996a) induced callus from the leaf of a strawberry plant (cv. Shikinari) by using LS medium containing 3% sucrose, 0.2% Gellangum, 0.1 mg L^{-1} BA and 1 mg L^{-1} 2,4-D. The tissue was incubated under a 16:8 h light-dark cycle. A cell suspension culture was initiated by transferring friable callus to liquid LS medium containing 3% sucrose, 1 mg L^{-1} 2,4-D and 0.1 mg L^{-1} BA. Mori and Sakurai published at least two further papers in the same year (Mori and Sakurai, 1996b; Mori et al., 1996) and again in 2001 (Mori et al., 2001) reporting on anthocyanin production in strawberry cell suspensions by using the same technique/recipe, but adding a conditioned medium (filtered culture medium) as reported previously (Mori et al., 1994). Seki et al. (1999) also used a LS solid medium for the FAR cell line (strawberry) to produce anthocyanin in the dark.

2.5.2 Aralia cordata (Traditional Japanese vegtable also known as "Udo")

Calli were induced from the leaves and stems of *Aralia cordata*. Culturing was done on MS agar medium supplemented with 3% sucrose, 1 mg L^{-1} 2,4-D and 0.1 mg L^{-1} kinetin. Incubation was in the dark, after which calli were maintained by periodic transfer to fresh media in a light-dark cycle. Cell lines were selected afterwards for further experimental procedures (Sakamoto et al., 1994).

2.5.3 Vitis sp.

Do and Cormier (1991) used Gramborg B5 medium supplemented with 250 mg L^{-1} casein hydrolysate, 0.1 mg L^{-1} NAA, 0.2 mg L^{-1} kinetin and 88 mM sucrose for their grape cell suspension. Seven day-old cultures were transferred to a basal medium supplemented with various concentrations of sucrose and mannitol for experimental procedures. However, it was

not reported on which part of the grape/ vine was used for the induction of calli to start off with.

Suzuki (1995) received the cell suspension used for his studies on anthocyanin accumulation due to pH and osmotic stress, as a gift. Thus no mention is given on the exact origin of the grape cells. The cell were however cultured in a myo-inositol, thiamine-HCL, 2,4-D and kinetin. Sub-culturing was done in continuous light conditions.

Decendit and Merillon (1996) investigated effective conditions for cell growth and polyphenol production (tannins and anthocyanin) by using a suspension culture derived from *V. vinifera*. The callus used to establish the cell suspension culture was provided by an external source. The suspension cultures were maintained under continuous fluorescent light. The maintenance medium contained B5 macro elements, MS microelements and vitamins, supplemented with sucrose, casein hydrolysate, NAA and kinetin. Merillon et al. (1998) made use of the methodology developed by Decendit and Merillon (1996) to investigate the regulation of polyphenol synthesis by sugars.

Macheix et al. (1995) used pulp fragments to generate calli, and thus cell suspensions of *V. vinifera* cv. Gamy Freaux in order to study the enhancement of anthocyanin synthesis in grape cell suspensions. The culturing medium consisted of Gamborg macro-elements, Murashige and Skoog microelements and Morel vitamins, supplemented with sucrose, casein hydrolysate and the growth factors kinetin and NAA. The pH was adjusted to 6.

2.5.4 Pyrus communis L. cv. Passe Crassane (European Pear)

Pech et al. (1979) developed pear fruit callus cultures from the outer pulp (receptacle). One of these cultures used were initiated in 1972 from young fruit, 45 days after full bloom, and another in 1975 from mature fruit picked at harvest. Calli from the same cultivar were also initiated from stem and leaf petioles in 1975 from one-month-old shoots. The medium used for culturing contained mineral nutrients from Murashige and Skoog (1962), sucrose, asparagin, ascorbic acid, thiourea, a vitamin solution containing Capanthotenate, inositol, biotin, nicotinic acid, thiamin and pyridoxin. Growth factors used were 2,4-D and 6-benzylaminopurine (BAP). The culture established in 1972 was used in various subsequent studies to, for example,

study the senescence of pear fruit cells cultured in a continually renewed, auxin-deprived medium (Pech and Romani, 1979; Pech et al., 1982), the stimulation of cyanide-resistant respiration in suspension cultures of senescent pear fruit cells by cycloheximide (Romani et al., 1981), ethylene production by pear fruit suspension cultures (Romani and Puschmann, 1983; Romani et al., 1985), and protein synthesis, and metabolic and respiratory responses of the suspensions cells (Lelievre et al., 1987, Romani et al., 1990, Kader et al., 1992). Pech and Romani (1979) developed cell culture methodology that permitted renewal of the medium without removal of cells.

3. Anthocyanin

Anthocyanins are water-soluble vacuolar flavonoid pigments that colour various plant organs, including leaves and fruits (Harborne and Grayer, 1988). Anthocyanins are found predominantly in outer cell layers such as the epidermis and the cell layers directly beneath the epidermis (Mazza and Miniati, 1993; Lancaster et al., 1994).

3.1 Biosynthesis - General

Biosynthesis of flavonoids and anthocyanins have been widely studied and is well understood, with the exception of a few enzymatic steps (Macheix et al., 1990). It has been the theme of various reviews (e.g. Heller and Forkman, 1988; Lancaster, 1992; Neill, 2002; Davies, 2009).

Anthocyanins are synthesized from two major precursory pathways: the phenylpropanoid pathway via the Shikimic acid pathway (in order to produce the amino acid phenylalanine) and the malonic acid pathway with the production of 3 molecules of malonyl-CoA (Hermann, 1995). The conversion of phenylalanine to trans-cinnamate, mediated by phenylalanine ammonia-lyase (PAL), is considered the first committed step in the synthesis of phenolic compounds. Phenylalanine from the Shikimate pathway (see Figure 1) is condensed with the 3 molecules malonyl-CoA (a 3 carbon unit derived from acetyl-CoA) by the enzyme chalcone synthase (CHS) to form chalcone (Koes et al., 1994). Chalcone is subsequently isomerized by enzymes such as chalcone isomerase (CHI) to the colourless pigment naringenin (a flavanone). Naringenin is oxidized by the enzymes flavanone hydroxylase (FHT / F3H),

flavonoid 3' hydroxylase and flavonoid 3'5'- hydroxylase. The products are subsequently reduced by the enzyme dihydroflavonol 4-reductase (DFR), to the corresponding leucoanthocyanidins. Leucoanthocyanidins are the direct precursors of the anthocyanidins, although the enzymatic steps catalyzing the conversion are not well understood (Heller and Forkmann, 1993). Anthocyanidin synthase (ANS) is believed to catalyze the 2-oxoglutarate-dependent oxidation of leucoanthocyanidin to 2-flavan-3,4-diol, which can then readily be converted to anthocyanidin by acidification (Heller and Forkmann, 1993). The resulting anthocyanidins, which are unstable, are further glycosylated by enzymes such as UDPGalactose: flavonoid-3-o –glycosyltransferase (UFGT). This results in the final and more stable anthocyanins. The colour intensity and stability of anthocyanins are determined by the number and position of hydroxyl groups, methyl groups, sugars and acylated sugars substituted to the molecule (Mazza and Miniati, 1993).

3.2 Regulation of Anthocyanin Biosynthesis in Pear

Most fruit experience a peak in anthocyanin synthesis during ripening, i.e. towards the harvest period (Saure, 1990). PAL activity increases with the accumulation of phenolic compounds including anthocyanin in many plant and fruit types including apple fruit (Lister et al., 1996). In apple fruit and grape berries, red colour development and thus anthocyanin accumulation, is seemingly regulated by the activity of the last enzyme of the biosynthetic pathway, UFGT (Ju et al., 1999; Kondo et al., 2002; Ban et al., 2003). UFGT activity has been strongly correlated with red colour developing in maturing apples (Lister et al., 1996). Steyn et al. (2004a) reported that UFGT activity is not likely to be the limiting factor for anthocyanin synthesis in pear peel, since UFGT activity in 'Rosemarie' and 'Bon Rouge' pears was found to increase during fruit development whereas red colour decreased.

Pear fruit attain their highest anthocyanin concentration midway between anthesis and harvest (Steyn et al., 2004a). The anthocyanin concentration, and red colour, decreases towards harvest due to a combination of decreasing synthesis, degradation at high temperatures and dilution (Steyn et al., 2004a, b).This means that the fruit colour at harvest will be the result of the competing factors, namely maximum anthocyanin concentration reached vs. the severity of colour loss and dilution towards harvest.

Environmental factors that contribute to anthocyanin accumulation in pear include temperature and light, although high temperatures together with high light, contribute to the fading of red colour due to degradation of anthocyanin (Steyn et al., 2004b).
Light is essential for anthocyanin synthesis in most fruit and plant tissues (Mancinelli, 1983). The rate of anthocyanin synthesis in apples increases linearly with the level of light energy they are subjected to (Proctor, 1974). The anthocyanin concentration of apples within the raceme, is determined by the bearing position and position within the tree canopy and relates to the light levels that they received (Awad et al., 2000). Light is also a culprit when it comes to degradation of anthocyanin (Francis, 1989; Steyn et al., 2004b). Steyn et al. (2004b) reported that under limiting conditions for anthocyanin synthesis, light will probably contribute more to anthocyanin degradation in pear peel than to synthesis. Shading of 'Sensation Red Bartlett' pears during the month before harvest, decreased anthocyanin degradation (Dussi et al., 1995). Apple fruit, in contrast, require high light intensities during the ripening stage which is the stage of maximum anthocyanin accumulation (Macheix et al., 1990; Saure, 1990).

Whereas colour development can be induced in detached apple fruit (Curry, 1997), Marais et al. (2001b) has not been able to induce anthocyanin synthesis in detached pear fruit. This makes it more difficult to determine optimum temperatures for anthocyanin synthesis for pear fruit.

Reay (1999) reported that anthocyanin accumulation in detached 'Granny Smith' apples benefited from induction at low temperatures (4 ºC), while subsequent accumulation of anthocyanin required irradiation at higher temperatures (20 ºC). Anthocyanin synthesis in all apple cultivars that have been studied thus far benefited from low temperatures (Curry, 1997; Reay, 1999; Marais et al., 2001a). PAL, together with other enzymes forming part of the anthocyanin biosynthesis pathway including CHS and CHI, have been shown to be low-temperature inducible in apple fruit (Faragher, 1983; Tan, 1980). Pears generally do not increase in red colour in response to low temperatures (Steyn et al., 2004a; Steyn et al., 2005). There is, however, the exception of 'Rosemarie', which does require low temperature for red colour development. Steyn et al. (2004a) reported that PAL and UFGT activity as well as red colour increased with the passing of a cold front. PAL and UFGT activity showed a strong negative correlation with daily minimum temperatures. This suggests induction of

anthocyanin synthesis at low temperatures. In ‘Bon Rouge’, enzyme activity and red colour did not increase in response to low temperatures (Steyn et al., 2004b).

3.3 Anthocyanin Synthesis in the Laboratory

Anthocyanin accumulation in suspension cultures has been studied with cell cultures derived from many different horticultural commodities including strawberries (Mori and Sakurai, 1996a, b), flowers (e.g. *Centaurea cyanus*) (Kakegawa et al., 1987), carrots (Ozeki, 1996), grapes (Macheix et al., 1995), poplar (Tholakalabavi et al., 1997), apples (Li et al., 2004) and pears (Pech et al., 1979).

Light seems to be an important factor when it comes to anthocyanin synthesis even with cell suspension cultures. Generally, anthocyanin synthesis is prevented when cultures are kept in the dark (Kakegawa et al., 1987). However, when cultures are irradiated with ultraviolet (UV) and white light, anthocyanin synthesis is induced (Kakegawa et al., 1987; Seki et al, 2000). Anthocyanins usually only accumulate in small amounts within cultured cell lines (Seki et al., 1999), and as mentioned above, requires strong light irradiation for synthesis. Some plant cell cultures, however, have been reported to produce anthocyanins in the dark (e.g. *Daucus carota*, *Vitis* hybrid and *Aralia cordata*), although only very low levels were attained (Dougall et al., 1980; Yamakawa et al., 1983). Producing anthocyanin at a commercially viable level (as natural colourant) has proved a difficult task (Sakamoto et al., 1994). It is also expensive to operate a photo-bioreactor, which produces a high light intensity. Thus for commercial applications, it would be preferable to produce anthocyanin in the dark. Seki et al. (1999) reported a cell line from strawberry callus that produced anthocyanin at high enough levels in the dark to be considered for the industrial production of anthocyanin. Seki et al. (2000) concluded in a later report that anthocyanin production in strawberry cells does not only depend on light intensity, but requires a light and dark cycle with second- or hour-scale periods. This was an enhancement on an earlier study that concluded that the production of anthocyanin in strawberry cells was greatly induced by high light intensity (Mori et al., 1993; Seki et al., 2000).

Increasing the osmotic potential of the culture medium by increasing the sucrose concentration or by adding manitol to the culture medium (*Vitis vinifera*) caused a significant

increase in anthocyanin accumulation in pigmented cells (Do and Cormier, 1991; Suzuki, 1995; Tholakalabavi et al., 1997). Suzuki (1995) reported increasing anthocyanin accumulation in cultured cells with increasing D-mannitol concentrations and thus osmotic stress. The proportion of pigmented cells to non-pigmented cells also increased, but cell growth was repressed with increasing osmolarity of the media. Increasing sucrose concentrations in suspension cultures proved to stimulate anthocyanin accumulation (Cormier et al., 1990; Yamakawa et al., 1983; Matsumoto et al., 1973), and has been regarded as providing a good carbon source. The stimulation of the methylation due to the higher osmotic potential and, therefore, the stability of anthocyanins has been described as the possible and most likely reason for this effect (Do and Cormier, 1991). Anthocyanin production was reduced in LS, MS and B5 basal mediums in both light and dark conditions when the sucrose concentrations used exceeded 5 % (v/w) (Sakamoto et al., 1994). Sucrose concentration of 9 % and 12 % (v/w) respectively, resulted in growth reduction in all media. The higher sucrose concentration limits anthocyanin accumulation probably because of the higher osmotic strength of the media, which could negatively affect the water content of the vacuole. Sakamoto et al. (1994) reported an optimum sucrose concentration for the highest anthocyanin production to be 2 % for LS medium in the dark and 2 % for B5 medium in the light, respectively. The best overall conditions for anthocyanin production in light and dark were on LS medium with a sucrose concentration of 4 % and 2 %, respectively.

Anthocyanin production purportedly benefits from a higher ratio of NO^{-3} / NH^{+4} (reported but no data shown), although in the dark, cell growth is increased if N is decreased to 20% of the total nitrogen of the standard medium (Sakamoto et al., 1994). Both cell growth and anthocyanin concentration decrease with increasing pH of the basal medium (Suzuki, 1995). Anthocyanin production was higher in media with a low pH (4.5) than in neutral media (pH 7). The mechanisms of anthocyanin induction could possibly differ in response to the conditions used to induce osmotic stress and various pH values of the media (Suzuki, 1995). However, Furusaki and Zhang (1997) reported that although a pH of 4.5 to 5.0 was favorable for cell growth and anthocyanin synthesis after inoculation – with no lag phase or adaptation period - the maximum anthocyanin production of suspended strawberry cells was obtained at pH 8.7.

Stuart and Street (1969) used filtered culture medium to stimulate cell growth of a subsequent culture. Their supposition is that conditioning factors are produced and released by cultured plant cells into the culturing medium. These cultured cells release metabolites into the medium during the lag phase prior to initiation of cell division to sufficient levels for growth to initiate, i.e. cell cycle initiation. These conditioning factors then promote cell growth and cell division. Mori and Sakurai (1996b) reported that anthocyanin accumulation could be enhanced by using 'Conditioned Medium'.

Sakuta et al. (1994) reported on the regulatory mechanisms of biosynthesis of anthocyanin in relation to cell division activity in *Vitis* sp. suspension cultures. Inhibition of cell division by means of a DNA inhibitor (aphidicolin) or reduction of phosphate concentration in the medium resulted in rapid accumulation of anthocyanin coinciding with the cessation of cell division. CHS and PAL activity increased to high levels when transfers were done to fresh medium, but decreased thereafter and remained at low activity levels during the exponential phase of cell division. When cell division ceased, PAL and CHS activity increased to high levels and remained at these high levels for the duration of anthocyanin accumulation (Sakuta et al., 1994). A deficiency in inorganic phosphate during culture led to growth reduction, anthocyanin production and increased dihydroflavanol reductase (DFR) activity in the cell suspension (Macheix et al., 1995).

4. Discussion

Research on the use of plant cell cultures (including callus cultures and cell suspension cultures) have increased exponentially over the last half century, with a wide range of applications and implications for agricultural, horticulture and forestry.

Cell suspension cultures from different fruit types have been established and used for studying anthocyanin accumulation under different conditions. However, opposing conditions are required for induction of anthocyanin synthesis and for maintaining active cell division for culture growth (Ozeki, 1996). Hence, in most cultured plant cells, the activities of secondary metabolism (including the flavonoid pathway) are much lower than in differentiated organs and tissues of intact plants (Ozeki, 1996).

Pear cell suspension cultures have been widely used in research. Hence, in theory it seems possible to establish a pear cell suspension culture for studying temperature effects on anthocyanin accumulation in pear. As mentioned, pear fruit do not develop red colour once removed from the tree and so it is a difficult task to establish an optimum temperature range for colour development in pear fruit. Although pear cell suspensions may allow the study of anthocyanin accumulation in pear fruit, optimum temperatures for anthocyanin synthesis in culture may differ from optimum temperatures for anthocyanin synthesis in fruit. In the literature cited for this review, most cell suspension cultures are maintained at 25 ºC seeing that the temperature effect on anthocyanin synthesis was not studied. Optimum temperatures for anthocyanin accumulation in the epidermal layer of different apple cultivars were reported tot be in the range of 23 ºC tot 27 ºC (Curry, 1997; Arakawa and Bakhshi, 2006). Although these temperatures appear to correlate with the optimum temperature for anthocyanin synthesis in cultures, induction at low temperatures may be required as in intact apples (Reay, 1999; Curry, 1997). Also, the secondary metabolic pathway of cultured cells may differ from that in differentiated organs and intact plants. Thus, optimum temperatures for anthocyanin accumulation in intact fruit (differentiated organ) may very likely differ from optimum temperatures for anthocyanin accumulation in a culture of cells (from the same fruit). The effect of temperature, not falling in the optimum range for anthocyanin accumulation on a suspension culture, may be different from the effect of temperatures on the whole fruit.

Few of the publications on pear cell suspensions provide a complete list of the exact materials and methods that were followed. This is because most of the cell suspension cultures were obtained from elsewhere. In one of the oldest publications on pear cell suspension cultures (Pech et al., 1979), it is mentioned that the callus cultures derived from the outer pulp of pears was already established in 1972 and 1975, with the procedures being described elsewhere. Subsequent papers all refer to the already established callus cultures from 1972 or the paper of Pech et al. (1979) for detail on the establishment of the cultures. In most of the work published, the researchers do, however, explain the conditions under which the suspensions were held and sub-cultured. It may prove difficult and time consuming to validate and repeat these studies if an established strain of suspension cultures is not readily accessible. Also, establishing a suspension culture will require access to culture facilities, and arduous study

and training in the concepts and techniques of general biotechnology, sterile techniques, media preparation, tissue cultures, callus development and cell suspension cultures.

