RED COLOUR DEVELOPMENT AND LOSS IN PEAR FRUIT

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DECLARATION

I, the undersigned, hereby declare that the work contained in this dissertation is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

SUMMARY

Downgrading of fruit due to insufficient red colour has limited the profitability of lucrative blushed pear cultivars (*Pyrus communis* L.). In 'Rosemarie', poor fruit colour has been ascribed to pre-harvest red colour loss during periods of high temperature. The regulation of colour development in pears has not been studied and, in addition, little is known about anthocyanin degradation in attached fruit.

Changes in colour were recorded and phenylalanine ammonia-lyase (PAL) and UDPGalactose: flavonoid-3-*o*-glycosyltransferase (UFGT) activities assessed in response to cold fronts and during fruit development in order to establish the regulation of colour development in red and blushed pear cultivars. Best red colour was generally attained a month or more before harvest whereafter red colour faded towards harvest. Unlike in some other fruits, UFGT activity apparently did not limit colour development whereas fading of red colour towards harvest might relate to decreasing PAL activity. 'Rosemarie' colour fluctuated considerably, increasing with cold fronts and decreasing during intermittent warmer periods, while red colour was more stable in other cultivars. PAL and UFGT activities in 'Rosemarie' increased in response to low temperatures, but were unaffected in 'Bon Rouge'. We concluded that anthocyanin synthesis in 'Rosemarie' requires low temperatures while colour development in 'Bon Rouge' and probably also other cultivars is primarily regulated by endogenous factors.

Detached pome fruit were used to study temperature and light effects on anthocyanin degradation and fruit colour and to assess the modifying effect of anthocyanin concentration on colour loss. Anthocyanin degradation and red colour loss increased linearly between 10°C and 30°C. Irradiation further increased the rate of degradation and colour loss. The rate of colour loss depended on anthocyanin concentration, being much faster in fruit with high compared to fruit with low pigment levels. This was ascribed to the exponential relationship between anthocyanin concentration and hue at high pigment levels and the linear relationship at lower pigment levels. Anthocyanin degradation and pre-harvest red colour loss in 'Rosemarie' was quantitatively confirmed and corresponded with a warm period during fruit development. Based on these data, we attributed the susceptibility of 'Rosemarie' to

pre-harvest colour loss to low anthocyanin concentrations in its peel that allow the visualisation of net anthocyanin degradation at high temperatures.

Overhead evaporative cooling (EC) as measure to improve red colour in blushed pears was evaluated. 'Rosemarie' fruit that received pulsed EC applications from two weeks before harvest at air temperatures exceeding 28ºC were redder than control fruit at harvest. EC had no effect on 'Forelle' colour. Though EC could be used to improve 'Rosemarie' fruit colour in warm production areas, its effect was relatively small compared to colour change in response to temperature.

Lastly, we assessed the photoprotective function of anthocyanin in pear peel. Photoinhibition was evident in exposed faces of pears under natural conditions. The extent of photoinhibition increased with decreasing redness of peel and was maintained after photoinhibitory treatment. Although anthocyanin was apparently able to afford photoprotection at 40ºC, we argued against this as a general function. There were indications that photoprotection was associated, but not necessarily due to light attenuation by anthocyanin.

OPSOMMING

Afgradering van vrugte vanweë onvoldoende rooi kleur beperk die winsgewendheid van blospeercultivars (*Pyrus communis* L.). In die geval van 'Rosemarie' word swak kleur toegeskryf aan vooroes rooikleurverlies gedurende warm periodes. Die regulering van kleurontwikkelling in pere is nog nie ondersoek nie terwyl min bekend is oor antosianiendegradasie aan die boom.

Om die regulering van kleurontwikkelling in rooi- en blospeercultivars vas te stel, is veranderinge in kleur en in die aktiwiteit van fenielalanien ammonia-liase (FAL) en UDPGalaktose: flavonoied-3-*o*-glikosieltransferase (UFGT) gemeet gedurende vrugontwikkelling en in reaksie op koue fronte. Pere was op hul rooiste 'n maand of langer voor oes. Hierna het rooi kleur afgeneem tot met oes. Anders as in sommige ander vrugsoorte het UFGT aktiwiteit nie kleurontwikkeling beperk nie. Die afname in rooi kleur tot met oes mag egter verband hou met 'n gelyktydige afname in FAL aktiwiteit. 'Rosemarie' kleur het aansienlik gefluktueer in reaksie op temperatuur. Rooi kleur het toegeneem met koue fronte en afgeneem in die warmer periodes tussen fronte. Rooi kleur was meer stabiel en klaarblyklik minder afhanlik van lae temperature in ander peercultivars. Die noodsaaklikheid van lae temperature vir kleurontwikkelling in 'Rosemarie' is bevestig deur 'n toename in ensiemaktiwiteit in reaksie op koue fronte. Lae temperature het geen effek gehad op ensiemaktiwiteit in 'Bon Rouge' nie.