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Table 1. Composition of MS and B5 basal media (Gamborg and Phillips, 1995)

Component	MS		B5	
Major salts	*mg L^{-1}*	*mM*	*mg L^{-1}*	*mM*
NH_4NO_3	1650	20.6		
KNO_3	1900	18.8	2500	25
$CaCl_2.2H_2O$	440	3.0	150	1.0
$MgSO_4.7H_2O$	370	1.5	250	1.0
KH_2PO_4	170	1.25		
$(NH_4)_2SO_4$			134	1.0
$NaH_2PO_4. H_2O$			150	1.1
Minor salts	*mg/L*	*µM*	*mg/L*	*µM*
KI	0.83	5.0	0.75	4.5
H_3BO_3	6.2	100	3.0	50
$MnSO_4.4H_2O$	22.3	100		
$MnSO_4.H_2O$			10	60
$ZnSO_4.7H_2O$	8.6	30	2.0	7.0
$NaMoO_4.H_2O$	0.25	1.0	0.25	1.0
$CuSO_4.5H_2O$	0.025	0.1	0.025	0.1
$CoCl.6H_2O$	0.025	0.1	0.025	0.1
Na_2EDTA	37.3	100	37.3	100
$FeSO_4.7H_2O$	27.8	100	27.8	100
Vitamins and Organics				
myo-Inositol	100	555	100	555
Nicotinic acid	0.5	4	1.0	8
Pyridoxine HCL	0.5	2.5	1.0	5
Thiamine HCL	0.1	0.3	10	30
Glycine	2.0	27		
Sucrose	30 g		20 g	
pH	5.8		5.5	

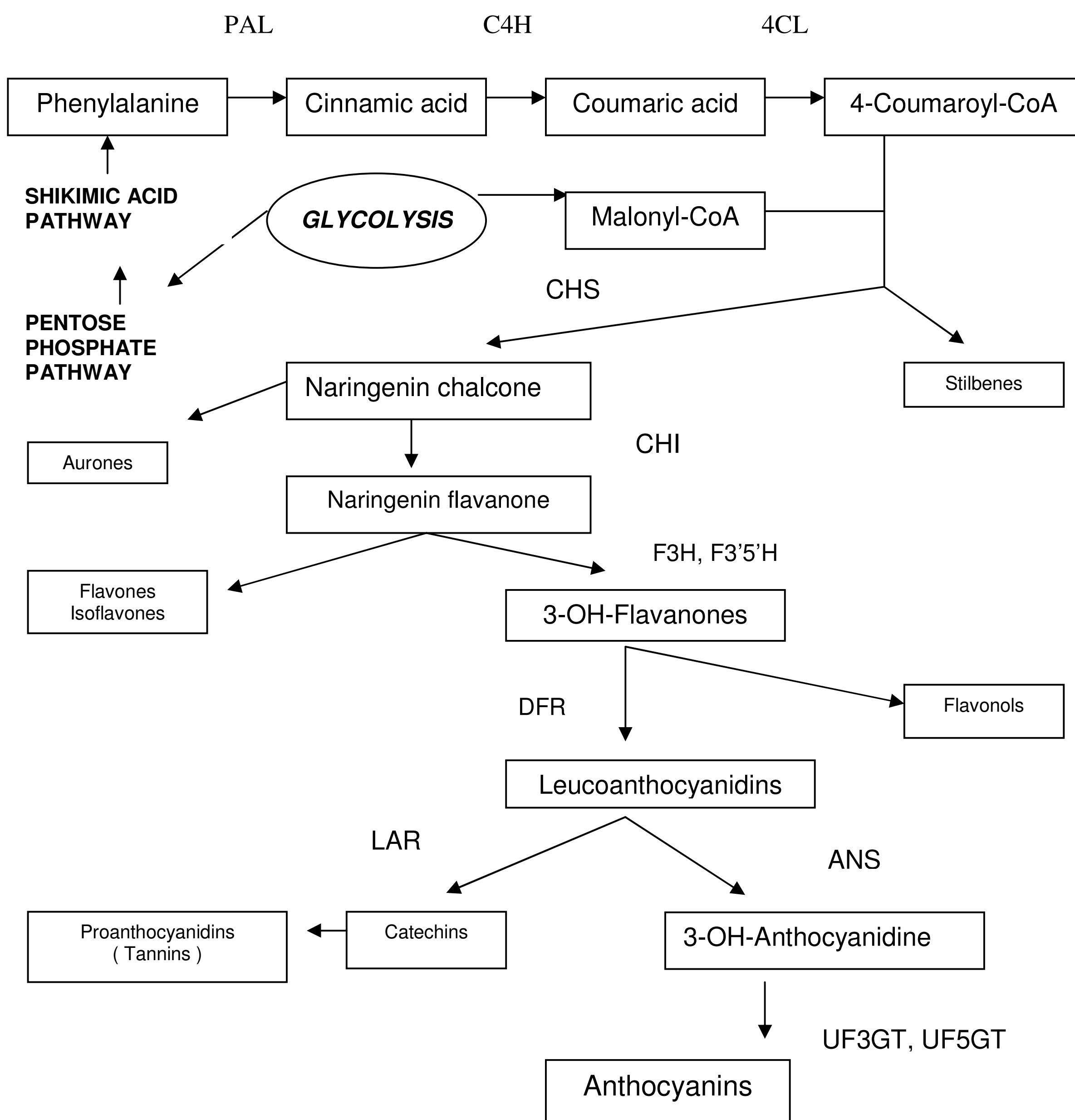

Figure 1. The flavonoid pathway based on the review by Neill (2002), modified from Shirley (1996). Abbreviations: PAL, phenylalanine ammonia-lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumarate:CoA ligase; CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone 3-hydroxylase; F3'H, flavonoid 3-hydroxylase; DFR, dihydroflavonol reductase; ANS, anthocyanin synthase (leucoanthocyanidin dioxygenase); UFGT, UDPGalactose: flavonoid-3-o-glycosyltransferase. CHS is considered as the first enzyme of the flavonoid biosynthetic pathway. PAL form part of the phenylpropanoid pathway.

PAPER 1: OPTIMUM DAY TEMPERATURES FOR RED COLOUR DEVELOPMENT IN APPLE FRUIT

Abstract

This study focused on determining the optimum day-time temperatures for anthocyanin accumulation for red and bi-coloured apple cultivars grown in South Africa. 'Royal Gala' (RG), 'Fuji' (FJ), 'Braeburn' (BB), 'Early Red One' (ERO) and 'Cripps' Pink' (CP) were sampled from two distinctly different production areas in the Western Cape. Peel discs were punched from the shaded sides of fruit, placed on Peltier temperature plates set to a temperature range from 16 to 31 ºC with 3 ºC intervals and exposed to photosynthetic photon flux (PPF) of 550 to 650 µmol m^{-2} s^{-1} for 72 hours where after their change in hue was determined. During the 2007/2008 growing season, the study mainly focused on determining optimum temperatures for red colour development at the peak of anthocyanin synthesis, i.e., from two weeks before the onset of commercial harvest. During the 2008/2009 season, the effect of temperatures on anthocyanin synthesis were evaluated at regular intervals throughout fruit development. Although apples from Ceres generally developed redder colour than apples from Grabouw, the response to temperature was the same in both areas. Colour development generally showed a quadratic response to temperature with reddest colour developing from 17 to 25 ºC. The response to temperature was less clearly defined in ERO where colour developed over a broader temperature range. The temperature optima for red colour development were appreciably lower in 2008/2009 compared to 2007/2008 for RG, FJ and BB suggesting that prior climatic conditions affect the potential to synthesise anthocyanin. Cultivars and their strains showed the same response to temperature with regard to red colour development. The optimum temperature for red colour development increased during fruit development in CP, but not in RG, BB, FJ and ERO. All cultivars showed an increase in the extent of red colour development from early fruit development until approximately halfway towards commercial harvest.

Introduction

Fruit colour plays an important role in the international markets. Preference is generally given to the better-coloured apple fruit and thus fruit colour will be a factor when it comes to grading

fruit for packing and exporting (Kevany et al., 2003; Reay, 1999). According to local packing specifications (Department of Agriculture, Forestry and Fisheries, 2010), Class 1 fruit of bi-coloured cultivars, e.g. Braeburn, Fuji, Royal Gala and Cripps' Pink require a minimum of 40 to 50% red blush coverage.

Two periods of colour development in the epidermal tissue of apple fruit can be distinguished. The first period occurs during the cell division phase, even in non-red apple cultivars, such as Golden Delicious and Granny Smith (Lancaster, 1992; Saure, 1990). The second period of colour development occurs from approximately halfway during fruit development until harvest and again some non-red cultivars are capable of some anthocyanin formation at this later stage of fruit development (Curry, 1997; Reay, 1999). Most fruit experience a peak in anthocyanin synthesis during ripening (Steyn, 2009).

Light and temperature are the major factors that determine the extent of red colour development in apple fruit (Lancaster, 1992; Reay & Lancaster, 2001; Saure 1990). Light is not considered to be limiting to red colour development in South Africa (Pretorius and Wand, 2003). Anthocyanins in ripening apples are apparently induced at low temperatures (<10 ºC) (Curry, 1997) and synthesis takes place under irradiation at mild temperatures (20 ºC to 27 ºC) in detached, mature apples (Curry 1997; Reay, 1999; Saure, 1990 citing Nauman, 1964). Faragher (1983) found that maximum anthocyanin accumulation in attached immature 'Jonathan' apples occurred at 12 ºC and for mature fruit at 16 to 24 ºC. The effect of temperature on red colour development is cultivar dependent (Saure, 1990). Curry (1997) reported that different apple cultivars have different optimum temperatures for optimum colour development (i.e., 21, 23 and 25 ºC for Braeburn, Gala and Fuji, respectively). Detached climacteric 'Red Chief Delicious' apples had a higher optimum temperature for anthocyanin accumulation (27 ^{o}C) compared to pre-climacteric apples (25 ^{o}C). Reay (1999) found two thirds less anthocyanin synthesis in 'Granny Smith' peel at a day temperature of 30 ºC compared to 20 ºC. A 3 h high temperature (30 ºC) pre-treatment also reduced subsequent anthocyanin synthesis at 20 ºC (Reay, 1999). Apart from inhibiting anthocyanin synthesis, high temperature and irradiation also accelerates anthocyanin degradation. Irradiation of well-coloured 'Cripps' Pink' apples for 144 h at 37 ºC resulted in a ≈50% decrease in anthocyanin and a 19º hue increase (Marais et al., 2001b).

Considering the importance of low night and mild day temperatures for anthocyanin synthesis in apple peel, it is not surprising that the high temperatures experienced in warm production areas such as the Western Cape province give rise to poor red colour development (Reay, 1999; Wand et al., 2005; Wand et al., 2002). Since different cultivars have different optimum temperatures for anthocyanin synthesis, not all may be equally suited for all production areas. Knowing the optimum temperature for colour development for different cultivars can provide a good indication of where these cultivars can be grown.

This study focused on determining the optimum day-time temperatures for anthocyanin accumulation for red and bi-coloured apple cultivars grown in South Africa, viz. Royal Gala, Braeburn, Fuji, Early Red One and Cripps' Pink. During the 2007/2008 growing season, the study mainly focused on determining optimum temperatures for red colour development at the peak of anthocyanin synthesis from 2 weeks before scheduled harvest until commercial harvest. Redder strains of some of these cultivars were also evaluated to determine whether their enhanced anthocyanin synthesis is due to a shift in the optimum temperature for anthocyanin synthesis. Fruit were harvested from two different production areas to assess whether growing conditions may influence the temperature requirements for anthocyanin synthesis. During the 2008/2009 season, the effective temperatures for anthocyanin synthesis were evaluated at regular intervals throughout fruit development.

Materials and method

Plant material:

'Royal Gala' (RG), 'Royal Beaut' (RB), 'Early Red One' (ERO), 'Braeburn Braestar' (BB), 'Braeburn Frasier' (BF), 'Fuji' (FJ), 'Fuji Raku Raku' (FR) and 'Cripps' Pink' (CP) fruit were obtained from Oakvalley Estate in Grabouw (latitude: 34º 08' S, longitude: 19º 02' E, Altitude: 300 m), while RG, ERO, BB, FJ and CP fruit were obtained from Vastrap in Ceres (latitude: 33º 14' S, longitude: 19º 14' E, Altitude: 890 m). Both these regions are in the Mediterranean-type climate of the Western Cape Province of South Africa. Average daily minimum and maximum temperatures recorded for these regions during the relevant months of the 2007-08 and 2008-09 seasons are presented in Table 1 and 2, respectively. Apples were picked at random before 1100 HR from the same orchard row on each of the harvest dates with one

apple picked from the inner canopy of each of six (RG, RB and CP) or 12 trees. Only apples with a green/shaded side were picked. Fruit were placed in a cooler bag for transport to our laboratory and stored in the dark at 4 ºC for 72 h to induce anthocyanin synthesis (Curry, 1997).

2007/2008 season: Fruit were harvested on 28 January, 1 February and 8 February for RG and RB; 29 February, 7 March and 14 March for ERO, BB, BF, FJ, FR; and 4 April, 11 April and 18 April for CP. For RG, RB and CP six peel discs (15 mm in diameter, 5 mm thick) were punched from the shaded side of each apple to yield a total of 36 discs. Discs were randomly placed on the 12 peltier plates (5 cm x 6.5 cm) of a Celtec, constructed according to the design of Burke and Mahan (1993), with a H_2O-moistend filter paper between the discs and the plate. Each plate contained 3 discs for each of RG-Ceres, RG-Grabouw, RB, CP-Ceres and CP-Grabouw. Each Peltier plate was covered with thin (0.5 mm) 100% crystal clear polyethylene wrap (Glad Wrap™, Glad products, Glad South Africa, Randburg, South Africa). A few holes were made in the plastic with a toothpick to prevent the build-up of CO_2 and, possibly, ethylene and to reduce condensation of water on the inside of the plastic.

For FJ, FR, BB , BF and ERO, one disc was punched from the shaded side of each apple to yield a total of 12 discs for each of FJ-Ceres, FJ-Grabouw, FR, BB-Ceres, BB-Grabouw, BF, ERO-Ceres and ERO-Grabouw. Discs were randomly placed on the 12 peltier plates of the Celtec as ascribed above so that each cultivar area combination was represented by 1 disc per plate.

Temperature treatments:

Set temperatures of 16 ºC, 19 ºC, 22 ºC, 25 ºC, 28 ºC and 31 ºC were randomly assigned to the 12 plates of the Celtec. The Celtec was placed in a growth cabinet set at 12 ºC with 2 overhead lamps (400W High Pressure Sodium; SON-T; Osram Mgbh, Munich, Germany) providing irradiance of 550 to 650 µmol m^{-2} s^{-1} photosynthetic photon flux (PPF) measured with a quantum meter (LI-189; Li-Cor, Lincoln, Nebraska, USA) at disc level. Disc temperature was measured with an infrared thermometer (Raynger MX4, Raytek Corporation, Santa Cruz, USA) to ensure that the required temperatures were maintained. Peel temperatures of discs

in each plate, as well as the moisture level of the filter paper on which discs were placed, were assessed at least twice daily.

Colour measurement:

Disc hue angle was measured with a chromameter (NR-3000; Nippon Denshoku, Tokyo, Japan) before and again 96 h after placement on the Celtec. Hue angle (0º = red-purple, 90º = yellow, 180º = blue-green and 270º = blue) is the most relevant measurement to express the differences in color development in this study (McGuire, 1992).

Maturity indexing:

Firmness (by means of a penetrometer using an 8 mm plunger) and starch breakdown (by brushing iodine on a freshly cut apple half to stain the internal starch and therefore indicating the level of unconverted sugar) of apples were assessed. Internal ethylene levels were measured at harvest during the 2007/2008 season, which proved the fruit to be in the desired pre-climacteric state, i.e., core ethylene <0.5 µl L^{-1}.

2008/2009 season: RG, BB, FJ, ERO and CP apples were harvested from Grabouw and Ceres from shortly after fruit set until commercial harvest. The respective harvest dates are presented in Table 3. Twelve apples were harvested on each date for every area cultivar combination with one disc punched from the green, shaded side of each apple. The same Celtec setup as for 2007/2008 was used and each area cultivar combination was represented by one disc per temperature plate. Hue angles of discs were measured before and 96 h after being placed on the Celtec. Fruit firmness and starch breakdown was assessed as mentioned above, while fruit diameter was also measured.

Statistical analysis.

The data were analyzed with the General Linear Models (GLM) procedures of Statistica version 9 (Statsoft Inc., Tulsa, Oklahoma), and this data analysis software was also used to fit nonlinear curves to data in order to calculate the inflexion point for each temperature curve according to the equation $Y = a^0(a^1 + a^2x - 0.5\ x^2)$

Results

Red colour development before commercial harvest in 2007/2008.

No area-temperature or strain-temperature interaction occurred (Tables 4 and 5). Therefore, the main effects are presented.

During the peak period of anthocyanin synthesis in 2007/2008, BB and FJ apples from Ceres showed a greater hue decrease during 72 h irradiation, i.e., a greater increase in red colour, compared to fruit from Grabouw (Fig. 1a). ERO and CP from Ceres appeared to increase more in red colour than fruit from Grabouw, but these area differences were not significant ($p<0.1$) (Table 4).

Cultivars and their strains showed the same response to temperature with regard to red colour development (Fig. 1c). RB increased more in red colour than RG whereas FJ Raku Raku developed less red colour than standard FJ (Fig. 1c). No difference in colour development was observed between standard BB and BF (Table 5).

According to a quadratic trend line fitted to the data of RG (R^2 = 0.89), FJ, (R^2 = 0.90) BB (R^2 = 0.92), ERO (R^2 = 0.98) and CP (R^2 = 0.79), the temperature optima (inflexion point) for red colour development were 25.16 ± 0.52 ºC, 22.72 ± 0.44 ºC, 21.13 ± 0.90 ºC, 18.73 ± 1.93 ºC and 22.92 ± 0.37 ºC, respectively (Table 6). There was no difference in red colour development in RG at 22 to 28 ºC while the least red colour developed at 16 and 19 ºC (Fig. 2a). Less red colour developed at 31 ºC than at 25ºC. In FJ, there was no difference in red colour development at 22 and 25 ºC, but less red colour developed at 16, 19 and 28 ºC. At 31 ºC the least red colour developed (Fig. 2b). Red colour development in BB occurred maximally between 19 and 25 ºC with less red colour developing at 28 and 31 ºC. At 16 ºC less red colour developed than at 22 to 25 ºC but more than at 31 ºC (Fig. 2c). In ERO, red colour development occurred maximally at 16 to 25 ºC with less red colour developing at 28 and 31 ºC (Fig. 2d). In CP, red colour development occurred maximally at 22 ºC with less red colour developing at 16, 19, 25, 28 and 31 ºC (Fig. 2e). At 19 and 28 ºC more red colour developed than at 16 and 31 ºC. Red colour development was more at 25 ºC than at 31 ºC.

Red colour development during the 2008/2009 season.

Three-way interaction between temperature, stage of fruit development and area did not occur and there was also no interaction between area and temperature or between stage of fruit development and area (Table 7).

RG and FJ from Ceres increased more in red colour than fruit from Grabouw during the 2008/2009 season, BB and ERO did not show a area difference while CP from Grabouw appeared to increase more in colour than CP from Ceres, although the difference was not significant (p=0.0761) (Fig. 1b).

According to a quadratic trend line fitted to the data of RG (R^2 = 0.94), FJ, (R^2 = 0.94) BB (R^2 = 0.93) and ERO (R^2 = 0.80), the temperature optima for red colour development in 2008/2009 were 18.39 ± 2.08 ºC, 17.41 ± 2.16 ºC, 19.12 ± 1.42 ºC and 21.75 ± 1.29 ºC, respectively (Table 6). Hence, the optimum temperatures for red colour development were lower for RG, FJ and BB and higher for ERO in 2008/2009 compared to 2008 (Table 6). Although CP was the only cultivar to show a significant change in temperature optima for red colour development during fruit development (Table 6), temperature optima during the last three harvests in 2009 were determined in order to compare temperature optima between seasons. Even then, temperature optima for RG (20.44 ± 1.89 ºC) and FJ (18.62 ± 2.10 ºC) were still appreciably lower than in the previous season. The temperature optimum for BB (19.95 ± 1.28 ºC) was comparable between seasons while ERO had a higher temperature optimum during 2008/2009 (21.70 ± 1.27 ºC) than in 2008 (Table 6). There was no difference in red colour development in RG, FJ and BB at 16 to 22 ºC while the least red colour developed at 28 and 31 ºC (Fig. 3a, b and c). Less red colour developed at 25 ºC than at 19 ºC (RG), 16 to 22 ºC (FJ) and 19 to 22 ºC (BB). ERO showed a comparable change in hue over the entire temperature range (Fig. 3d).

Interaction between the stage of fruit development and temperature was found for CP, but not for RG, BB, FJ and ERO (Table 7). The latter cultivars all showed an increase in red colour development from early fruit development until approximately halfway towards commercial harvest (Fig. 4a, b, c and d). CP apples harvested on 21 Nov 2008 showed a similar small change in hue to irradiation over the entire temperature range (Fig. 5a). The optimum

temperature for red colour development gradually increased during fruit development, but more gradually after 20 March until the final collection date (3 April 2009). According to a quadratic trend line fitted to the CP data on 16 January 2009 (R^2 = 0.93), 20 February 2009 (R^2 = 0.84), 20 March 2009 (R^2 = 0.82) and 3 April (R^2 = 0.93), the temperature optima for red colour development were 13.91 ± 6.12 ºC, 20.23 ± 0.84 ºC, 21.21 ± 0.67 ºC and 22.34 ± 0.46 ºC respectively (Fig. 5b). There was no difference in red colour development on 16 January 2009 between 16 to 22 ºC while the least red colour developed at 28 and 31 ºC (Fig. 5a). Less red colour developed at 25 ºC than at 19 ºC. There was no difference in red colour development on 20 February 2009 at 19 to 22 ºC while the least red colour developed at 28 and 31 ºC (Fig. 5a). Less red colour developed at 16 and 25 ºC than at 19 ºC. There was no difference in red colour development on 20 March 2009 at 19 to 22 ºC while the least red colour developed at 28 and 31 ºC (Fig. 5a). Less red colour developed at 16 and 25 ºC than at 22 ºC. There was no difference in red colour development on 3 April 2009 at 22 to 25 ºC while the least red colour developed at 31 ºC (Fig. 5a). Less red colour developed at 16, 19 and 28 ºC than at 22 ºC.

Discussion

Our data indicate that redder strains of bi-coloured apple cultivars do not owe their enhanced pigmentation to a higher temperature optimum for anthocyanin synthesis. Instead, their redder colour (as observed in the field) is most likely due to a general up regulation of anthocyanin synthesis transcription factors (Espley et al., 2007). FJ Raku Raku developed significantly less red colour than standard FJ, Royal Beaut developed redder colour than standard RG while BB and BB Frasier did not differ in colour. However, fruit of these strains did appear redder when viewed in the orchard at harvest compared to their respective parents (personal observation). It is uncertain why the colouring potential of FJ Raku Raku and BB Frasier was less under laboratory conditions than in the orchard.

In both seasons, FJ from Ceres developed significantly redder colour than FJ from Grabouw. ERO showed the same trend, although area differences were not significant. BB and RG from Ceres developed redder colour in 2007/2008 and 2008/2009, respectively. CP from Ceres and Grabouw did not differ in their ability to develop red colour.

Since the extent of anthocyanin synthesis in apples of the same cultivar can be affected by cultural practices such as pruning, thinning, irrigation, fertilization, especially excess nitrogen, rootstocks (Saure, 1990) and since fruit were collected from only one farm in each area, the observed regional differences in the ability to develop red colour should be interpreted with caution. However, on the whole, it does appear that fruit from Ceres generally developed redder colour than fruit from Grabouw. It is possible that the warmer Grabouw climate (Table 1) might inhibit anthocyanin synthesis compared to the cooler Ceres climate. Reay (1999) found that a 3 h pre-treatment at 30 ºC reduced subsequent anthocyanin synthesis at 20 ºC by two thirds compared to pre-treatment at 20 ºC. To validate our results, these experiments have to be repeated with a minimum of three experimental orchards per cultivar from each production area.

Light is required for anthocyanin synthesis in apple (Lancaster, 1992; Saure, 1990). However, due to radiant heating of apple peel exposed to high irradiance (Schrader et al., 2003), the optimum temperatures for red colour development *in situ* should be lower than the temperatures reported here. In addition, detached apples have a higher temperature optimum for anthocyanin synthesis than attached fruit, possibly as a result of accelerated ripening (Ritenour and Khemira, 1997). Previously, Faragher (1983) as well as Diener and Naumann (1981) found that the temperature optimum for anthocyanin accumulation in whole detached apples increased during fruit development. In the current study, the optimum temperatures of detached apple cultivars seemed to differ between seasons, but not between Ceres and Grabouw (Table 7). CP in 2008/2009 was the only cultivar that showed a gradual increase in optimum temperature for colour development as the season progressed with the optimum temperature around harvest being similar for both seasons. The increase in optimum temperature during fruit development concurs with previous research by Faragher (1983). The optimum temperature for RG, FJ and BB red colour development was lower in 2008/2009 than during the 2007/2008 harvest period. The biggest difference in optimum temperature between 2007/2008 and 2008/2009 was for RG (25.1 ± 0.5 ºC vs. 18.4 ± 2 ºC) and FJ (22.7 ± 0.4 ºC vs. 17.4 ± 2.2 ºC). Although there were no interaction between stage of fruit development and temperature throughout the 2008/2009 season, the optimum temperatures for colour development in RG, FJ and BB for the last three harvest dates was slightly higher than the average over fruit development. However, the temperature optima for colour

development in 2008 compared to 2009 are still higher for RG (25 ± 0.5 ºC vs. 20.1 ± 4.2 ºC) and FJ (22.7 ± 0.4 ºC vs. 18.8 ± 2.6 ºC). The shift in optimum temperature for colour development occurred in both Ceres and Grabouw. Although climatic conditions may have played a role in the shift in optimal temperature for red colour development in RG and FJ, there is no apparent causal correlation between optimum temperatures for colour development and monthly average temperatures (Table 1 and 2). Reay and Lancaster (2001) found that apart for temperature, maturity and the previous exposure to light are major modifying factors in the accumulation of anthocyanin in 'Gala' and 'Royal Gala'.

Except for ERO, each cultivar evaluated showed a distinct quadratic response to temperature with regard to red colour development. In general, red colour development decreased ≤17 ºC and ≥25 ºC. This is in agreement with Faragher (1983) who reported that the optimum temperature range for red colour development in green mature 'Jonathan' apples was 16 ºC to 24 ºC. Christie et al. (1994) reported on the greater anthocyanin accumulation at 25 ºC following a period of low temperature (10 ºC) in maize seedlings. Anthocyanin synthesis was less in seedlings kept at 5 ºC, which is likely a consequence of both transcriptional and translational failure or possibly due to a higher temperature requirement for post-transcriptional steps. Temperatures above 30 ºC may decrease red colour development by accelerating the degradation of anthocyanins (Kevany et al., 2003; Marais et al., 2001; Reay, 1999). Some cultivars (e.g. ERO) seem to develop good colour over a broad temperature range while other cultivars (most notably FJ and CP) have a much narrower optimum temperature range. Cultivars with a narrow range of optimum temperatures as well as those with a low optimum temperature will be most inclined to develop poor colour in warmer production areas. Due to slightly lower temperature optima in 2009 and a limited number of temperatures that could be evaluated, the quadratic response to temperature was not as clearly defined in 2008/2009 (Fig. 4). Curry (1997) indicated that the early season cultivar, Gala, had a lower temperature optima for red colour development than the later season cultivar, Fuji. Although it appears that RG may have a higher optimum for anthocyanin synthesis, our data does not indicate the same distinction between early and late season cultivars. The optimum temperatures for red colour development in pre-climacteric 'Gala' (23 ºC), FJ (25 ºC) and BB (21 ºC) reported by Curry (1997) were determined by measuring the actual fruit peel temperatures with an infrared thermometer, for fruit harvested 7 days prior to

commercial harvest. Bakhshi and Arakawa (2006) studied red colour development in FJ, Jonathan and Orin at 10 ºC, 17 ºC, 24 ºC and 30 ºC and found an temperature optimum of 24 ºC for all three cultivars. Reay and Lancaster (2001) found that 'Gala' and 'Royal Gala' accumulated more anthocyanin at 20 ºC than at 10 ºC. These wide temperature intervals do not allow distinction of temperature optima between different cultivars.

Diener and Naumann (1981) found that riper apples had a higher optimal temperature range for colour development. They subsequently suggested that each ripening stage had a different optimal temperature regime for colour formation. Faragher (1983) also reported that the optimum temperature for anthocyanin synthesis increased during fruit development in 'Jonathan' apples from 12 ºC in immature apples to 16 to 24 ºC in mature apples. This would seemingly explain why anthocyanins accumulate more readily towards harvest in apple peel. In this study, RG, FJ, BR and ERO did not show any interaction between temperature and the stage of fruit development, i.e., each cultivar coloured at the same temperature optimum throughout the season. However, the extent of red colour development did increase during fruit development until about the beginning of February where after it stabilized. Bakhshi and Arakawa (2006) also did not find an increase in the optimum temperature for anthocyanin accumulation during fruit development, but the large temperature intervals (7 ºC) used in their study would make it difficult to detect small shifts in optimal temperatures. On the contrary, the optimum temperature for red colour development in CP clearly increased during fruit development in the 2008/2009 season (Fig. 5) from ≈14 ºC in January 2009 to 22.5 ºC in April 2009 in accordance with the data of Faragher (1983) and Diener and Naumann (1981).

It should be kept in mind that although apples in a pre-climacteric state, i.e. ethylene production <0.5 $\mu l l^{-1}$ (Curry, 1997), were used in this study, the temperature trials were done using peel discs, i.e., wounded material that produces ethylene. Wounded apple tissue shows an increase in ethylene synthesis (Burg and Thiman, 1959). Ethylene stimulates anthocyanin synthesis in apple peel (Faragher and Brohier, 1984; Saure, 1990). Temperature also plays a significant role in ethylene production. Burg and Thiman (1959) found that the optimal temperature over a two hour period for ethylene production in apple was 32 ºC and the Q_{10} between 10 and 25 ºC was 2.8. However, at 31 ºC, and even at 28 ºC, hardly any red colour developed in any of the cultivars, suggesting that wounding-related ethylene synthesis does

not invalidate the results of this study. Previous research on colour development in peel discs (Curry 1997) did not refer to this issue.

Conclusion

In general, red colour in the cultivars evaluated developed maximally between 17 ºC and 25 ºC, except for ERO that developed red colour over a broad temperature range. The optimum temperature for red colour development in RG, FJ, BR and ERO remained constant throughout fruit development, but increased during fruit development in CP in the 2008/2009 season. The extent of red colour development did increase during fruit development. Although it appeared that apples from Ceres generally developed redder colour than fruit from Grabouw, a regional difference in the ability to accumulate anthocyanin needs to be validated using fruit from more orchards in each region. The optimum temperatures for anthocyanin synthesis in detached apples did not differ between Ceres and Grabouw, but did seem to differ between seasons. This suggests that growing conditions affects the potential for anthocyanin synthesis. Finally, our data indicate that redder strains of bi-coloured apple cultivars do not owe their enhanced pigmentation to higher temperature optima for anthocyanin synthesis.

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Table 1: Temperatures recorded by the Eikenhof weather station in Grabouw (latitude: 34º 13' S, longitude: 19º 05' E, altitude 365 m) and the Paardekloof weather station in Ceres (latitude: 33º 26' S, longitude: 19º 26' E, altitude 878 m) during the 2007/2008 production season.

Area	2007 / 2008 Production season temperatures (ºC)									
	December		January		February		March		April	
	Day	Night	Day	Night	Day	Night	Day	Night	Day	Night
Grabouw										
Average	25.1	14.4	26.4	15.0	26.4	15.3	26.1	13.7	23.3	11.7
Highest	36.2	18.7	33.1	18.5	33.9	20.6	34.7	18.0	33.4	18.7
Lowest	17.8	8.0	17.3	11.8	21.2	11.5	17.2	9.2	15.2	7.3
Ceres										
Average	24.6	12.2	27.4	15.5	26.0	13.6	25.6	12.5	21.5	7.8
Highest	32.0	17.5	33.1	22.0	32.0	20.0	32.0	19.5	30.5	18.0
Lowest	17.5	5.0	18.5	9.5	18.0	8.5	15.0	7.0	11.5	0.1

Table 2: Temperatures recorded by the Eikenhof weather station in Grabouw (latitude: 34º 13' S, longitude: 19º 05' E, altitude 365 m) and the Paardekloof weather station in Ceres (latitude: 33º 26' S, longitude: 19º 26' E, altitude 878 m) during the 2008/2009 production season.

Area	2008 / 2009 Production season (ºC)													
	October		November		December		January		February		March		April	
	Day	Night	Day	Night	Day	Night	Day	Night	Day	Night	Day	Night	Day	Night
Grabouw														
Average	21.1	10.4	22.1	11.8	25.6	14.0	25.3	14.45	27.6	15.8	27.0	14.1	24.6	12.9
Highest	30.3	14.6	31.2	14.3	35.7	18.1	30.9	17.17	33.2	18.5	36.8	23.4	35.2	16.2
Lowest	13.6	4.5	14.3	8.2	18.3	8.5	19.7	12.02	19.1	12.3	16.5	9.4	16.0	10.0
Ceres														
Average	20.2	7.3	22.1	9.5	25.4	12.3	24.1	10.4	26.3	13.2	26.8	*	22.9	9.4
Highest	27.5	14.0	30.5	16.5	34.0	17.5	29.5	17.0	32.5	20.0	27.0	*	32.0	16.5
Lowest	9.5	0.0	12.5	4.5	16.5	2.5	19.5	6.5	20.0	9.0	26.5	*	11.5	4.00

* Data missing

Table 3: Harvesting dates of apple cultivars during the 2008/2009 season.

Harvest date	Cultivar				
	Royal Gala	Fuji	Braeburn	Early Red One	Cripps' Pink
21-Nov-08	X	X	X		X
28-Nov-08	X	X	X	X	
9-Jan-09	X	X	X	X	
16-Jan-09	X	X	X		X
13-Feb-09	X	X	X	X	
20-Feb-09	X	X	X		X
6-Mar-09	X	X	X	X	
20-Mar-09		X	X		X
3-Apr-09			X		X

Table 4: Significance values for the change in hue of 'Royal Gala', 'Braeburn', 'Fuji', 'Early Red One' and 'Cripps' Pink' peel discs harvested from Ceres and Grabouw in 2007/2008 and irradiated at 550 to 650 µmol m^{-2} s^{-1} for 72 h at different temperatures.

Pr > F	Royal Gala	Fuji	Braeburn	Early Red One	Cripps' Pink
Area (A)	0.6768	<0.0001	0.0009	0.0695	0.0867
Temperature (T)	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
A x T	0.0851	0.4731	0.5679	0.8177	0.1519

Table 5: Significance values for the change in hue of peel discs of 'Royal Gala', 'Fuji' and 'Braeburn', as well as their respective strains at different temperatures in 2007/2008.

Pr > F	Royal Gala	Fuji	Braeburn
Strain (S)	0.0470	0.0027	0.3459
Temperature (T)	0.0103	<0.0001	0.0006
S x T	0.2901	0.7626	0.4533

Table 6: The temperature at which the maximum change in hue angle occurred for each cultivar (i.e., inflection point), harvested weekly for three weeks prior to commercial harvest in 2007/2008, throughout the 2008/2009 season and the last three harvests in 2009, according to a polynomial fitted trendline and therefore representing the optimum temperature for red colour development for each of these cultivars.

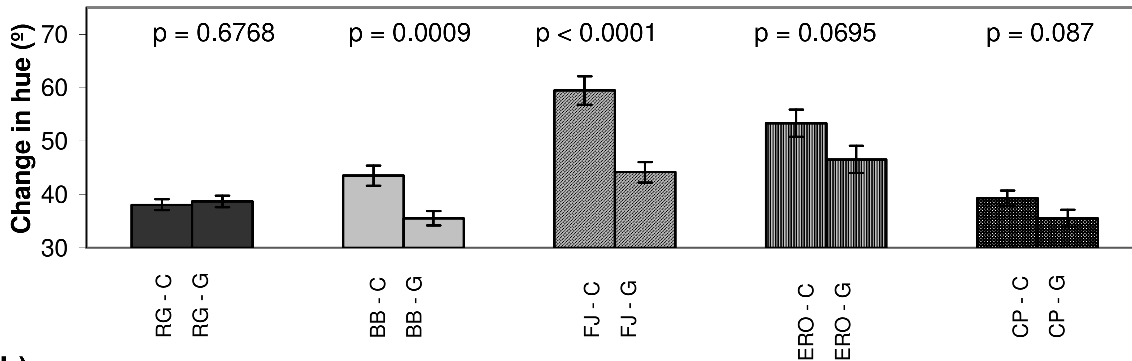
Cultivar	Inflection point 2007/2008 (ºC)	Inflection point 2008/2009 (ºC)	Inflexion point for the last 3 harvests in 2009 (ºC)
Royal Gala	25.16 ± 0.52	18.39 ± 2.08	20.44 ± 1.89
Braeburn	21.13 ± 0.90	19.12 ± 1.42	19.95 ± 1.28
Fuji	22.72 ± 0.44	17.41 ± 2.16	18.62 ± 2.10
Early Red One	18.73 ± 1.93	21.75 ± 1.29	21.70 ± 1.27
Cripps' Pink	22.92 ± 0.37	*	21.26 ± 0.65

* 'Cripps' Pink at different inflection points at different stages during fruit development. Presented in Fig. 5b.

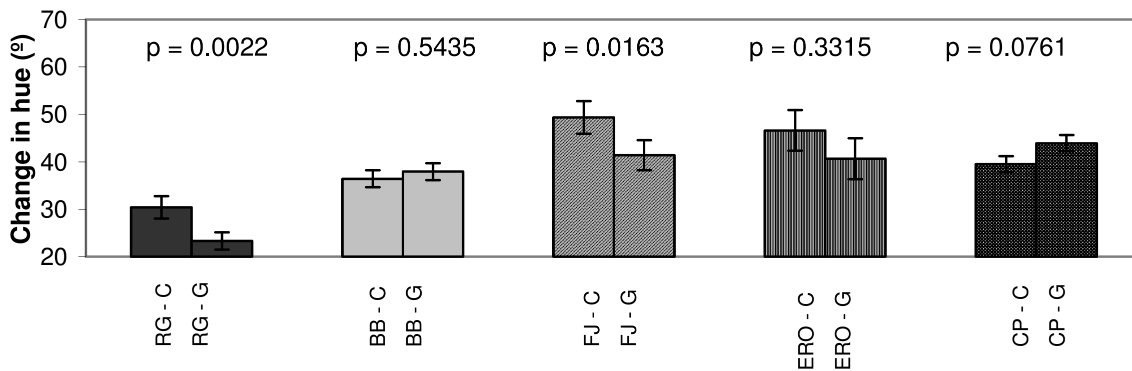
Table 7: Significance values for the change in hue of 'Royal Gala', 'Braeburn', 'Fuji', 'Early Red One' and 'Cripps' Pink' peel discs punched from fruit harvested at Grabouw and Ceres throughout the fruit development in 2008/2009 and irradiated at 550 to 650 µmol m^{-2} s^{-1} for 72 h at different temperatures.