Appels en pere is gebruik om die effek van temperatuur en lig op antosianiendegradasie en vrugkleur te ondersoek. Die modifiserende effek van antosianienkonsentrasie op kleurverlies is ook ondersoek. Antosianiendegradasie en rooi kleurverlies het lineêr toegeneem tussen 10° en 30°C. Beligting het degradasie en kleurverlies verder versnel. Die tempo van kleurverlies was afhanklik van antosianienkonsentrasie. Kleurverlies was aansienlik vinniger in vrugte met hoë pigmentvlakke, in vergelyking met vrugte met lae pigmentvlakke vanweë die eksponensiële verwantskap tussen antosianienkonsentrasie en kleurskakeringswaardes (hue values) by hoë pigmentvlakke en die lineêre verwantskap by lae pigmentvlakke. Antosianiendegradasie en vooroes rooikleurverlies in 'Rosemarie' is kwantitatief bevestig en het saamgeval met 'n warm

periode tydens vrugontwikkelling. Gebaseer op hierdie data is die gevoeligheid van 'Rosemarie' vir vooroes rooikleurverlies toegeskryf aan lae antosianienkonsentrasies wat die sigbaarheid van netto antosianiendegradasie by hoë temperature verhoog.

Die gebruik van oorhoofse evaporatiewe verkoeling (EC) om rooi kleur van blospere te verbeter is ge-evalueer. 'Rosemarie' vrugte wat evaporatief verkoel is bo 28ºC vanaf twee weke voor oes, was rooier as kontrole vrugte by oes. 'Forelle' kleur het nie gereageer op EC nie. Die effek van EC op vrugkleur was relatief klein in vergelyking met die effek van temperatuur. Al kan EC 'Rosemarie' kleur verbeter in warm produksiestreke sou dit meer effektief wees om 'Rosemarie' se verbouing te beperk tot koeler klimaatstreke.

Laastens is die vermoë van antosianien om peerskil teen fotoinhibisie te beskerm ondersoek. Fotoinhibisie was aanwesig in vrugskil wat direk blootgestel was aan sonlig in die boord. Die omvang van fotoinhibisie het toegeneem met 'n afname in rooi pigmentasie van vrugskil. Die verband tussen skilkleur en fotoinhibisie was steeds aanwesig na blootstelling aan ligstres by 10º en 40ºC. Ons het egter geredeneer teen 'n algemene funksie vir antosianien in fotobeskerming by hoë temperature. Verder was daar aanduidings dat, alhoewel geassossieer met rooi skilkleur, beskerming teen ligstres nie noodwendig te wyte was aan antosianien nie.

Reinette, Pa Basie, Ma Hermien en Herma.

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CONTENTS

LITERATURE REVIEW 1:

RED COLOUR DEVELOPMENT AND LOSS IN POME FRUIT.

Downgrading due to insufficient red colour has limited the profitability of blushed pear cultivars in the Western Cape region of South Africa (Huysamer, 1998). Little is known about the regulation of red colour development in pears. Here, we review the regulation of colour development by endogenous and environmental factors in pome fruit. Although mostly referring to the extensive apple literature as covered in reviews by Lancaster (1992) and Saure (1990), we also refer to the limited literature available on pears. Since insufficient red colour of blushed pear fruit has been ascribed to pre-harvest colour loss (Huysamer, 1998), relevant literature on the degradation of anthocyanin will also be reviewed. The biosynthetic pathway of anthocyanins is presented to facilitate discussion of the regulation of synthesis.

Genotypic differences in skin colour.