Pr > F	Royal Gala	Fuji	Braeburn	Early Red One	Cripps' Pink
Fruit development stage (FD)	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Area (A)	0.0022	0.0163	0.5435	0.3315	0.0761
Temperature (T)	<0.0001	<0.0001	<0.0001	0.5452	<0.0001
FD x A	0.0889	0.5231	0.6259	0.7290	0.3908
FD x T	0.1839	0.0801	0.1288	0.4863	0.0001
A x T	0.6627	0.9106	0.8851	0.9337	0.1148
FD x A x T	0.9119	0.9981	0.9990	0.9499	0.2247

(a)



(b)



(c)

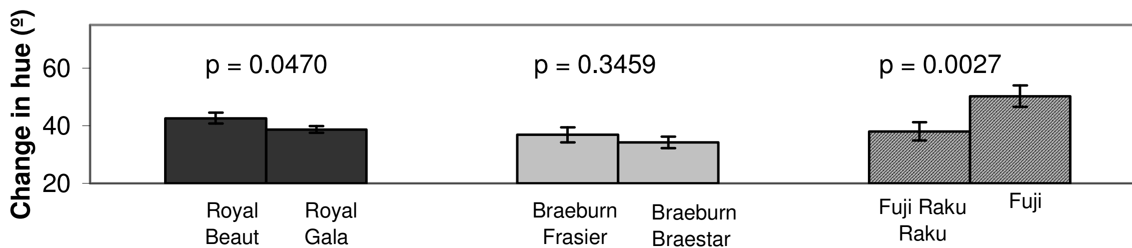


Figure 1: Change in the hue angle of 'Royal Gala' (RG), 'Braeburn' (BB), 'Fuji' (FJ), 'Early Red One' (ERO) and 'Cripps' Pink' (CP) peel discs punched from apples harvested from Grabouw (G) and Ceres (C) in **(a)** 2007/2008 and **(b)** 2008/2009 following irradiation at 550 to 650 µmol m^{-2} s^{-1} for 72 h. **(c)** Change in hue of RG, BB and FJ peel discs compared with their respective strains namely 'Royal Beaut', 'Braeburn Frasier' and 'Fuji Raku Raku' in 2007/2008. Since hue angle decreases with increasing redness of peel, a greater change in hue during irradiation indicates a greater increase in red colour.

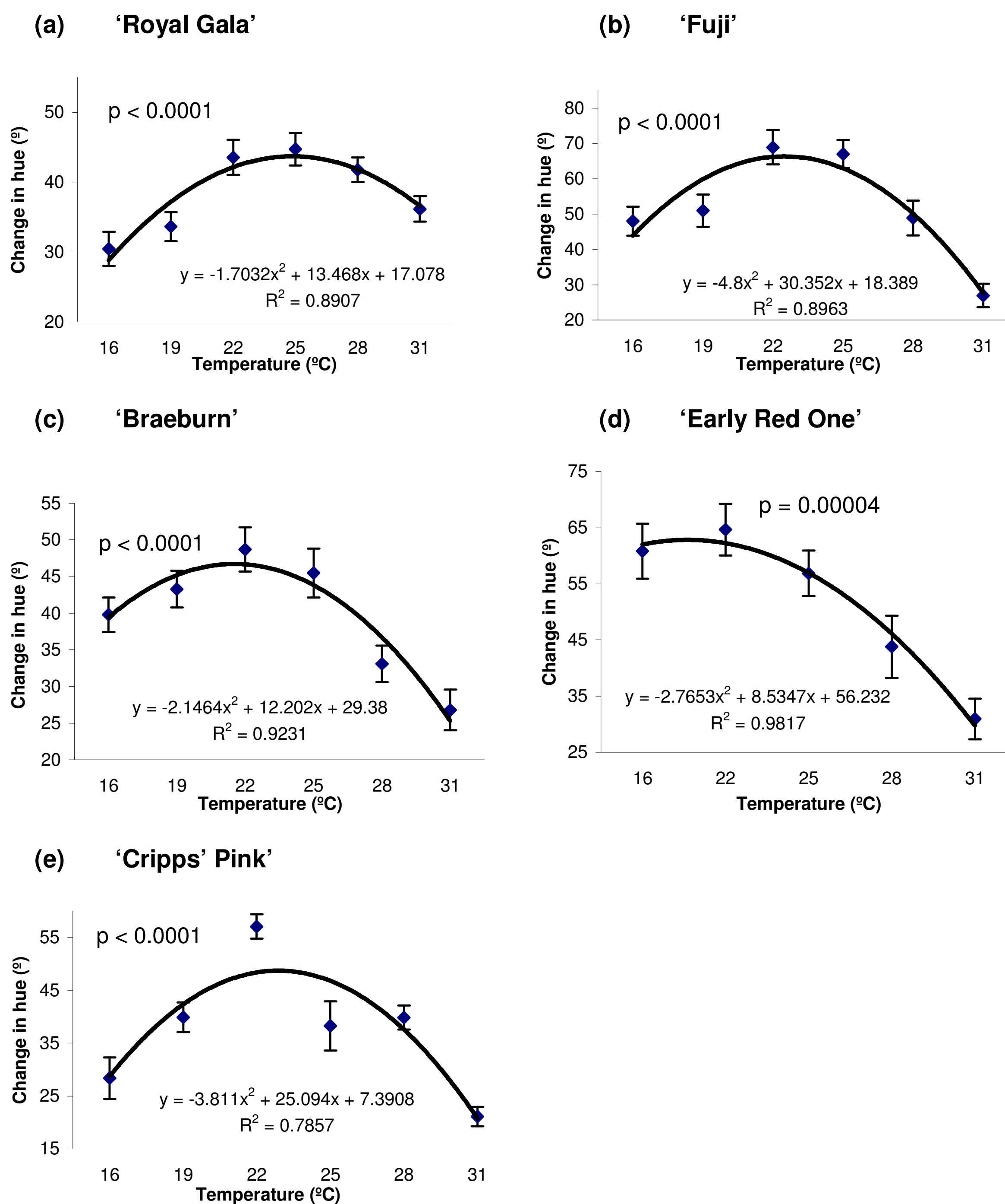
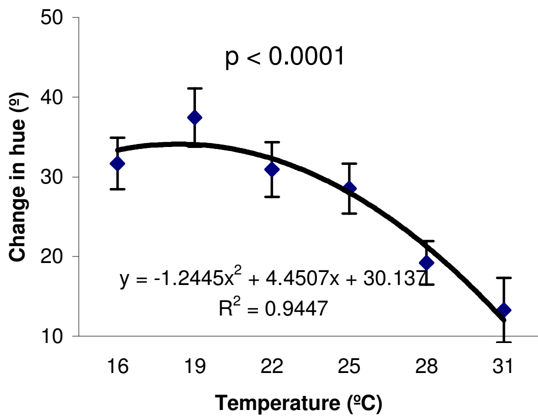
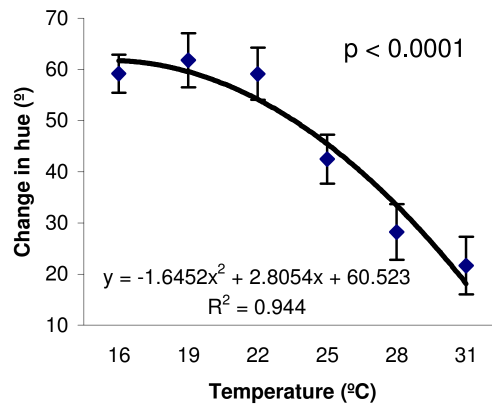


Figure 2: Change in the hue angle of **(a)** 'Royal Gala', **(b)** 'Fuji' **(c)** 'Braeburn', **(d)** 'Early Red One' and **(e)** 'Cripps' Pink' peel discs at different temperatures. Discs were punched from fruit harvested three weeks prior to commercial harvest in 2007/2008 and irradiated at 550 to 650 µmol m^{-2} s^{-1} for 72 h. Since hue angle decreases with increasing redness of peel, a greater change in hue during the 72 h irradiation indicates a greater increase in red colour (n = 12).

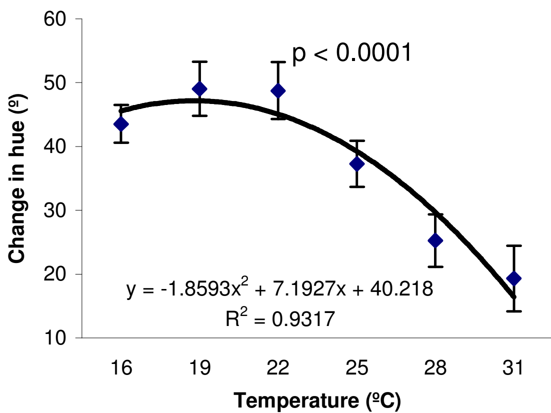
(a) **‘Royal Gala’**



(b) **‘Fuji’**



(c) ‘Braeburn’



(d) ‘Early Red One’

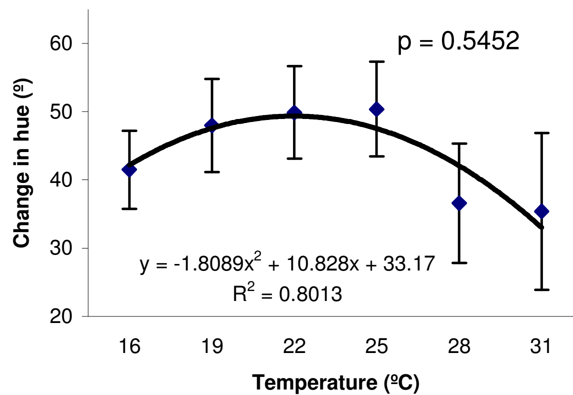


Figure 3: Change in hue angle of **(a)** ‘Royal Gala’ (n = 28), **(b)** ‘Fuji’ (n = 32), **(c)** ‘Braeburn’ (n =36) and **(d)** ‘Early Red One’ (n = 16) at different temperatures. Peel discs were punched from fruit harvested at regular intervals during 2008/2009 and irradiated at 550 to 650 µmol m^{-2} s^{-1} for 72 h. Since hue angle decreases with increasing redness of peel, a greater change in hue during the 72 h irradiation indicates a greater increase in red colour.

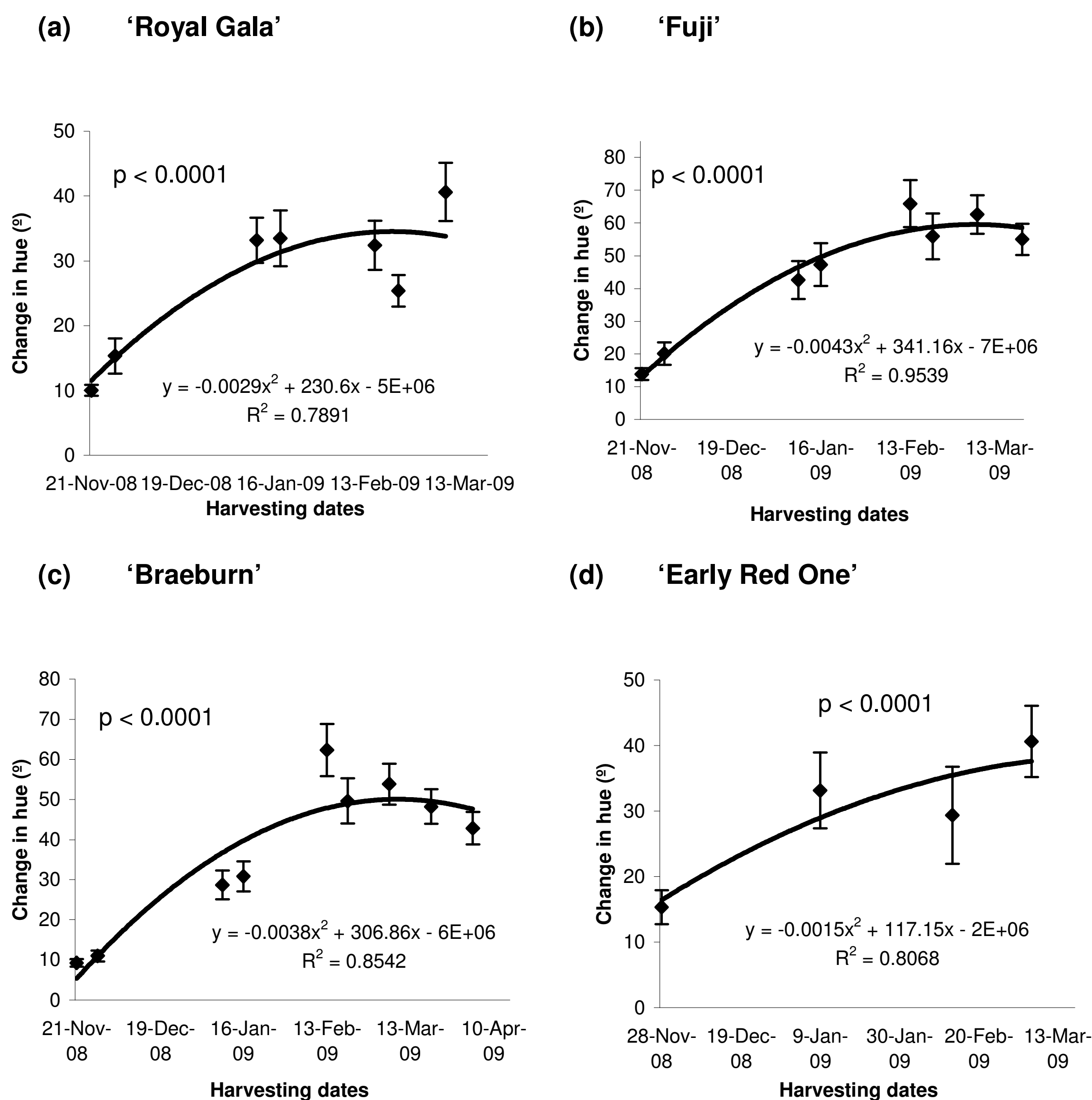
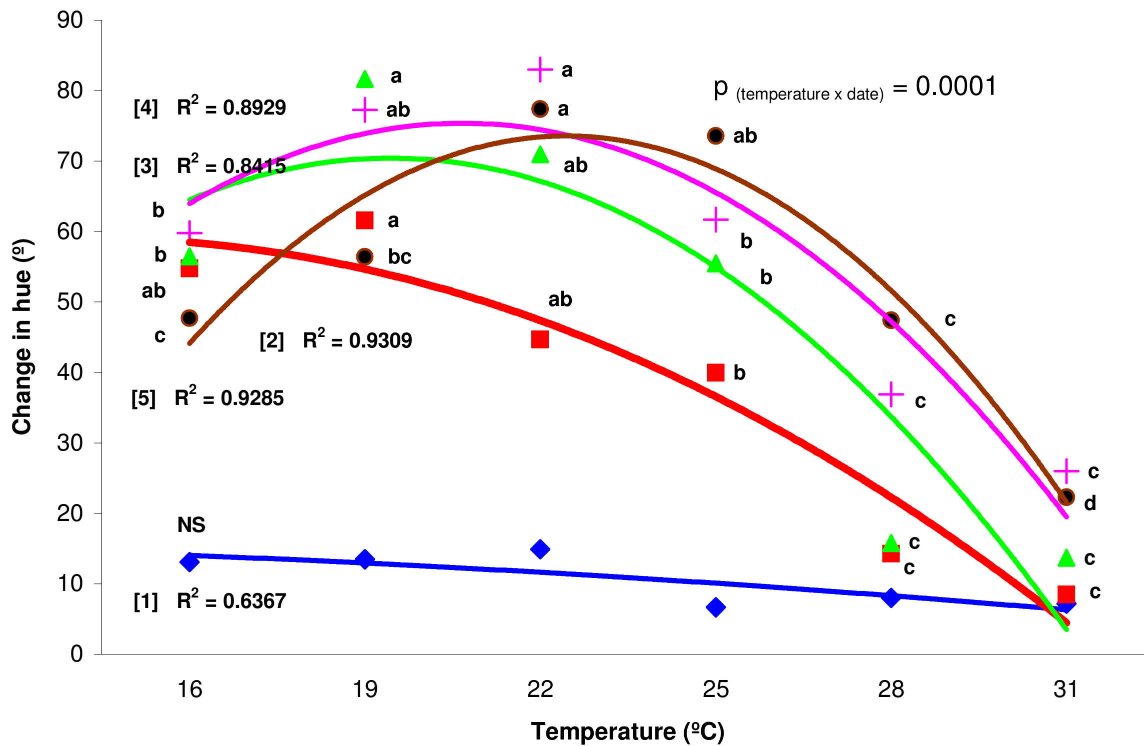


Figure 4: Change in hue angle of **(a)** 'Royal Gala', **(b)** 'Fuji', **(c)** 'Braeburn' and **(d)** 'Early Red One' peel discs during fruit development in 2008/2009. Peel discs were irradiated at 550 to 650 µmol m^{-2} s^{-1} for 72 h. Since hue angle decreases with increasing redness of peel, a greater change in hue during the 72 h irradiation indicates a greater increase in red colour (n = 24).

(a)



(b)

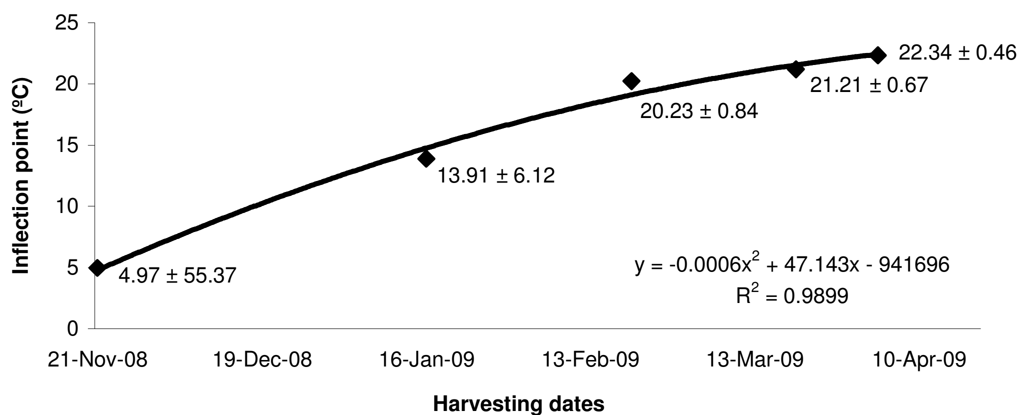


Figure 5: (a) Change in hue angle of 'Cripps' Pink' peel discs punched from fruit harvested on (1) 21 November 2008, (2) 16 January 2009, (3) 20 February 2009, (4) 20 March 2009 and (5) 3 April 2009 at different temperartures. Discs were irradiated at 550 to 650 µmol m^{-2} s^{-1} for 72 h. **(b)** The inflection point of each date-temperature curve presented in (a). Since hue angle decreases with increasing redness of peel, a greater change in hue during the 72 h irradiation indicates a greater increase in red colour.

PAPER 2: OPTIMUM NIGHT TEMPERATURES FOR RED COLOUR DEVELOPMENT IN APPLE FRUIT

Abstract

The aim of this study was to determine the optimum night-time temperatures for anthocyanin synthesis in red and bi-coloured apple cultivars grown in South Africa. 'Royal Gala' (RG), 'Fuji' (FJ), 'Braeburn' (BB), 'Early Red One' (ERO) and 'Cripps' Pink' (CP) were sampled at regular intervals during fruit development from two production areas, viz. Grabouw and Koue Bokkeveld, in the Western Cape. Peel discs were punched from the shaded sides of fruit and placed on Peltier temperature plates set to a temperature range from 0 to 20 ºC at 4 ºC intervals without light exposure for 48 hours. Following the low temperature treatments, all discs were exposed to a photosynthetic photon flux (PPF) of 550 to 650 µmol m^{-2} s^{-1} at 23 ºC for a further 48 hours where after their change in hue was determined. Except for RG from Ceres that appeared to benefit from induction at 4ºC, none of the cultivars responded positively to low temperature induction with regard to red colour development. The lack of a response was observed throughout fruit development and in fruit from both production areas. In fact, in the case of FJ, red colour development seemed to decrease with a decrease of inductive temperature. Possible reasons for the failure to observe a stimulating effect of low temperature on red colour development are discussed.

Introduction

South Africa's apple production areas are considered to be among the warmer production areas in the world and this may contribute to fruit not developing sufficient red colour. In addition to light, which is considered a prerequisite for the biosynthesis of anthocyanin, the major factor that affects anthocyanin synthesis in ripening apples is temperature (Saure, 1990; Lancaster, 1992). It has long been known that cool night temperatures increase anthocyanin accumulation (Saure, 1990 citing Naumann, 1964). Low temperatures also seem to stimulate anthocyanin synthesis in immature fruit. Diener and Naumann (1981) found the highest accumulation of anthocyanin in immature fruit at the lowest combination of day and night temperatures (2 / 12 ºC vs. 12 / 24 ºC), while the converse was true for mature apples.

More recent work by Curry (1997) and Reay (1999) showed that anthocyanin synthesis in apple benefits from induction at low temperatures (2 and 4 ºC in the respective studies) followed by irradiation at higher temperatures (25 and 20 ºC, respectively). Work done in maize showed the same two-step temperature requirement for anthocyanin synthesis with induction at low temperature (10 ºC) followed by maximum synthesis in light at mild temperature (25 ºC) (Christie et al., 1994). Low temperatures are also associated with anthocyanin synthesis in vegetative tissues in various plants (Steyn et al., 2002).

Differences in the degree of red colour development of the same apple cultivar, but from different production areas can possibly be explained by differences in night temperatures even when fruit experience the same optimum day-time temperatures (Reay, 1999). Hence, it would be advantageous to know the temperature range for optimum induction of anthocyanin synthesis in various cultivars, since this may allow more informed decisions with regard to cultivar choice in different production areas. This study focused on determining the optimum night-time temperatures for anthocyanin synthesis throughout fruit development in red and bi-coloured apple cultivars grown in South Africa, i.e., Royal Gala, Braeburn, Fuji, Early Red One and Cripps' Pink. Fruit were harvested from two different production areas during the 2008/2009 season to assess whether growing conditions may influence the temperature requirements for anthocyanin synthesis.

Materials and Methods

Plant material:

'Royal Gala' (RG), 'Early Red One' (ERO), 'Braeburn Braestar' (BB) 'Fuji' (FJ), and 'Cripps' Pink' (CP) fruit were obtained from Oakvalley Estate in Grabouw (latitude: 34º 08' S, longitude: 19º 02' E, altitude: 300 m), and from Vastrap in Ceres (latitude: 33º 14' S, longitude: 19º 14' E, altitude: 890 m) in the Mediterranean-type climate Western Cape Province of South Africa. Average daily minimum and maximum temperatures recorded for these regions during the relevant months of the 2008-09 seasons are presented in Table 1. Apples were picked at random before 1100 HR from the same orchard row on each of the harvest dates (Table 2) from shortly after fruit set until commercial harvest. Apples were picked from the inner canopy of trees. Only apples with a green/shaded side were picked. Fruit were placed in a cooler bag for transport to our laboratory. Twelve apples were

harvested on each date for every area cultivar combination. One disc (15 mm in diameter, 5 mm thick) was punched from the green, shaded side of each apple upon arrival at our laboratory on the same day of harvest. Discs were randomly placed on the 12 peltier plates (5 cm x 6.5 cm) of a Celtec, constructed according to the design of Burke and Mahan (1993), with a H_2O-moistend filter paper between the discs and the plate. Each area cultivar combination was represented by one disc per plate. Hue angles of discs were measured before and 96 h after being placed on the Celtec. Discs were covered with thin (0.5 mm) 100% crystal clear polyethylene wrap (Glad Wrap™, Glad products, Glad South Africa, Randburg, South Africa) that was fastened over the peltier plates. A few holes were made in the plastic with a toothpick to prevent the build-up of CO_2 and, possibly, ethylene and to reduce condensation of water on the inside of the plastic.

Temperature treatments:
The Celtec was placed in a growth cabinet set at 12 ºC with 2 overhead lamps (400W High Pres Sodium; SON-T; Osram Mgbh, Munich, Germany) providing irradiance of 550 to 650 µmol m^{-2} s^{-1} photosynthetic photon flux (PPF) measured with a quantum meter (LI-189; Li-Cor, Lincoln, Nebraska, USA) at disc level. Set temperatures of 0 ºC, 4 ºC, 8 ºC, 12 ºC, 16 ºC and 20 ºC were randomly assigned to the 12 plates of the Celtec for 48 h in the dark, after which the overhead lamps were switched on and the temperature of all plates kept at 23 ºC for a further 48 h. Disc temperature was measured (Raynger MX4, Raytek Corporation, Santa Cruz, USA) to ensure that the required temperatures were obtained.

Colour measurement:
Disc hue angle was measured with a chromameter (NR-3000; Nippon Denshoku, Tokyo, Japan) before and again 96 h after placement on the Celtec. Hue angle (0º = red-purple, 90º = yellow, 180º = blue-green and 270º = blue) is the most relevant measurement to express the differences in colour development in this study (McGuire, 1992).

Maturity indexing:
Firmness (by means of a penetrometer using an 8 mm plunger) and starch breakdown (by brushing iodine on a freshly cut apple half to stain the internal starch and therefore indicating the level of unconverted sugar) of apples were assessed at maturity.

Statistical Analysis.

The data were analyzed with the General Linear Models (GLM) procedures of Statistica version 9 (Statsoft Inc., Tulsa, Oklahoma), and this data analysis software was also used to fit nonlinear curves to data in order to calculate the inflexion point for each temperature curve according to the equation $Y = a^0(a^1 + a^2 x - 0.5\ x^2)$.

Results

The interaction between area, stage of fruit development and temperature for change in hue was not significant for any of the cultivars (Table 3). RG showed an interaction between production area and temperature (Figure 1a) with RG from the Grabouw area showing no difference in ability to develop colour over the temperature range, whereas peel discs from Ceres developed redder colour at 4 ºC than at other temperatures. The other cultivars did not show an interaction between production area and temperature (Table 3).

Only ERO showed an interaction between the stage of fruit development and temperature (Figure 1b). ERO harvested on 30 January 2009 developed redder colour at 20 ºC than at 4 ºC and 16 ºC. Redder colour developed at 0 ºC than at 16 ºC and at 8 ºC and 12 ºC than at 4 ºC and 16 ºC. ERO harvested on 13 March 2009 developed redder colour at 16 ºC than at other temperatures. ERO harvested on 12 December 2008 showed no difference in ability to develop colour over the temperature range.

FJ, BB and ERO showed an interaction between the stage of fruit development and production area (Table 3; Fig. 2a, b and c). No interaction between the fruit development stage and production area occurred for RG and CP. ERO harvested from Ceres on 12 December 2008 and 30 January 2009 developed redder colour than when harvested on13 March 2009. ERO from Grabouw harvested on 30 January 2009 and 13 March 2009 developed redder colour than when harvested on 12 December 2008. ERO harvested on 12 December 2008 coloured the same over the entire temperature range. FJ harvested from Ceres on 30 January, 6 February and 13 March 2009 developed redder colour than when harvested on 12 and 19 December 2008. FJ harvested from Grabouw on 30 January and 6 February 2009 developed redder colour than when harvested on 12 December 2008 and 13

March 2009. Redder colour developed on 19 December 2008 than on 13 March 2009. BB harvested from Grabouw on 12 December 2008 developed less red colour than on other harvesting dates. BB from Ceres harvested on 6 February 2009 developed redder colour than fruit harvested on 12 December 2008, 19 December 2008 and 13 March 2009. Fruit harvested on 30 January 2009 developed redder colour than fruit harvested on 12 and 19 December 2008.

RG peel discs from fruit harvested on 19 December 2008 developed redder colour than discs of fruit harvested on 12 December 2008, 30 January 2009 and 6 February 2009 (Table 4). Peel discs from fruit harvested on 12 December 2008 and 30 January 2009 developed redder colour than discs of fruit harvested on 6 February 2009. CP harvested on 19 December 2008 and 10 April 2009 developed redder colour than when harvested on 6 February and 13 March 2009 (Figure 2d). FJ, BB and CP peel discs developed a comparable level of red colour over the entire temperature range (Figure 3a, b and c).

Discussion

A favorable effect of low temperatures on anthocyanin accumulation is generally noted in field observations, as has been reported in different fruit (Steyn et al., 2009) including apple (Creasy, 1968; Uota, 1952) and pear (Steyn et al., 2004). Uota (1952) found that a higher percentage of red surface colour correlated with low average night temperatures. Apparently a few nights with temperatures in the range 2 to 5 ºC followed by warm sunny days promotes red colour development (Jackson, 2003). Tan (1980) found that whole 'Red Spy' apples receiving a 6 ºC pre-treatment, either in light or darkness, followed by 25 ºC in light accumulated much more anthocyanin than apples kept at 25 ºC during the pre-treatment. However, Gurnsey and Lawes (1999) noted that cool night temperatures only need to be below 18 ºC from a few weeks before harvest to enhance apple fruit colour.

Two periods of anthocyanin production have been identified during fruit growth in apple. The first, smaller peak occurs during cell division (Lancaster, 1992) shortly after fruit set (<2 cm fruit diameter). The second, larger peak in anthocyanin synthesis occurs during ripening (Macheix et al., 1990; Saure, 1990). However, the daily change in hue of apples and pears strongly correlates with average daily temperature as was seen with 'Cripps' Pink' apples

(Paper 3) and ‘Rosemarie’ pears (Steyn et al. 2004). Hue decreased with the onset of a cold front and increased again during warmer periods. Thus it appears as if the general pattern of colour development might be linked to relatively lower temperatures during early fruit development and decreasing night temperatures during fruit maturity. The data reported here does not indicate an increased sensitivity to temperature during early and late fruit development.

Inconsistent results were found for red colour development in response to temperature, stage of fruit development and production area. The ability of RG to accumulate anthocyanin did not seem to relate to the stage of fruit development. FJ peaked in red colour development between the end of January and beginning of February, where after the accumulation of anthocyanin in FJ from Grabouw, but not Ceres, decreased considerably. BB from Ceres showed a similar trend as FJ from Grabouw, while BB from Grabouw showed a similar trend as FJ from Ceres. The effect of fruit development stage on the colour response of ERO from Ceres and Grabouw resulted in a shared accumulation peak by the end of January. In contrast, CP reached an absolute lowest anthocyanin accumulation potential at the beginning of February where after it increased.

Curry (1997), also using apple peel discs placed on a Celtec, found that pre-cooling at low temperature increased anthocyanin accumulation in ‘Red Chief Delicious’ and ‘Fuji’. The amount of anthocyanin in discs pre-cooled at 2 ºC was almost double compared to discs kept at 22 ºC. The optimum day temperature for anthocyanin synthesis was not affected by the induction at low temperature. Reay (1999) reported that a temperature combination of 4 ºC followed by 20 ºC was the most effective at inducing accumulation of anthocyanin in whole, detached ‘Granny Smith’ apples. In our study, only RG from Ceres appeared to have benefited from pre-cooling at 4 ºC. None of the other cultivars showed induction at low temperature. In fact, in contrast to Curry (1997), red colour development in FJ seemingly decreased with a decrease in induction temperature.

We were faced with several practical obstacles when working with low temperatures on the Celtec and these may explain the inconsistent results obtained. The adhesion of the sticky tape used to fasten the clear polyethylene wrap over the peltier plates was poor at the lower

temperatures. This may have resulted in dehydration of some samples, although no apparent dehydration was observed. Condensation was high at low temperatures despite the few holes made in the polyethylene wrap, thereby decreasing irradiance that the discs might have received.

Overhead lamps were switched on simultaneously with the commencement of the 23 ºC day cycle of the experiment. Discs at the lower inductive temperatures would take more time to reach 23 ºC than discs that received higher inductive temperatures. Environmental stresses such as low temperatures lower the photosynthetic rate at a given irradiance, thereby increasing the degree to which absorbed light is excessive. Chlorophyllous tissues that receive more light energy than can be used for photochemistry, undergo a decrease in quantum efficiency of photosynthesis, better known as photoinhibition (Adams et al., 2008; Long et al., 1994). Under severe conditions, chloroplasts generate ROS, which may have a destructive effect on cellular components including chlorophyll (Alscher et al., 1997). Photobleaching of discs was observed on a few occasions. However, photobleaching of chlorophyll would not necessarily decrease the ability of apple skin to synthesize anthocyanin (personal communication, WJ Steyn).

We considered that the inconsistent results could be due to the use of peel discs instead of whole fruit. Most researchers have used whole, detached apples to study temperature effects on colour development (Creasy, 1968; Diener and Naumann, 1981; Faragher, 1983; Kevany et al., 2003; Marais et al., 2001; Reay, 1999; Reay and Lancaster, 2001; Tan, 1980). However, Curry (1997) utilized peel discs for his comprehensive study on the effect of temperature on red colour development in apple, to all appearances, with great success (also see Paper 1).

Conclusion

None of the low temperature pre-treatments, with the possible exception of 4 ºC in RG from Ceres, increased red colour development at any stage during fruit development in red and bi-coloured apple cultivars. Thus no effective inductive temperature range for optimum colour development could be confirmed or established for any of the cultivars evaluated. We suggest

that the experiment is repeated with whole apples to rule out the possibility that the lack of a temperature response may relate to the use of peel discs on peltier temperature plates.

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Table 1: Temperatures recorded by the Eikenhof weather station in Grabouw (latitude: 34º 13' S, longitude: 19º 05' E, altitude 365 m) and the Paardekloof weather station in Ceres (latitude: 33º 26' S, longitude: 19º 26' E, altitude 878 m) during the 2008/2009 production season.

Area	2008 / 2009 Production season (ºC)													
	October		November		December		January		February		March		April	
	Day	Night	Day	Night	Day	Night	Day	Night	Day	Night	Day	Night	Day	Night
Grabouw														
Average	21.1	10.4	22.1	11.8	25.6	14.0	25.3	14.6	27.6	15.8	27.0	14.1	24.6	12.9
Highest	30.3	14.6	31.2	14.3	35.7	18.1	30.9	17.2	33.2	18.5	36.8	23.4	35.2	16.2
Lowest	13.6	4.5	14.3	8.2	18.3	8.5	19.7	12.0	19.1	12.3	16.5	9.4	16.0	10.0
Ceres														
Average	20.2	7.3	22.1	9.5	25.4	12.3	24.1	10.4	26.3	13.2	26.8	*	22.92	9.4
Highest	27.5	14.0	30.5	16.5	34.0	17.5	29.5	17.0	32.5	20.0	27.0	*	32.0	16.5
Lowest	9.5	0.0	12.5	4.5	16.5	2.5	19.5	6.5	20.0	9.0	26.5	*	11.5	4.00

* Data missing

Table 2: Harvesting dates of apple cultivars during the 2008/2009 season.

Harvest date	Cultivar				
	Royal Gala	Fuji	Braeburn	Early Red One	Cripps' Pink
12-Dec-08	X	X	X	X	
19-Dec-08	X	X	X		X
30-Jan-09	X	X	X	X	
6-Feb-09	X	X	X		X
13-Mar-09		X	X	X	X
10-Apr-09					X

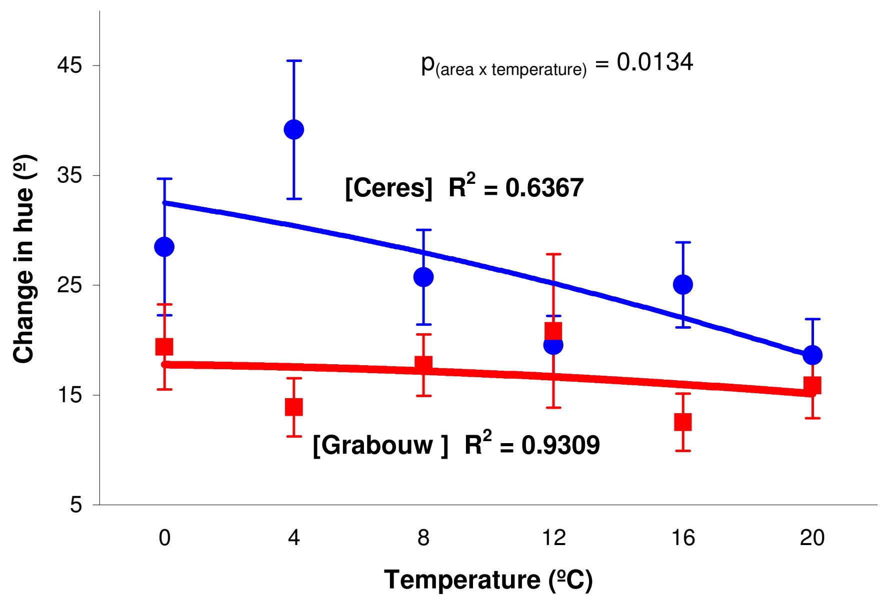
Table 3: Significance values for the change in hue of 'Royal Gala', 'Braeburn', 'Fuji', 'Early Red One' and 'Cripps' Pink' peel discs punched from fruit harvested at Grabouw and Ceres throughout the fruit development in 2008/2009 and irradiated at 550 to 650 µmol m^{-2} s^{-1} for 48 h at 23 ºC following a 48 h cold treatment at different temperatures.

Pr > F	Royal Gala	Fuji	Braeburn	Early Red One	Cripps' Pink
Fruit development stage (FD)	<0.0001	<0.0001	<0.0001	0.0162	0.0011
Area (A)	<0.0001	0.6969	0.5758	0.0361	0.2169
Temperature (T)	0.1339	0.0625	0.4426	0.4944	0.1246
FD x A	0.2802	<0.0001	0.0391	0.0050	0.1735
FD x T	0.4360	0.0639	0.3880	0.0047	0.1647
A x T	0.0134	0.3406	0.7156	0.2388	0.5973
FD x A x T	0.1043	0.7148	0.4572	0.4921	0.7782

Table 4: Change in hue angle of 'Royal Gala' peel discs punched from fruit harvested four times during fruit development stages in 2008/2009. Dics were irradiated at 550 to 650 µmol m^{-2} s^{-1} for 48 h at 23 ºC following a 48 h cold treatment. Since hue angle decreases with increasing redness of peel, a greater change in hue during the 48 h irradiation indicates a greater increase in red colour (n = 24).