Different shades of red in pome fruit peel are thought to arise through the visual blending of the red anthocyanins dissolved in the vacuole and the green to yellow chlorophyll and carotenoids residing in the plastids (Lancaster et al., 1994). The relative concentrations and distribution of each pigment type within the skin determines its contribution to fruit colour. Generally, fruit with better red colour contains more anthocyanin, has more pigmented cell layers, more red cells per layer and larger vacuoles (Awad et al., 2000; Lancaster et al., 1994). Anthocyanins in apples are located in the epidermis and adjacent hypodermal layers (Lancaster et al., 1994). In pears, two to five pigmented hypodermal layers are typically underlying an unpigmented epidermis and one or two unpigmented hypodermal layers (Dayton, 1966) suggesting that plastid pigments might contribute more to pear compared to apple colour.

Anthocyanin-containing cells of apple cultivars and genotypes displaying different shades of red colour did not display a red shift in maximum absorption of anthocyanin, which is characteristic of co-pigmentation with other molecules and has a blueing effect. This result led Lancaster et al. (1994) to conclude that co-

pigmentation does not contribute to apple colour. Though not studied, this probably also applies to pears. The contribution of colour intensification due to self-association of anthocyanin pigments (Goto, 1987) to apple and pear colour has not been determined. Unlike in some other fruit kinds where qualitative differences in anthocyanin structural forms account for colours ranging from orange to purple, apples and pears only contain the red cyanidin pigments (Macheix et al., 1990). Cyanidin 3-galactoside constitutes the major portion of total anthocyanins in apples and pears (>85% in apples, less in pears) (Dussi et al., 1995 Francis, 1970; Lancaster, 1992). Cyanidin 3-arabinoside is the only secondary pigment in pears. In addition, apples also contain cyanidin 3-glucoside and trace amounts of acylated and other cyanidin pigments.

Anthocyanin biosynthesis.

The major precursory pathways as well as the individual enzymatic steps of the anthocyanin biosynthetic pathway are presented in Fig.1 based on the reviews by Lancaster (1992) and Saito and Yamazaki (2002). The basic flavonoid structure arises from the aromatic amino acid, phenylalanine, synthesised via the shikimic acid pathway, and from acetate units contributed by malonyl-CoA via acetate-CoA and glycolysis. In what is seen as the first committed step in phenolic metabolism, phenylalanine is converted to trans-cinnamate in a reaction catalysed by phenylalanine ammonia lyase (PAL). Trans-cinnamate is further converted to 4-coumaroyl-CoA. Conversion of phenylalanine to 4-coumaroyl-CoA constitutes general phenyl propanoid metabolism, which apart from flavonoids, also gives rise to phytoalexins, UV protectants, feeding repellents and cell-wall components (Hahlbrock and Scheel, 1989). Condensation of 4-coumaroyl-CoA with three molecules malonyl-CoA mediated by chalcone synthase (CHS) gives rise to tetrahydroxychalcone, the first specific flavonoid structure. The conversion of tetrahydroxychalcone to naringenin, a flavonone, is catalysed by chalcone isomerase (CHI). Dihydrokaempherol is formed by hydroxylation in the 3-position by flavonone-3-hydroxylase. Subsequent steps are postulated to entail conversion of dihydrokaempherol to dihydroquercetin by flavonoid 3'-hydroxylase, followed by conversion to leucocyanidin by dihydroflavonol reductase. The conversion of leucocyanidin to cyanidin has not been characterised until recently when Saito and Yamazaki (2002) clarified the biochemical mechanism of anthocyanidin synthase.

Ultimately, UDPGalactose: flavonoid-3-*o* –glycosyltransferase (UFGT) catalyses the attachment of a sugar (galactose and arabinose in the case of apples and pears) to the anthocyanin aglycone, considerably increasing its stability against degradation. All the enzymatic steps with the exception of the ultimate step occur in the cytosol. The glycosylation reaction probably takes place during the transport of cyanidin through the tonoplast. Once in the acidic environment of the vacuole, anthocyanins attain their characteristic colour through hydration (Saito and Yamazaki, 2002).

It is uncertain to what extent the activity of primary metabolic pathways such as glycolysis can influence colour development. However, the positive relationship between carbohydrate levels and anthocyanin accumulation is well documented. Anthocyanins synthesis is induced by exogenous sugars and treatments that increase carbohydrate levels (Jeannette et al., 2000; Vestrheim, 1970). Furthermore, there are indications that the supply of phenylalanine can regulate flux through the phenylpropanoid and flavonoid pathways (Da Cunha, 1987). Faust (1965) found that anthocyanin accumulation in apples concurred with increased flux through the pentose phosphate pathway. It is uncertain whether the availability of malonyl-CoA can also limit anthocyanin biosynthesis (Lancaster, 1992).