Harvest date	Change in hue angle
12-Dec-08	22.47 ± 2.65 b
19-Dec-08	29.37 ± 3.25 a
30-Jan-09	19.93 ± 2.11 b
6-Feb-09	13.88 ± 1.99 c

(a) 'Royal Gala'



(b) 'Early Red One'

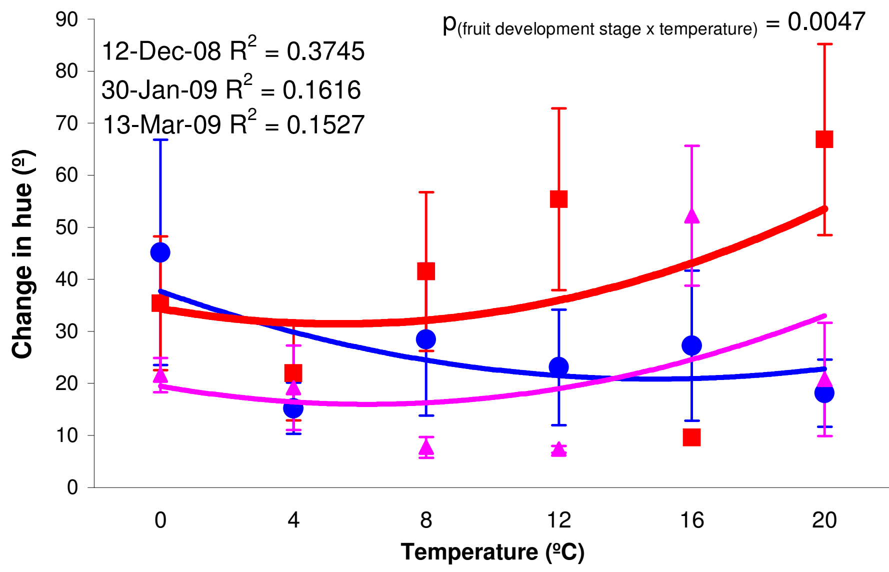


Figure 1: Change in hue angle of **(a)** 'Royal Gala' (n = 8) and **(b)** 'Early Red One' (n = 4) peel discs harvested from Ceres and Grabouw during 2008/2009 and irradiated at 550 to 650 µmol m^{-2} s^{-1} for 48 h at 23 ºC following a 48 h cold treatment at different temperatures. The significant temperature*area and temperature*stage of fruit development interactions are presented for 'Royal Gala' and 'Early Red One', respectively. Since hue angle decreases with increasing redness of peel, a greater change in hue during the 48 h irradiation indicates a greater increase in red colour.

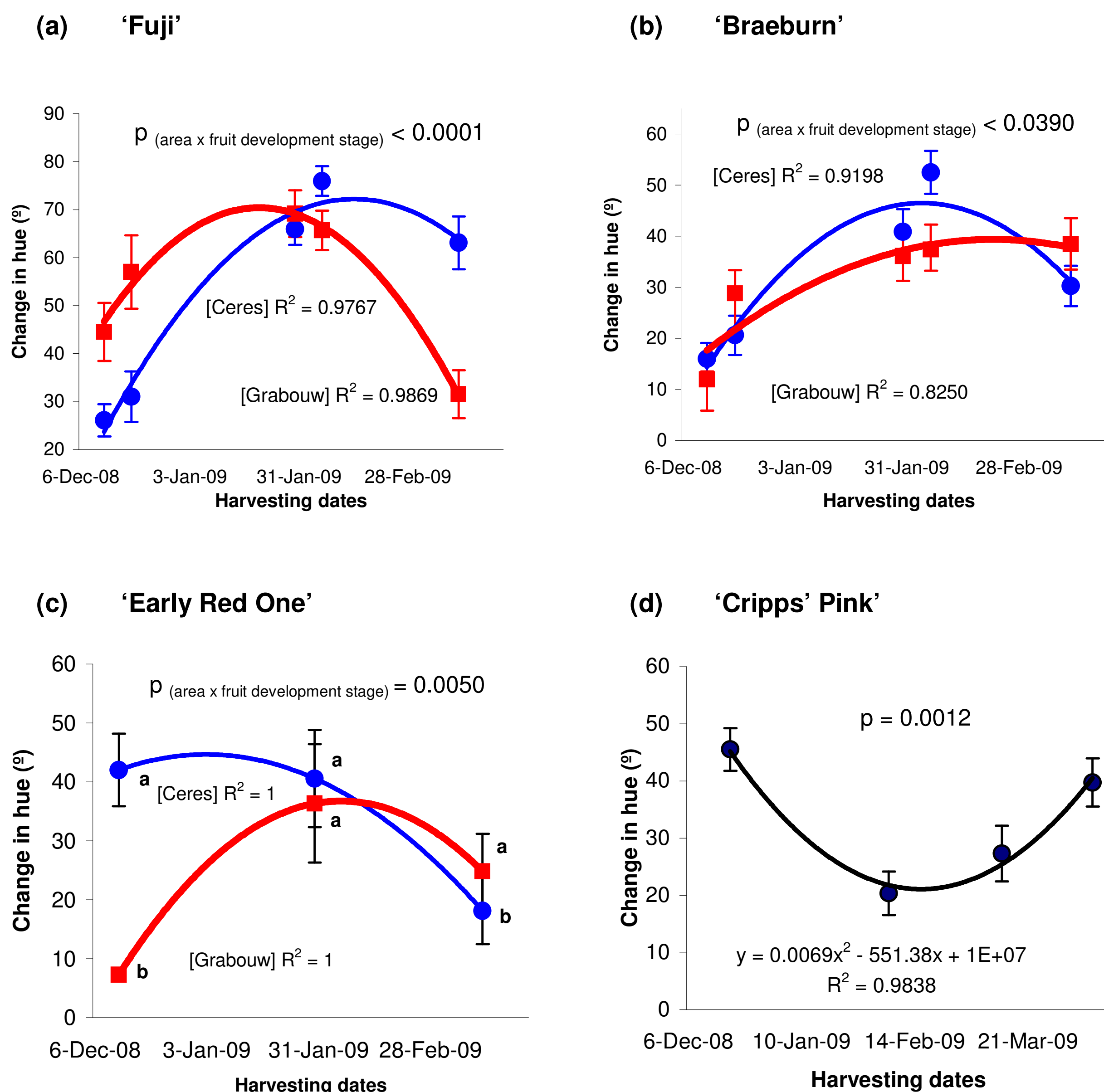


Figure 2: Change in hue angle of **(a)** 'Fuji', **(b)** 'Braeburn', **(c)** 'Early Red One' and **(d)** 'Cripps' Pink' peel discs harvested from Ceres and Grabouw during 2008/2009 and irradiated at 550 to 650 μmol m^{-2} s^{-1} for 48 h at 23 ºC following a 48 h cold treatment at different temperatures. The temperature*area interactions are presented except for 'Cripps' Pink' for which the interaction was not significant. Since hue angle decreases with increasing redness of peel, a greater change in hue during the 48 h irradiation indicates a greater increase in red colour (n = 12 with exception of CP where n = 24).

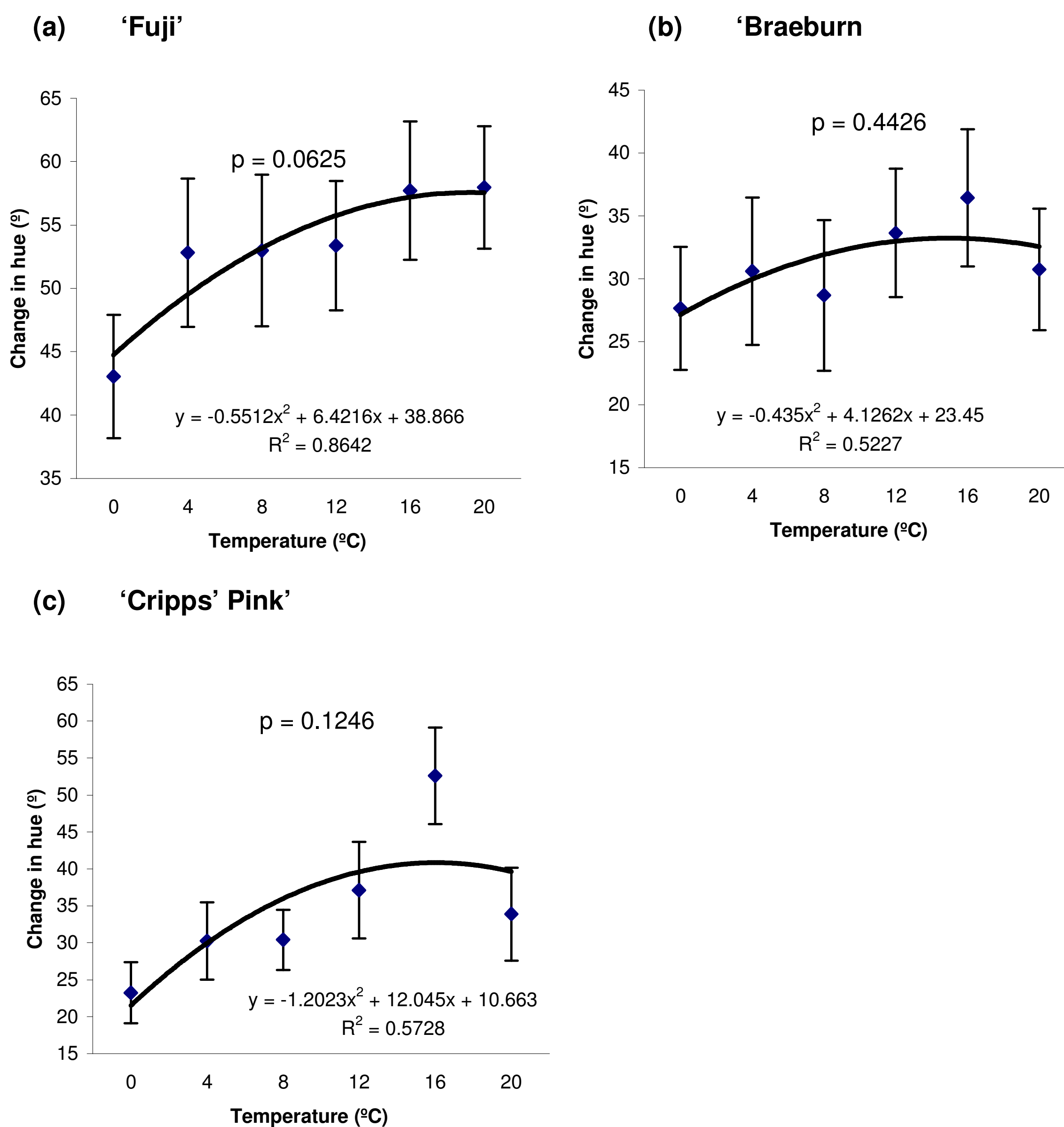


Figure 3: Change in hue angle of **(a)** 'Fuji', **(b)** 'Braeburn' and **(c)** 'Cripps' Pink' peel discs harvested during 2008/2009 and irradiated at 550 to 650 µmol m^{-2} s^{-1} for 48 h following a 48 h cold treatment at different temperatures. Since hue angle decreases with increasing redness of peel, a greater change in hue during the 48 h irradiation indicates a greater increase in red colour (n = 20).

PAPER 3: THE ROLE OF ANTHOCYANIN ACCUMULATION DURING EARLY APPLE FRUIT DEVELOPMENT.

Abstract

'Cripps' Pink' apples were used to investigate three hypothesis based on the premise that anthocyanins in immature apple fruit protect apple peel from photoinhibition and photooxidative damage during conditions of increased light stress. Firstly we wanted to establish whether anthocyanins accumulate fast enough to provide photoprotection during sudden cold snaps. The rate of colour change was measured in respons to a passing cold front. The average hue angle of 'Cripps' Pink' apples decreased from 80 to 47º between on 16 March 2009 and 20 March 2009, the duration of the cold front, and increased again with increasing daily temperature. The rate at which the anthocyanin light screen is deployed appears to be sufficient to provide photoprotection during a cold snap. We also considered that protection of fruit peel against photoinhibition during cold snaps lowers the risk of subsequent high light and high temperature- induced damage to fruit peel when temperatures increase again after the cold snap. We found that apple peel incurred significantly more photoinhibition at low (16 ºC) compared to mild (24 to 32 ºC) and high (40 ºC) temperatures under high irradiace. The recovery rate was temperature-dependent, being the slowest at low temperatures and increasing with temperature. Unfortunately, we could not prove that photoinhibition incurred during cold snaps predisposes fruit peel to photothermal damage when temperatures increase again after the cold snap. Lastly we hypothesized that the sensitivity of fruit peel to photoinhibition increases during fruit development. The response of 'Cripps' Pink' apple peel photochemistry (F_v/F_m, ϕ_{PSII}, qP and qNP) to a range of temperatures (16, 24, 32 and 40 ^oC) and irradiance levels (140, 470 and 1400 µmol m^{-2} s^{-1} photosynthetic photon flux (PPF)) was assessed during the 2008/2009 season. The photoapparatus in 'Cripps Pink' peel appears to be particularly sensitive to light stress at low temperature (16 ºC) throughout the season with significant photoinhibition occurring even at a moderate temperature of 24 ºC. The sensitivity of the fruit peel to photoinhibition increased throughout the season at lower irradiance levels, but remained the same at higher irradiance.

Introduction

The red color of apple fruit is caused by the accumulation of anthocyanins, mostly in the outer layers of the skin (Lancaster et al., 1994). Two periods of anthocyanin production have been identified during fruit growth in apple. The first, smaller peak occurs during cell division (Lancaster, 1992) shortly after fruitset (<2 cm fruit diameter). Even some non-red apple cultivars that are typically yellow or green at maturity produce anthocyanins at this stage, e.g. Golden Delicious (Saure, 1990) and Granny Smith (Lancaster et al., 2000). The anthocyanins that accumulate during this stage are lost during fruit growth. The second, larger peak in anthocyanin synthesis takes place during ripening (Macheix et al., 1990; Saure, 1990). Little is known about anthocyanin accumulation during early fruit growth in apple due to its economical non-significance and only speculated biological significance.

Hatier and Gould (2009) reviewed the potential functions of anthocyanins in plants, viz. defense against herbivory and pathogen attack, attracting frugivores for seed dispersal and insect vectors for flower pollination, as well as protection against osmotic stress, UV-B radiation, photoinhibition and photooxidation (either through shielding of visible light or quenching of reactive oxygen species (ROS)). These functions are not necessarily all applicable to apple fruit. Steyn (2009) argued that anthocyanins in fruit may serve to attract seed dispersers, signal fruit quality, and protect fruit against seed predators, and photoinhibition and oxidative damage caused by high levels of visible light. Lancaster (1992) suggested that high levels of flavonoids and anthocyanins in apple fruitlets filter out UV light during cell division and thereby prevent nuclear aberration. However, Solovchenko and Schmitz-Eiberger (2003) found that anthocyanins in 'Braeburn' apple had no additional effect in UV-B protection over and above other phenolic compounds present in the skin.

Anthocyanin synthesis in plants generally coincides with periods of high excitation pressure and increased potential for photo-oxidative damage (Steyn et al., 2002). The same appear to be true for apple fruit (Steyn et al., 2009). Anthocyanins in ripening apples are induced at low temperatures (<10 ºC) (Curry, 1997; Reay, 1999; Saure, 1990) and synthesis takes place under irradiation at mild temperatures (20 ºC to 27 ºC) (Curry 1997; Reay, 1999; Paper 1). Immature apples of at least some cultivars seem to accumulate anthocyanins at lower temperatures than mature fruit. According to Faragher (1983), the optimum temperatures for

anthocyanin accumulation in immature and mature ‘Jonathan’ apples under continuous irradiation are 12 ºC and 16 ºC to 24 ºC, respectively. Optimum temperatures for color development in ‘Cripps’ Pink’ increased from 5 ºC to 22 ºC during fruit development (Paper 1). Daily changes in the hue of ‘Rosemarie’ pears strongly correlated with average daily temperature (Steyn et al., 2004). Hue decreased with onset of a cold front by up to 6° per day and increased during warmer periods with up to 5° per day.

Chlorophyllous tissues that receive more light energy than can be used in photochemistry undergo a decrease in quantum efficiency of photosynthesis, better known as photoinhibition (Adams et al., 2008; Long et al., 1994). When photoinhibited, chloroplasts generate ROS that, when in superabundance, may potentially destroy thylakoid membranes, damage DNA and denaturate proteins associated with photosynthetic electron transport (Alscher et al., 1997). ROS production increases in response to stresses such as low temperature (Prassad et al., 1994; Prassad, 1996) and have been implicated in photoinhibition (Hull et al., 1997) and cellular damage (Wise, 1995).

Chlorophyll bleaching in red zones of apple fruit containing anthocyanins was much lower than in green zones and protection increased with pigment concentration (Merzlyak and Chivkunova, 2000). Cheng and Ma (2004) reported that anthocyanin accumulation coincided with the upregulation of the xanthophyll cycle and ascorbate-glutathione cycle after exposure of shaded apple peel to full sunlight. The higher photoprotective capacity of sun-exposed peel of red ‘Anjou’ pear compared with green ‘Anjou’ is mainly attributable to its higher anthocyanin concentration and, to a lesser extent, to a larger xanthophylls cycle pool size and higher activity of some antioxidant enzymes (Li et al., 2008).

Our premise in this paper is that anthocyanins in immature apple fruit protect apple peel from photoinhibition and photooxidative damage during conditions of increased light stress. Three hypotheses were based on this premise. The first hypothesis was that anthocyanins accumulate fast enough to provide photoprotection during sudden cold snaps. Secondly, we considered that protection of fruit peel against photoinhibition during cold snaps lowers the risk of subsequent high light and high temperature induced damage to fruit peel when temperatures increase again after the cold snap. Lastly, we hypothesized that the sensitivity

of fruit peel to photoinhibition increases during fruit development, thereby explaining why anthocyanins apparently accumulate at lower temperatures in immature apples.

Materials and Method

Plant material.

All experiments were conducted, or fruit for experiments obtained, from a North-South planted 'Cripps' Pink' apple orchard at Welgevallen experimental farm in the Mediterranean-type climate Stellenbosch region (Lat: 33º 58' S; Long: 18º 50' E) of the Western Cape province, South Africa.

Experiment 1: Rate of colour change in response to temperature.

One sun-exposed fruit was tagged on the Eastern side on each of 28 trees in a single orchard row. A cold front passed over the Western Cape from 17 to 20 March 2009. Hue measurements were taken three times daily (0700 HR, 1300 HR, and 1900 HR) from 16 March until 22 March 2009 at the reddest position on the fruit using a chromameter (NR-3000, Nippon Denshoku, Tokyo, Japan). Hue angles relevant to this experiment ranged between 30 º (red) and 100 º (yellow-green). A hue angle of 15 º indicates that less green light is reflected than red light and the tissue will appear red to the human eye. Climatic data were obtained from a weather station ±4 km from the trial site.

Experiment 2: Response of apple peel photochemistry to temperature and light.

The response of 'Cripps' Pink' apple peel photochemistry to a range of temperatures in combination with varying light intensities was assessed during the 2008/2009 season. This experiment was repeated four times during fruit development at 60, 100, 130 and 159 days after full bloom (dafb). Commercial harvest was expected at 175 dafb. Fruit were sampled at random from the same row, one fruit per tree, before 0730 HR. The fruit were brought to our laboratory, dark adapted for 30 min at 20 ºC and transferred to growth chambers set at 16, 24, 32 or 40 °C for 30 min before onset of measurement in order for the fruit peel to reach the desired temperature. F_v/F_m (maximum quantum yield of fluorescence), actual PSII efficiency (ϕ_{PSII}), photochemical quenching (qP) and non-photochemical quenching (qNP) were measured with a pulse modulated fluorimeter (FMS2, Hansatech Instruments Ltd., Norfolk, England) within the growth chambers. Measurements were taken at increasing actinic

irradiance (140, 470 and 1400 μmol m^{-2} s^{-1}PPF) on a 2-cm thick disc that was cut from the sun-exposed side of fruit just prior to measurement. The order in which replicates of the treatments were measured was randomized. Calculations of F_v/F_m, ϕ_{PSII}, qP and qNP were done according to Van Kooten and Snel (1990) and Maxwell and Johnson (2000). Additional measurements included average fruit size for each experimental repeat and anthocyanin and plastid pigment concentrations.

Pigment analysis: Anthocyanin analysis was done by weighing 0.2 g freeze dried sample in a centrifuge tube and adding 5 ml cold solvent consisting of methanol and 3 mol·L^{-1} HCl (95:5 by volume) to the sample. The solution was stirred at 4 ºC for 1 h, followed by centrifugation at 10,000 g_n for 10 min at 4 ºC. The supernatant was poured into a glass vial, closed and kept in the fridge. The pellet was resuspended in 5 ml cold solvent and vortexed for a few seconds. It was then again centrifuged for 10 min at 10,000 g_n at 4 ºC. The second supernatant was added to the first and mixed well. The extract was filtered through 0.45 μm syringe filter into a glass vial. Absorbance of the supernatant was measured at 530 nm and 630 nm (UV-visible spectrophotometer Cary 50Bio, Varian Ltd, Walton-on-Thames, UK). Due to the presence of chlorophyll, the absorption at 530 nm is corrected by the equation: $A_{530} - 0.24 \times A_{653}$ (Murray and Hackett, 1991).

Chlorophyll and carotenoid analyses were done by weighing 0.2 g dry sample in a centrifuge tube and adding 4 ml cold solvent (80% acetone) to the sample. The solution was stirred at 4 ºC for 24 h, followed by centrifugation at 10,000 g_n for 15 min at 4 ºC. The supernatant was decanted into a glass vial, closed and kept in the fridge. The pellet was resuspended in 4 ml cold solvent and vortexed for a few seconds. It was then again centrifuged for 15 min at 10,000 g_n at 4 ºC. The second supernatant was added to the first and mixed well. The extract was filtered through 0.45 μm syringe filter into a glass vial. Absorbance of the supernatant was measured at 470 nm, 645 nm and 662 nm and chlorophyll and carotenoid concentrations determined according to Lichtenthaler (1982).

Experiment 3: Predisposal of apple peel to heat damage by prior low temperature stress.

This experiment was conducted during the 2007/2008 season. Shaded green 'Cripp's Pink' apples were randomly picked from the inside of the tree canopy from the same row before

0730 HR on 17 January 2008. Fruit were randomly assigned to three treatments (LOW-16, MILD-28 and Control) with seven replications of two fruit each and dark adapted at 20 ºC for 30 min. Hereafter, F_v/F_m was measured with a pulse modulated fluorimeter (FMS2, Hansatech Instruments Ltd., Norfolk, England) on the greenest sides of the fruit. Fruit for the low (LOW-16) and mild temperature treatments (MILD-28) were placed in growth chambers for 3 h set at 16 ºC and 28 ºC, respectively. Growth chambers were fitted with 2 overhead lamps (400 W; SON-T; Osram Mgbh, Munich, Germany) producing a constant PPF of 600 to 700 µmol m^{-2} s^{-1} (quantum meter LI-189; Li-Cor, Lincoln, Nebraska, USA) at the level of the fruit. Control fruit were also placed in the growth chamber set at 16 ºC, but were shaded from light by means of cardboard covers. After these pre-treatments, apples were again dark adapted and F_V/F_M measured. Apples from all three treatments were subsequently subjected to high temperature (37.5 ± 1.5 ºC) and high PPF (900 to 1350 µmol m^{-2} s^{-1}) for 2 h, followed by dark adaptation and measurement of F_V/F_M.

On 26 February 2008, 48 'Cripps' Pink' apples were picked and dark adapted as described above. A peel disc (15 mm in diameter, 5 mm thick) was punched from the greenest side of each apple. Discs were randomly assigned to treatments, i.e. LOW-10 (10 ºC), MILD-25 (25 ºC) and CONTROL (10 ºC and kept dark), with three replicates of four discs per treatment. Discs were placed on the 12 independently controlled peltier plates (5 cm x 6.5 cm) of a CELTEC constructed according to the design of Burke and Mahan (1993), with a H_2O-moistend filter paper between the discs and the plate. The CELTEC was placed in a growth chamber with 2 overhead lamps (400 W) producing a PPF of 700 to 800 µmol m^{-2} s^{-1}at fruit level. Temperature plates, containing the discs, were covered with thin (0.5 mm) 100% crystal clear polyethylene wrap (Glad Wrap™, Glad South Africa, Randburg, South Africa). A few holes were made in the plastic with a toothpick to prevent the build-up of CO_2 and, possibly, ethylene and to reduce condensation of water on the inside of the plastic. After 3 h, discs were removed from the CELTEC and placed in petri dishes containing water. F_V/F_M measurements were done after 30 min dark adaptation and again after 22 h recovery at 20 ºC in the dark. All the peel discs were subsequently transferred back to the CELTEC and subjected to a stress treatment of 45 ºC for 2 h at PPF of 700 to 800 µmol m^{-2} s^{-1}. F_V/F_M measurements were taken after dark adaption and again after 12 h recovery.

Statistical Analysis.

The data were analyzed with the General Linear Models (GLM) procedures of SAS Enterprise Guide 3.0. (SAS Institute, 2004, Cary, N.C, U.S.A). Orthogonal contrasts were fitted where appropriate.

Results

Experiment 1: The rate of color change in response to low temperature.

After the maximum temperature reached 36 ºC on 15 March, temperatures decreased considerably (av. Max. and min. temperature 24.5 ºC and 10.5 ºC, respectively) from 16 to 20 March in response to a cold front on 16 March (Fig. 1). Radiation levels were high over most of the measurement period, except for 16 March, which was an overcast day. The average hue angle of 'Cripps' Pink' apples decreased from 80 to 47º between 0700 HR on 16 March and 1900 HR on 20 March, the duration of the cold front. The fastest decrease in hue (0.65º h^{-1}) was measured on 18 March which was a clear, cool day (max. temperature = 24 ºC), following a cold night (min. temperature = 7.6 ºC). Hue angle started to increase again on 21 March (max. temperature 33 ºC) and increased markedly between 0800 HR and 1900 HR on 22 March (0.39º h^{-1}) when the daily temperature reached 28 ºC.

Experiment 2: Effect of temperature and light on apple peel photochemistry.

Fruit size increased linearly from 33 mm to 56 mm between 60 and 159 dafb. 'Cripps' Pink' apple peel did not contain anthocyanins prior to 159 dafb and even at 159 dafb, the level of anthocyanins was still too low to be of statistical significance (Table1). Chlorophyll and carotenoid concentrations showed a quadratic decrease from 60 dafb until 159 dafb. At 60 dafb chlorophyll and carotenoid concentrations were higher than at 100, 130 and 159 dafb. At 100 dabf, chlorophyll and carotenoid concentrations were higher than at 130 and 159 daf. Although the chlorophyll:carotenoid ratio did not differ significantly at the different fruit development stages, the ratio did decrease linearly during fruit development.

No interaction was found between temperature and dafb for any of the fluorescence parameters. F_v/F_m of 'Cripps' Pink' apples did not differ significantly at the onset of the experiment at all four stages of fruit development (Table 2). Actual photochemical efficiency (ϕ_{PSII}) decreased linearly with a decrease in temperature from 40 to 16 ºC. However, ϕ_{PSII} was

repressed to similar low levels at 16 and 24 ºC at 1400 µmol m^{-2} s^{-1}. There was no difference in ϕ_{PSII} between 40 and 32 ºC at 140 and 1400 µmol m^{-2} s^{-1}. ϕ_{PSII} showed a gradual, albeit insignificant (p = 0.0543), increase with dafb at 1400 µmol m^{-2} s^{-1}. However, at 140 and 470 µmol m^{-2} s^{-1}, ϕ_{PSII} showed a quadratic response to dafb, first decreasing from 60 to 130 dafb and then increasing again to 159 dafb. ϕ_{PSII} at 140 µmol m^{-2} s^{-1} was significantly lower at 130 dafb compared to 60 and 100 dafb, with the latter also differing significantly. At 470 µmol m^{-2} s^{-1}, ϕ_{PSII} was significantly lower at 130 compared to 60 dafb.

qP decreased linearly with temperature at all three light levels and also decreased linearly with dafb at 140 µmol m^{-2} s^{-1}. No differences between stages of fruit development were apparent at the higher light levels (Table 3). At 140 and 470 µmol m^{-2} s^{-1}, qP was higher at 40 ºC and 32 ºC compared to 24 ºC and 16 ºC while at 1400 µmol m^{-2} s^{-1}, qP was higher at 40 ºC than at 24 ºC and 16 ºC and higher at 32 ºC than at 16 ºC. qP was higher at 24 ºC than at 16 ºC at all three light levels. At 140 µmol m^{-2} s^{-1}, qP was lower at 130 and 159 than at 60 and 100 dafb.

qNP decreased linearly with increasing temperature at 140 µmol m^{-2} s^{-1} and 470 µmol m^{-2} s^{-1} while no differences between temperatures were observed at 1400 µmol m^{-2} s^{-1} (Table 4). At 140 µmol m^{-2} s^{-1}, qNP was higher at 16 ºC and 24 ºC compared to 32 ºC and 40 ºC. At 470 µmol m^{-2} s^{-1}, qNP was lower at 32 ºC than 16 ºC and also lower at 40 ºC than at 16 ºC and 24 ºC. At 140 µmol m^{-2} s^{-1}, qNP was lower at 60 dafb than at later development stages. At 470 µmol m^{-2} s^{-1} qNP was lower at 60 dafb than at 159 dafb while at 1400 µmol m^{-2} s^{-1}, qNP was lower at 130 dafb than at the other development stages.

Experiment 3: Predisposal of 'Cripps Pink' apple peel to heat damage by prior low temperature stress.

The initial maximum quantum efficiency (F_v/F_m) of the fruit used for this experiment did not differ significantly at the onset of the experiment (Table 5). F_v/F_m decreased considerably after MILD-28 (0.471) and LOW-16 pre-treatment (0.398) whereas the control fruit remained at 0.840. Heat stress decreased F_v/F_m of all three treatments. F_v/F_m of MILD-28 (0.284) and LOW-16 (0.288) did not differ significantly, but were lower than the control (0.447).

The experiment was subsequently repeated making use of fruit peel discs. F_v/F_m of peel discs did not differ significantly at the onset of the experiment (Table 6). LOW-10 treatment significantly decreased F_v/F_m (0.040) compared to MILD-25 (0.112) whereas control discs were not affected by the pretreatment. F_v/F_m of LOW-10 and MILD-25 increased during the 24 h recovery period to 0.075 and 0.197 respectively, but still differed significantly in the same order as after the pretreatment. Heat stress had a severe effect on $F_v/F_{m,}$ to such an extent that none of the treatments differed significantly. F_v/F_m did not increase markedly during the 12 h recovery period.

Discussion

The rate of color change in response to low temperature.

Changes in the hue angle of 'Cripps' Pink' apples during a cold front were recorded in order to assess the rate at which anthocyanin photoprotection can be deployed. According to Iglesias (1999), a linear relationship exists between anthocyanin concentration in apple peel and hue angles ranging from 120º to 35º. Hence, a change in hue angle over this range is evident of a corresponding change in anthocyanin level. Not many studies have been done on the rate of anthocyanin accumulation in response to a decrease in temperature. Steyn et al. (2009) reported that the hue angle of 'Cripps Pink' apples decreased by ≈9.75º.day^{-1} on the second day of a cold front and hue angle decreased by 27º over the four days that the cold snap lasted. The fastest decrease in hue angle in this study also occurred on the second day of the cold snap with a 33º decrease in hue angle over the four day duration of the cold front. The rapidity of the hue angle decrease suggests that anthocyanin photoprotection may be employed before the photo-apparatus incurs any significant photodamage under conditions of high excitation pressure. Hence, anthocyanin accumulation may provide effective short-term photoprotection under conditions when other photoprotective measures are less effective or may take too long to deploy.

Environmental stresses such as low temperatures lower the photosynthetic rate at a given irradiance, thereby increasing the degree to which absorbed light is excessive. This increases the need for energy dissipation through, amongst others, de-epoxidation of violaxanthin to zeaxanthin as part of the xanthophyll cycle (Demmig-Adams and Adams, 1996) and the up

regulation of nonenzymatic radical scavengers (i.e. glutathione, ascorbic acid and cartenoids) and antioxidant enzymes (i.e. superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), ascorbate peroxidase (APX) and glutathione reductase (GR)) (Almeselmani et al., 2006). However, many of these protective measures are rate-limited by temperature.

In summer, zeaxanthin levels gradually increase in response to increased excitation levels to reach a peak at midday (Demmig-Adams and Adams, 1996). Arvidsson et al. (1997) found that temperature has a strong influence on both the rate and degree of maximal de-epoxidation in non-acclimated spinach leaves with only 50% conversion of violaxanthin detected at 4 °C, whereas at 25 °C and 37 °C the degree of conversion was 70% and 80%, respectively. The maximum rate of de-epoxidation in leaves of *Malva parviflore* L. and *Gossypium hirsutum* L. (cotton) showed a strong decrease with decreased temperature (Bilger and Björkman, 1991). Q_{10} measured around 15 ºC was in the range 2.1 to 2.6 in *Malva* leaves whereas it was as high as 4.6 in cotton leaves that developed at a daytime temperature of 30 ºC. Latowski et al. (2003) found that a decrease in temperature also resulted in a decrease in zeaxanthin production in leaves of *Lemma trisulca* L. The activity of SOD, CAT, APX and GR increased over a 3 day exposure of rice leaves to chilling treatment at 5 ºC followed by a 5 day recovery period to reach a maximum between 2 (GR) to 6 (APX, CAT and SOD) days after the onset of the chilling treatment (Kuk et al., 2003). Ma and Cheng (2004) reported that upon the sudden exposure of shaded apple peel to full sunlight, it took antioxidant enzymes, xanthophyll cycle carotenoids and soluble antioxidants ten days to reach the same levels as in sun-exposed fruits. The authors mentioned that red color increased concomitant with the increase in other protective measures, but unfortunately did not measure the rate or level of anthocyanin accumulation.

Plants acclimatize to the longer term stresses associated with seasonal changes in climate with longer term adaptations such as an increase in xanthophyll capacity and the persistence of high zeaxanthin levels throughout the day (Demmig-Adams and Adams, 1996; Katahata et al., 2005). These longer term adaptations may not be suitable for the transient cold snaps experienced during summer. Anthocyanins are rapidly degraded when favorable conditions for photosynthesis ensue after the passing of a cold front (Fig. 1; Steyn et al., 2004). Hence, the accumulation of anthocyanins in response to cold snaps does not have a lasting effect on

photosynthesis upon the return to warmer conditions. The prolonged presence of high levels of anthocyanins may negatively affect carbon assimilation (Creelman and Mullet, 1997; Jeanette et al., 2000; Steyn, 2002).

Effect of temperature and light on apple peel photochemistry.

The intrinsic (maximum) efficiency of PSII in dark-adapted fruit peel, remained at the same high level (> 0.76), indicative of healthy tissue (Maxwell and Johnson, 2000; Rosenqvist and Van Kooten, 2003), throughout fruit development. The photoapparatus in 'Cripps Pink' peel appears to be particularly sensitive to light stress at low temperature (16 ºC) throughout the season with significant photoinhibition occurring even at a moderate temperature of 24 ºC. The composition and biochemistry of fruit photosynthesis differs considerably from that of leaves (Blanke and Lenz, 1989). Steyn et al. (2009) suggested that the higher photosensitivity of fruit peel to high light and mild-to-low temperatures may relate to these differences. Steyn et al. (2009) further suggested that the apparent photosensitivity of fruit peel to low temperatures may also be an adaptation of sun-exposed fruit peel to the above ambient temperatures that fruit peel is exposed to due to radiant heating.

ϕ_{PSII} of 'Cripps' Pink' apple peel at 140 µmol m^{-2} s^{-1} decreased during fruit development, but increased again at 159 dafb (Table 2). At 470 µmol m^{-2} s^{-1}, ϕ_{PSII} were stable throughout fruit development with only a slight quadratic effect due to 60 dafb and 130 dafb differing statistically ($p = 0.044$). ϕ_{PSII} showed no response to stage of fruit development at 1400 µmol m^{-2} s^{-1} indicating that the apple peel is equally sensitive to high light conditions throughout fruit development. qP indicates the approximate oxidation of PSII and is the quenching of fluorescence caused by the potential for photochemistry, i.e., open PSII reaction centers (Rosenqvist and Van Kooten, 2003). qP typically decreases with increasing illumination as more plastoquinone (Q_A) of PSII becomes reduced. qP decreases due to closure of reaction centers resulting from saturation of photosynthesis by light (Maxwell and Johnson, 2000). Saturation occurs much faster at lower temperatures resulting in PSII receiving much more light that can be used in photochemistry as was also evident in this trial. However, except at 140 µmol m^{-2} s^{-1} where qP showed a linear decrease over fruit development, the reductive state of PSII where not apparently affected by the stage of fruit development. Non-photochemical quenching (qNP) relates to non-radiative dissipation of excitation energy as

heat through various mechanisms in the antenna and reaction center. (Rosenqvist and Van Kooten, 2003). qNP increased linearly with a decrease in temperature (Table 5) and also increased linearly during the fruit development at 140 µmol m^{-2} s^{-1} and 470 µmol m^{-2} s^{-1}, but not at 1400 µmol m^{-2} s^{-1}. Thus in this study it was found that at high irradiance, temperature and the stage of fruit development had no effect on the level of qNP.

Our data do not support the hypothesis that the sensitivity of sun-exposed apple peel to photoinhibition increases during fruit development. This is in agreement with the results of Li et al. (2008) for sun-exposed 'Anjou' pear peel. However, the shaded sides of 'Anjou' pear became more sensitive to light stress during fruit development. Thus it appears that sun-exposed apple and pear peel adapts to high PPF and that photoprotection is maintained throughout fruit development. The innate ability of apple peel to accumulate anthocyanin increases during fruit development (Curry, 1997; Paper 1). In 'Cripps' Pink', the optimum temperature for anthocyanin synthesis also increases during fruit development (Paper 1) as was also previously reported for 'Jonathan' apples (Faragher, 1983). Considering the sensitivity of apple peel to photoinhibition at low temperature and the stimulating effect of low temperature on anthocyanin synthesis, it could be considered that observed patterns of anthocyanin accumulation closely follows and reflects changes in the sensitivity of apple peel during fruit development. However, this does not seem to be the case.

Predisposal of 'Cripp's Pink' peel to heat damage by prior low temperature stress.

High levels of visible light combined with low temperatures have been implicated in the photooxidative destruction of chlorophyll (Henry et al., 1987). Steyn et al. (2009) recently showed that the severity of photoinhibition in 'Forelle' pear peel under high visible light increases with a decrease in temperature from 40 to 16 ºC. Photoinhibition occurs when fruit peel receives more light energy than can be used in photochemistry (Adams et al., 2008; Long et al., 1994). Under severe conditions, chloroplasts generate ROS with its destructive effect on cellular components (Alscher et al, 1997). Felicetti and Schrader (2008) characterized a type of sunburn caused by photooxidative damage induced by visible light at temperatures below 31 ºC. Considering the above, it is reasonable to suppose that young fruitlets, exposed to high irradiance and relative cool conditions in the early phase of development, may experience chronic photoinhibition and photooxidative damage that may

predispose the fruit to photooxidative sunburn later in the season under high temperatures and high light conditions. In agreement with previous research on leaves and fruit (Steyn et al., 2009), we found that apple peel incurred significantly more photoinhibition at low (16 ºC) compared to mild (24 to 32 ºC) and high (40 ºC) temperatures when irradiated with high light levels. Greer et al. (1986) reported photoinhibition to be the net difference between rate of damage and repair to reactions associated with photosystem II. The recovery rate is temperature dependent, being the slowest at low temperatures and increasing with temperature. Unfortunately, we could not prove that protection of fruit peel against photoinhibition during cold snaps lowers the risk of subsequent high light and high temperature-induced damage to fruit peel. This was because of the severe effect of the high temperature treatment on the photo apparatus that nullified earlier treatment differences. We suggest that these trials are repeated earlier during fruit development and making use of whole fruit and less severe high temperature stress.