Developmental regulation of anthocyanin synthesis.

Though the anthocyanin concentration of fruit peel may fluctuate in response to environmental conditions, the ability to colour is a function of developmental stage and is independent of climatic conditions (Saure, 1990). Juvenile fruit of most apple cultivars accumulate anthocyanins during the cell division stage that lasts up to six weeks after anthesis (Lancaster, 1992; Saure, 1990). Colour subsequently disappears, whether due to dilution or degradation is uncertain (Lancaster, 1992). Anthocyanin again accumulates during the maturation of red cultivars, though green cultivars will also develop some red colour under specific conditions (Ju et al., 1999; Lancaster, 1992; Saure, 1990). While the accumulation of anthocyanin in ripening fruit is thought to aid seed dispersal (Harborne, 1965), the function of anthocyanin in immature fruit remains uncertain. In contrast to apples, red and blushed pear cultivars attain their highest anthocyanin concentrations in immature fruit whereafter red colour gradually fades towards harvest (Dussi et al., 1997). Exceptions occur; Dussi et al.

(1997) described two cultivars in which red colour, as in apples, increased towards harvest. The significance of these pigmentation patterns is unknown.

Since research attention has focused on the economically more important ripening-associated phase of colour development, little is known about the regulation of anthocyanin synthesis in immature apple fruit (Lancaster, 1992). However, evidence suggests that the ripening-associated appearance of ethylene potentiates anthocyanin synthesis in maturating apple fruit. Inhibition of ethylene synthesis delayed the ethylene climacteric and red colour development of 'Jonagold' and 'Gala' apples (Wang and Dilley, 2001). Ethylene, though having no effect on maturity indices, increased anthocyanin accumulation in 'Jonagold' apples (Awad and De Jager, 2002). Similarly, application of a rapidly broken down ethylene-releasing compound to 'McIntosh' apples enhanced red colour development without stimulating fruit softening (Murphey and Dilley, 1988). Ripening, however, does not guarantee colour development. While there was no difference in the onset and rate of ethylene synthesis in 'Red Chief' apple at night temperatures of 11°C or 22°C, red skin colour was considerably reduced by the higher temperature (Blankenship, 1987). There has been no study on the developmental regulation of colour development in pears.

Environmental regulation of anthocyanin synthesis.

Saure (1990) listed several environmental and cultural factors that influence colour development in apples. Of these factors, light and temperature were the most important.

Light.

Light is usually a prerequisite for anthocyanin synthesis (Mancinelli, 1983). Apples are no exception as indicated by the ability to prevent anthocyanin synthesis by enclosing fruit in light-impermeable bags (Ju, 1998). Also, apples in the shaded interior of trees increase in redness in response to supplemental irradiation, but re-green upon removal of the light source (Proctor & Creasy, 1971). A similar light requirement for colour development in pears can be inferred from the strong degree of bi-colour observed in fruit of different red cultivars (Dussi et al., 1997). Significant

improvement in red colour of 'Forelle' pears in response to severe pruning correlated with increased light levels within the tree canopy (Dussi and Huysamer, 1995).

The rate of anthocyanin synthesis is a function of light energy received by the fruit (Bishop and Klein, 1975; Proctor, 1974). Anthocyanin concentrations in apples related to light levels experienced within the cluster, bearing position and position within the tree canopy while sun exposed surfaces of individual fruit also accumulated much higher anthocyanin concentrations than shaded skin (Awad et al., 2000). Heinicke (1966) reported an arbitrary minimum light level of 70% for adequate red colour development in apples. However, the minimum light requirement differs between cultivars and is influenced by other factors such as temperature. Cultivars that colour more easily require less light than those that are more difficult to colour (Proctor, 1974). In the absence of low temperature induction of anthocyanin synthesis, red colour in the interior of apple trees increased less than expected with increasing intensity of supplementary light (Proctor, 1974). Lancaster et al. (2000) found that previous light exposure or high pigment concentration may also reduce the ability of apples to further accumulate anthocyanin. Although light might be limiting on an inter-tree level, regional differences in the ability of fruit to colour could not be ascribed to differences in light energy levels and daylength (Steyn et al., 2001).