Conclusion

It is evident that photoapparatus in 'Cripp's Pink' peel remains sensitive to light stress at lower temperatures throughout the season. The rapidity at which apple peel increase and decrease in red color in response to changes in temperature, suggests that anthocyanins may be used to modulate light levels according to short term, transient changes in excitation pressure (Pietrini and Massacci, 1998). Fruit peel increased in sensitivity to photoinhibition during fruit development at low, but not at high irradiance. Hence, it is not possible to link the increase in the optimum temperature and extent of anthocyanin synthesis in 'Cripps' Pink' (Paper 1) and 'Jonathan' (Faragher, 1983) peel with an increase in sensitivity to light stress during fruit growth.

Although this study once more demonstrated the significant photodamage experienced at low temperatures versus mild and high temperatures, we could not prove that protection of fruit peel against photoinhibition during cold snaps lowers the risk of subsequent photothermal damage when temperatures increase again after the cold snap. Since the lack of results was due to the severity of the stress treatments, the experiment should be repeated with further fine tuning of experimental procedure and application at an earlier stage of fruit development before the hypothesis can be rejected.

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Table 1: Anthocyanin, chlorophyll and carotenoid concentrations in sun-exposed 'Cripps' Pink' apple peel during the 2008/2009 season. Means, separated by LSD (5%), are averages of 5 fruit.

Stage of fruit development (Dafb)	Fruit diameter (mm)	Carotenoid $\mu g\ g^{-1}$	Chlorophyll $\mu g\ g^{-1}$	Chl:Car	Anthocyanin $\mu g\ g^{-1}$
60	33 a [Z]	16.3 a	78.1 a	4.9 [ns]	0.0 [ns]
100	43 b	10.7 b	45.8 b	4.3	0.0
130	45 b	7.2 c	27.5 c	4.0	0.0
159	56 c	6.1 c	19.9 c	3.3	8.0
Pr > F	0.0002	0.0003	<0.0001	0.1928	0.4382
Linear	<0.0001	<0.0001	<0.0001	0.0416	0.2150
Quadratic	0.8623	0.0474	0.0054	0.8952	0.3449

[ns] Non-significant

[Z] Means followed by the same letter within the same column do not differ significantly at $P \leq 0.05$

Table 2: Effect of fruit age and temperature on the maximum quantum yield of PSII (F_v/F_m) and actual PSII efficiency (ϕ_{PSII}) at 140, 470 and 1400 µmol m^{-2} s^{-1} of sun-exposed 'Cripps' Pink' apple peel at four stages of fruit development during the 2008/2009 season. Means, separated by LSD (5%), are averages of 12 fruit.

Treatment	F_v/F_m	ϕ_{PSII}		
		140	470	1400
Temperature (ºC)				
16	0.783 ^{ns}	0.308 c ^{z}	0.208 d	0.081 b
24	0.797	0.385 b	0.298 c	0.104 b
32	0.782	0.524 a	0.386 b	0.134 a
40	0.780	0.557 a	0.437 a	0.154 a
Date (dafb)				
60	0.797 ^{ns}	0.518 a	0.351 a	0.105 ^{ns}
100	0.792	0.449 b	0.335 ab	0.114
130	0.760	0.389 c	0.304 b	0.122
159	0.793	0.413 bc	0.337 ab	0.135
Pr > F				
Temperature (T)	0.8787	<0.0001	<0.0001	<0.0001
T Linear	0.7112	<0.0001	<0.0001	<0.0001
T Quadratic	0.6585	0.1792	0.1919	0.8093
Date (D)	0.1609	<0.0001	0.0444	0.1490
D Linear	0.4441	<0.0001	0.1287	0.0543
D Quadratic	0.1867	0.0009	0.0385	0.5119
T * D	0.8806	0.1443	0.5009	0.5647

^{ns} Non-significant

^{z} Means followed by the same letter within the same column do not differ significantly at $P \leq 0.0$

Table 3: Effect of fruit age and temperature on photochemical quenching, which is the proportion of PSII 'open' reaction centers (qP), at 140, 470 and 1400 µmol m^{-2} s^{-1} of sun-exposed 'Cripps' Pink' apple peel at four stages of fruit development during the 2008/2009 season. Means, separated by LSD (5%), are averages of 12 fruit.

Treatment	qP		
	140	470	1400
Temperature (ºC)			
16	0.531 c [Z]	0.399 c	0.191 c
24	0.678 b	0.547 b	0.257 b
32	0.802 a	0.654 a	0.312 ab
40	0.853 a	0.714 a	0.361 a
Date (dafb)			
60	0.773 a	0.598 [ns]	0.263 [ns]
100	0.742 a	0.590	0.285
130	0.657 b	0.538	0.267
159	0.673 b	0.572	0.306
Pr > F			
Temperature (T)	<0.0001	<0.0001	<0.0001
T Linear	<0.0001	<0.0001	<0.0001
T Quadratic	0.0648	0.0843	0.8401
Date (D)	0.0010	0.2621	0.4383
D Linear	0.0002	0.1151	0.3763
D Quadratic	0.1065	0.1675	0.4079
T * D	0.1292	0.4627	0.6709

[ns] Non-significant

[Z] Means followed by the same letter within the same column do not differ significantly at P ≤ 0.05

Table 4: Effect of fruit age and temperature on non-photochemical quenching (qNP) at 140, 470 and 1400 µmol m^{-2} s^{-1} of sun-exposed 'Cripps' Pink' apple peel at four stages of fruit development during the 2008/2009 season. Means, separated by LSD (5%), are averages of 12 fruit.

Treatment	qNP		
	140	470	1400
Temperature (ºC)			
16	0.767 a [z]	0.831 a	0.905 [ns]
24	0.767 a	0.807 ab	0.908
32	0.668 b	0.768 bc	0.905
40	0.653 b	0.730 c	0.902
Date (dafb)			
60	0.610 b	0.760 b	0.912 a
100	0.739 a	0.789 ab	0.916 a
130	0.768 a	0.790 ab	0.886 b
159	0.761 a	0.811 a	0.912 a
Pr > F			
Temperature (T)	<0.0001	<0.0001	0.9501
T Linear	<0.0001	<0.0001	0.7350
T Quadratic	0.4900	0.4741	0.7009
Date (D)	<0.0001	0.0428	0.0118
D Linear	<0.0001	0.0102	0.4738
D Quadratic	0.0007	0.5266	0.1597
T * D	0.0866	0.7715	0.2163

[ns] Non-significant

[z] Means followed by the same letter within the same column do not differ significantly at $P \leq 0.05$

Table 5: Effect of subjecting ‘Cripps’ Pink’ apples picked on 17 January 2008 to a mild- (28 ºC) or low (16 ºC) temperature pre-treatment, followed by a heat stress treatment (37 ºC) on the maximal quantum efficiency of PSII photochemistry (F_v/F_m). T_0 = F_v/F_m before treatment, T_1 = F_v/F_m after 3 h exposure to low or mild temperature, T_2 = F_v/F_m after 3 h high temperature treatment. Means were separated by LSD (5%). (n = 14)

Treatment	T_0	T_1	% Reduction in F_v/F_m from T_0 to T_1	T_2	% Reduction in F_v/F_m from T_0 to T_2
Control (16 ºC)	0.852 ^{ns}	0.840 a ^{Z}	1.5 a	0.447 a	47.5 a
Mild (28 ºC + PPFD ^{X})	0.843	0.471 b	44.0 b	0.284 b	66.3 b
Low (16 ºC + PPFD)	0.853	0.398 c	53.2 c	0.288 b	66.2 b
Pr > F	0.4719	<0.0001	<0.0001	0.0074	0.0081

^{X} PPFD = 600 to 700 µmol m^{-2} s^{-1}

^{Z} Means followed by the same letter within the same column do not differ significantly at P ≤ 0.05

^{ns} Non-significant

Table 6: Effect of subjecting 'Cripps' Pink' peel discs from apples picked on 26 February 2008 to a mild- (25 ºC) or low temperature (10 ºC) pre-treatment, followed by a heat stress treatment (45 ºC) on the maximal quantum efficiency of PSII photochemistry (F_v/F_m). T_0 = F_v/F_m before treatment, T_1 = F_v/F_m after 3 h exposure to low or mild temperature, R_1 = F_v/F_m after 22 h recovery, T_2 = F_v/F_m after 2 h high temperature (45 ± 1.5 ºC), R_2 = F_v/F_m after 12 h recovery. Means were separated by LSD (5%). (n = 16)

Treatment	T_0	T_1	R_1	% Reduction in F_v/F_m from T_0 to T_1	T_2	R_2	% Reduction in F_v/F_m from T_0 to T_2
Control (25 ºC)	0.837 ^{ns}	0.829 a ^{Z}	0.820 a	2.8 a ^{Y}	0.050 ^{ns}	0.049 ^{ns}	94.2 $^{ns\ Y}$
Mild (25 ºC + PPFD ^{X})	0.857	0.112 b	0.197 b	76.9 b	0.029	0.063	92.6
Low (10 ºC + PPFD)	0.836	0.040 c	0.075 c	91.0 c	0.054	0.056	93.2
Pr > F	0.1217	<0.0001	<0.0001	<0.0001	0.3018	0.7417	0.7609

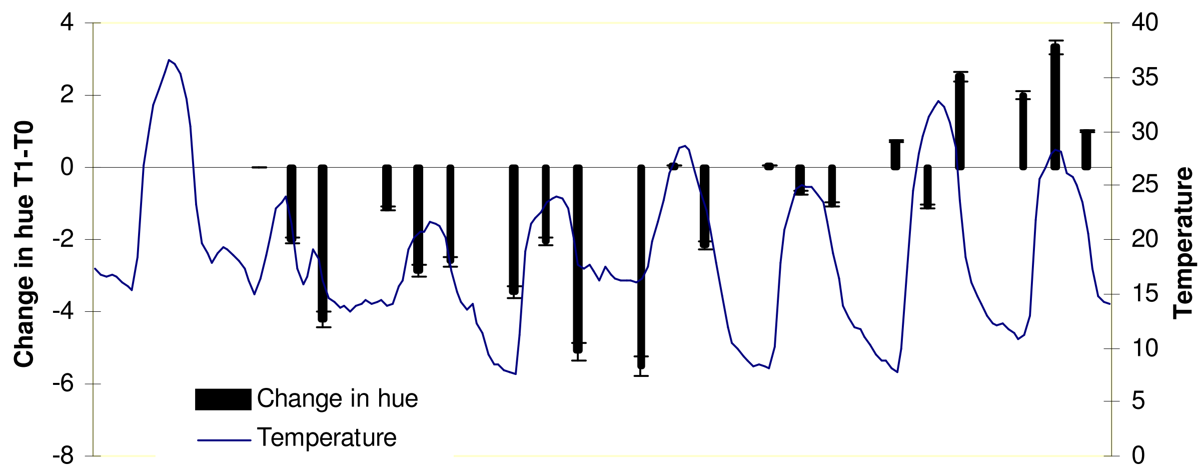
^{X} PPFD was measured to be at 600 to 700 µmol m^{-2} s^{-1}

^{Y} Reduction is determined in relation to F_v/F_m pre-treatment and F_v/F_m post recovery

^{Z} Means followed by the same letter within the same column do not differ significantly at P ≤ 0.05

^{ns} Non-significant

(a)



(b)

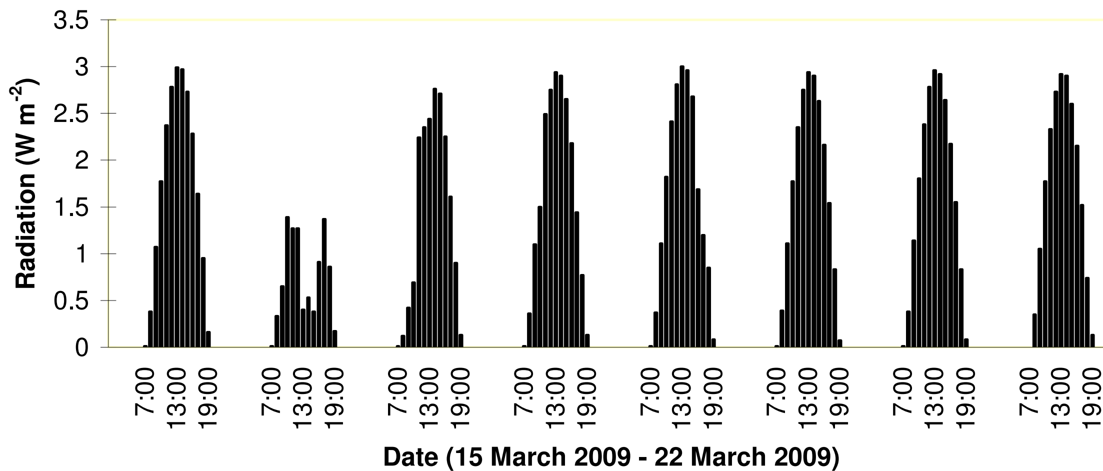


Figure 1: Change in hue angle of 'Cripps' Pink' apples in relation to **(a)** air temperature and **(b)** radiation levels experienced between 15 to 22 March 2009. Hue angle measurements commenced on 16 March 2009. Hue angle decreases with increasing redness. Therefore, a negative change in hue on this graph indicates an improvement in red color (n = 28).

GENERAL DISCUSSION AND CONCLUSION

The profitability of red and bi-coloured apples is dependent on the extent of red colour development before harvest. Red and bi-coloured apples require a certain minimum extent of red surface colour in order to qualify as Class one fruit. Fruit with less than the specified extent of red colour are downgraded and have to be sold in lower price markets or send for processing. It goes without saying that improving red colour development of red and bi-coloured apple cultivars and thereby increasing the proportion of the crop suitable for sale in more lucrative markets, is a primary objective in the production of these cultivars. Furthermore, it is well known that many apple cultivars, even some non-red ones, may develop a distinct red blush at times during fruit development. The significance of red colour development in immature fruit is not well understood. Temperature, apart from light, is the most limiting environmental factor for optimum red colour development (Lancaster, 1992; Saure, 1990). This is especially the case in the warm production areas of South Africa. Since different cultivars may have different temperature optima for anthocyanin synthesis (Curry, 1997), not all may be equally suited for all production areas. Knowing the optimum temperatures for red colour development in the various cultivars can provide a good indication of where these cultivars can be grown. Hence, the primary aim of our study was to determine the optimum day and night temperatures for red colour development in the five major red and bi-coloured cultivars grown in South Africa. We also considered the possible significance of early colour development in immature fruits.

In general, red colour in the cultivars evaluated developed maximally at a day-time temperature of 17 ºC to 25 ºC. However, it should be kept in mind that fruit surface temperatures in the orchard are generally higher than ambient air temperatures in the orchard (Schrader et al., 2003). Hence, the optimum daytime air temperatures for maximal red colour development would be lower than those reported in our study. Seasonal differences in optimum daytime temperatures for colour development was observed (Paper 1,Table 4), which implies that it may be difficult to make recommendations regarding areas that would not be suitable for the production of specific cultivars. It also suggests that growing conditions may affect the potential for anthocyanin synthesis. The optimum temperature for red colour

development in the various cultivars remained constant throughout fruit development, but increased during fruit development in CP during the 2008/2009 season as has been found for 'Jonathan' (Faragher, 1983). As expected from previous studies (Curry, 1997; Faragher, 1983; Reay, 1999; Saure, 1990), the extent of red colour development increased during fruit development. Finally, our data indicate that redder strains of bi-coloured cultivars do not appear to owe their enhanced pigmentation to higher temperature optima for anthocyanin synthesis. Although the temperature range established for optimal colour development seems to be in agreement with previous studies (Bakhshi and Arakawa, 2006; Curry, 1997; Faragher, 1983), we recommend the validation of our results including fruit from more than one orchard per production area. The effect of soil nutrient status, tree factors and cultural practices followed in the different orchards, and which may affect anthocyanin synthesis (Gurnsey and Lawes, 1999; Saure, 1990), could be assessed making use of the Celtec. These factors may explain differences in red colour development observed between cultivars, orchards, production areas and seasons.

It is been shown that maximal anthocyanin synthesis requires induction at low temperature (Curry, 1997; Christie, 1994). Hence, one could say that the daily minimum temperatures during the harvest period would be more decisive in determining red colour at harvest than the daily maximum temperatures. Unfortunately, no optimum inductive night temperature or temperature range for optimum colour development could be confirmed (vide Curry, 1997) or established for any of the cultivars evaluated. If we focus only on the results of RG from Ceres, it would appear that red colour development in RG may benefit from induction at 4 ºC. However, it is not scientifically and ethically correct to ignore the largely inconclusive results obtained for the other cultivars and for RG from Grabouw.

Curry (1997) also made use of apple peel discs on temperature controlled peltier plates to study the effect of inductive temperatures on anthocyanin synthesis. His results were much more conclusive and indicated that 'Fuji' and 'Red Delicious' peel discs accumulated considerably more anthocyanin if pre-treated at low temperature. Curry's experiment differed from our own in the larger dimensions of his peltier plates (7.5 cm x 7.2 cm) and in that he replicated peel discs on a single plate per temperature. Although not true replication, the use of a single plate with more discs of each cultivar per plate should have resulted in a smaller

variance. Curry did not make holes in the plastic film covering the samples, which we did in order to prevent condensation and the possible accumulation of ethylene at the risk of dehydration of samples. We did not observe any dehydration. The illumination source in our experiment was at shorter distance from the samples and provided considerably higher irradiance at the fruit surface. Hence, our irradiance levels would be more comparable to irradiation experienced under field condition. Since our day-temperature experiments gave satisfactory results, it is difficult to find reasons for the failure of the night-temperature experiment. We suggest that our technique for evaluating night temperatures is revised, maybe making use of whole apples instead of peel discs, before repeating the experiments.

In order to explain the role of anthocyanin synthesis during early fruit development, we argued that anthocyanins in immature apple fruit protect the peel from photoinhibition and photooxidative damage during conditions of increased light stress. Environmental stresses, such as low temperatures, lower the photosynthetic rate at a given irradiance, thereby increasing the degree to which absorbed light is excessive and increases the need for energy dissipation (Demmig-Adams and Adams, 1996). The rapidity by which the hue angle decreases in response to a sudden cold snap appears to be sufficient to provide photoprotection before the photo-apparatus incurs any significant photodamage under conditions of high excitation pressure. It remains to be determined whether the rapid accumulation of anthocyanin really is sufficient to avoid photoinhibition and subsequent photodamage in immature fruit. High temperature together with high irradiance can cause different types of sunburn (Felicetti and Schrader, 2008) depending on the photoprotective capacity of the fruit peel (Li et al., 2008). Unfortunately, we could not test the hypothesis that that protection of fruit peel against photoinhibition during cold snaps lowers the risk of subsequent high light and high temperature-induced damage to fruit peel. The severe effect of the high temperature treatment on the photo-apparatus nullified earlier treatment differences at low and mild temperature. We suggest that these trials are repeated at an earlier stage of fruit development, making use of whole fruit and less severe high temperature stress.

We found that apple peel incurred significantly more photoinhibition at low (16 ºC) compared to mild (24 to 32 ºC) and high (40 ºC) temperatures under high irradiance. The recovery rate

is temperature dependent, being the slowest after exposure to low temperature and increasing with an increase in the temperature that peel was exposed to.

Fruit peel increased in sensitivity to photoinhibition during fruit development at low, but not at high irradiance. Hence, we could not relate an increase in optimum temperature and the extent of anthocyanin synthesis during fruit development, as reported in previous studies (Farager, 1983), to an increase in sensitivity to light stress during fruit growth. Since the optimum temperature for red colour development in CP increased during fruit development, the lack of an increase in sensitivity of CP peel to light stres during fruit development is at odds with this idea.

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