While it is imperative that apple fruit are exposed to high light intensities during the second peak of anthocyanin synthesis to attain good colour before harvest, shading of 'Sensation Red Bartlett' pears reduced colour loss and the decrease of anthocyanin levels in the month before harvest (Dussi et al., 1995). Apparently, light either directly or via radiant heating increased anthocyanin degradation. Hence, light appears to have two opposing effects in pears, being both required for anthocyanin synthesis, but also apparently increasing red colour loss.

Temperature.

While the effect of temperature on colour development in pears has not been studied, low temperatures have long been known to increase and high temperatures to decrease red colour development in apples (Creasy, 1968; Uota, 1952). To date, colour development in all apple cultivars included in various studies benefited from low temperatures (Curry, 1997; Marais et al., 2001; Uota, 1952; Reay, 1999).

However, alternating low and high temperatures have been found to result in even greater accumulation of anthocyanin than continuous low or high temperature treatment (Marais et al., 2001). Studies indicated that anthocyanin synthesis benefited from induction at low temperatures (15°C) whether in light or dark, but subsequent accumulation of anthocyanin required irradiation at higher temperatures (20-25°C) (Curry, 1997; Reay, 1999).

The optimum induction temperature differed between apple cultivars. For 'Red Chief Delicious', 15°C was the optimum temperature (Curry, 1997). 'Delicious' fruit showed little response to pre-treatment for 48 hours at 2°C while, at the same temperature, anthocyanin synthesis in 'Fuji' apples increased by nearly 300% compared to the control (Curry, 1997). It should be borne in mind, however, that these induction studies were conducted in Washington State where a degree of induction might occur in the field prior to low temperature treatments. Hence, the effect of low temperatures should be even greater in warm production regions. In compliance, Cripps' Pink' apples from a warm region in the Western Cape region of South Africa benefited more from low temperature pre-treatment than apples harvested from a cold region where inductive low temperatures probably preceded collection of fruit (Marais et al., 2001). Temperatures in excess of 35°C inhibited subsequent anthocyanin synthesis (Curry, 1997). Likewise, three hours at 30°C, following on a 16-hour inductive treatment at 4°C, reduced subsequent anthocyanin accumulation in 'Granny Smith' apples during three days at 20°C by 40% (Reay, 1999).

The duration of the inductive low temperature treatment is also important (Curry, 1997). Colour development in 'Red Chief Delicious' apples only benefited from low temperature treatment if it exceeded 24 hours. Anthocyanin concentrations increased linearly with low temperature exposures up to 48 hours. Though night temperatures in major apple producing regions, such as Washington State in the USA, are suitable for colour development even during summer (Steyn et al., 2001), low temperatures would not prevail long enough to cause induction. However, low temperatures experienced over successive nights might have a cumulative effect, though this has not been studied.

The temperature optimum for anthocyanin synthesis during the light period varied between 20°C and 25°C in mature fruit of four apple cultivars studied with little anthocyanin produced at lower (15°C) or higher (35°C) temperatures (Curry, 1997). However, immature apples were found to have a lower optimum temperature for anthocyanin synthesis (<20°C) (Faragher, 1983; Arakawa et al., 1999). The optimal day temperature for anthocyanin synthesis did not change in the absence of induction, but the rate of anthocyanin synthesis could be 50% to 300% lower depending on the cultivar (Curry, 1997). It has to be kept in mind, however, that detached apples have a higher temperature optimum for anthocyanin synthesis than attached fruit (Saure, 1990). Also, as cautioned by Curry (1997), temperatures of exposed fruit peel may considerably exceed environmental temperatures due to radiant heating (Smart and Sinclair, 1976). Hence, the optimum environmental temperature for anthocyanin synthesis would probably be lower than those reported here.

Molecular regulation of anthocyanin synthesis.

Several reviews on molecular regulation of anthocyanin synthesis have been published in recent years, reflecting the considerable progress made in this field (Dooner et al., 1991; Forkmann, 1993; Holton and Cornish, 1995). From these reviews, it is clear that the induction of anthocyanin structural genes is coordinated and controlled by the action of regulatory genes. Regulatory genes are activated by developmental and environmental stimuli through mediation of signal transduction pathways of which the properties are only now being revealed (See Mol et al., 1996 for review). The site of regulation differs between species and on a tissue level.

In grape berries, a coordinated increase in the expression of seven genes of anthocyanin biosynthesis coincided with the onset of colour development (Boss et al., 1996). With the exception of UFGT, genes encoding earlier enzymes of anthocyanin biosynthesis were also expressed shortly after anthesis without any concomitant accumulation of anthocyanin. The authors postulated the presence of a ripening-associated regulatory gene. The pigmentation pattern of red and blushed apples suggests the presence of a similar regulatory gene.

Activities of PAL, CHS, CHI and UFGT have been studied in apple peel and correlated with changes in the levels of phenolics, flavonoids and anthocyanin during fruit development (Dong et al., 1995; Faragher and Chalmers, 1977; Ju et al., 1995a,b, 1999; Lister et al., 1996). Of these enzymes, PAL has been studied the most. Typically, PAL activity peaks in juvenile fruit of various fruit kinds, whereafter it gradually decreases towards harvest, only increasing again in fruits that accumulate anthocyanin or other phenolics during ripening (Macheix et al., 1990). Lister et al. (1996) found that PAL activity correlated with the second peak of anthocyanin synthesis in ripening 'Splendour' apple fruit. Post-harvest irradiation of 'Royal Gala' apples with ultraviolet and white light resulted in the accumulation of anthocyanin, and a 10- to 20-fold increase in the activity of PAL and CHI (Dong et al., 1995). The increase in PAL activity was ascribed to the induction of the PAL gene. In contrast, Ju et al. (1995b) found that increased PAL activity only corresponded with anthocyanin synthesis in immature fruit. Inhibition of PAL activity in ripening apples reduced the accumulation of phenolic acids, but had no effect on anthocyanin levels (Ju et al., 1995b). However, application of the PAL inhibitor to etiolated fruit reduced subsequent synthesis of phenolic acids and anthocyanin, thus indicating that PAL activity was only required when precursors were deficient.

The inconsistency of results with regard to PAL could result from its position up stream of a range of phenolic and flavonoid end products with diverse functions such as protection against UV-light, fungal infection and herbivory (McClure, 1975). This is illustrated by the following two examples. Firstly, wounding of apple fruit increased both PAL activity and anthocyanin accumulation in light, but in darkness a similar increase in PAL activity occurred in the absence of anthocyanin synthesis (Faragher and Chalmers, 1977). Secondly, fungal elicitation suppressed the inducing effect of UV-light on genes of the anthocyanin biosynthetic pathway in carrot cell cultures, but activity of PAL and the activity of the phenylpropanoid pathway remained high (Gläßgen et al., 1998). This indicates why enzyme activity, though prerequisite, does not necessarily guarantee or correlates with anthocyanin synthesis (Lister et al., 1996).

Similarly, UFGT displayed relatively high basal activity in green apple peel, probably due to the ability of this enzyme group to also glycosylate flavonols, which are

present at high levels in apple peel (Ju et al., 1995a). However, anthocyanin synthesis in grape berries was dependent on the expression of UFGT (Boss et al., 1996) while UFGT activity strongly correlated with colour development in ripening apples, despite the high basal activity (Ju et al., 1995a, 1999; Lister et al., 1996). CHI activity increased towards ripening in correlation with anthocyanin accumulation in apple peel, though this might also be related to a similar increase in the levels of flavonol glycosides (Lister et al., 1996).

Apart from Faragher (1983) and Tan (1980) who found much higher PAL activity in apples held at low (6-10°C) compared to high temperatures (>20°C), the molecular basis of low temperature induction of anthocyanin synthesis in apples has not been studied. Recent studies conducted on maize seedlings, however, indicated that low temperatures induce anthocyanin structural genes, but that subsequent enzyme action and accumulation of anthocyanin proceeded at higher temperatures in light (Christie et al., 1994). Low temperatures (10°C) increased the transcript abundance of anthocyanin structural genes and also induced expression of the *R* regulatory gene. The effectiveness of induction decreased at lower (5°C) and higher (15°) temperatures. However, induction alone was not sufficient to allow the accumulation of anthocyanin and seedlings had to be transferred to a higher temperature (25°C) to facilitate post-transcriptional events leading to anthocyanin synthesis. Seedlings kept at a constant temperature of 25°C did not accumulate anthocyanin. Seedlings kept at 15°C accumulated some anthocyanin since this temperature allowed induction as well as synthesis, albeit at lower rates. These results appear to also apply to apples and explain the stimulating effect of diurnal low and mild temperature regimes. Expression of PAL in parsley cell cultures in response to UV light and fungal elicitors ceased within an hour after transferring cultures to 37°C (Walter, 1989) indicating the possible mechanism by which high temperatures following on inductive treatment inhibit subsequent anthocyanin synthesis in apples (Curry, 1997; Reay, 1999).

Since maximal expression of red pigmentation is dependent on the prior induction of the anthocyanin pathway, it is clear why Uota (1952) found that night temperatures correlated better with anthocyanin accumulation than day temperatures.

Anthocyanin degradation.

Due to their reactive structures, anthocyanins are rapidly destroyed in food products causing discoloration in response to a wide variety of inducers (Francis, 1970). The most important factors found to influence colour stability were; the structure of the pigment, interaction with other compounds, pH, temperature and light (Francis, 1970). Evidence suggests that high temperatures and light are involved in colour loss in fruit, flowers and vegetative tissues.

Subjecting mature 'Cripps' Pink' apples to irradiation at 37°C for 144 hours resulted in red colour loss and a more than 50% reduction in anthocyanin concentration (Marais et al., 2001). High temperatures also accelerated anthocyanin degradation and colour loss in ripe eggplant fruit (*Solanum melongena* L.) (Sakamura and Obata, 1961). Shading of 'Sensation Red Bartlett' pears reduced colour loss and the decrease of anthocyanin levels in the month before harvest (Dussi et al., 1995), suggesting that light was involved in colour loss. Exposure to light greatly accelerated colour loss in Pomerac fruit (*Syzygium malaccense*) stored at 5°C (Sankat, et al., 2000). The colour of flower petals often fade or attain a bluish tint due to senescence associated pH changes or a reduction in pigment concentrations in response to heat stress during petal enlargement (Biran and Halevy, 1974; Oren-Shamir et al., 2001). Red colour loss in vegetative tissues also appeared to correspond with periods of high temperature (Nozzolillo et al., 1990; Oren-Shamir and Levi-Nissim, 1997). However, rapid loss of anthocyanins and red colour in vegetative tissues also occurred in response to developmental changes without the apparent involvement of environmental factors such as high temperature (Kubasek et al., 1992; Sherwin and Farrant, 1998).

Red colour loss in immature apple fruit in response to high temperatures has been attributed to degradation (Faragher, 1983), but the contribution of dilution was not taken into account. The relative contributions of degradation and dilution to the fading of pear red colour towards harvest are unknown.

Three enzyme groups, ß-glycosidases, peroxidases and polyphenoloxidases are able to degrade anthocyanins (López-Serrano and Barceló, 1999; Macheix et al., 1990; Piffaut et al., 1994). ß-glycosidase hydrolyses the sugar from the anthocyanin, which

subsequently exposes the unstable aglycone to degradation (Macheix et al., 1990). Degradation of anthocyanin induced by high temperatures or mediated by ß-glycosidase proceeded via the same pathway (Piffaut et al., 1994). H_2O_2, released as by product of cellular processes and during stress, may degrade anthocyanin through peroxidation, but apparently only attacks the aglycone (Macheix et al., 1990). Yamasaki (1997) recently proposed a role for anthocyanin in protection against H_2O_2, though other more numerous flavonoids may also fulfil this function (Yamasaki et al., 1997). Polyphenoloxidase oxidises phenols to quinones, which subsequently oxidise anthocyanins and convert back to the original phenol (Macheix et al., 1990). Since anthocyanin can also be degraded without mediation of enzymes (Attoe and Von Elbe, 1981), it is uncertain to what extent these enzyme groups are involved in the turnover of anthocyanin in attached fruit.

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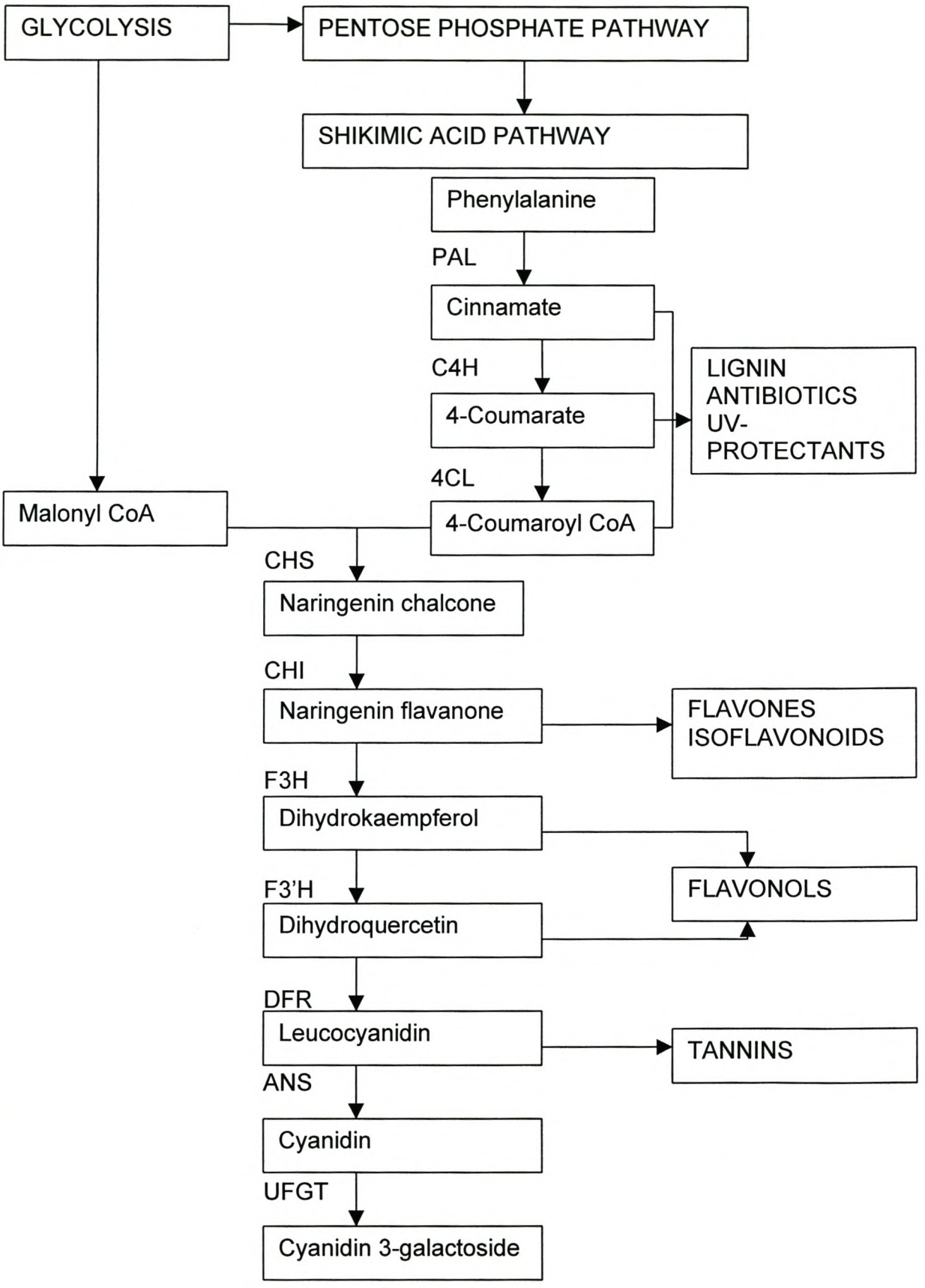
Fig. 1. The major precursory pathways as well as the individual enzymatic steps of the anthocyanin biosynthetic pathway based on the reviews by Lancaster (1992) and Saito and Yamazaki (2002). Abbreviations: PAL, phenylalanine ammonia-lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumarate:CoA ligase; CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone 3-hydroxylase; F3'H, flavonoid 3-hydroxylase; DFR, dihydroflavonol reductase; ANS, anthocyanidin synthase (leucoanthocyanidin dioxygenase); UFGT, UDPGalactose: flavonoid-3-*o*-glycosyltransferase. CHS is the first enzyme of the flavonoid biosynthetic pathway. PAL and enzymes prior to CHS form part of the phenylpropanoid pathway.



LITERATURE REVIEW 2:

ANTHOCYANINS IN VEGETATIVE TISSUES: A PROPOSED UNIFIED FUNCTION IN PHOTOPROTECTION

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Summary

The function of anthocyanins in green, vegetative tissues has always been a contentious issue. Here we evaluate their proposed photoprotective function since recent findings have shown that anthocyanins reduce photoinhibition and photobleaching of chlorophyll under light stress conditions. Anthocyanins generally accumulate in peripheral tissues exposed to high irradiance, although there are some exceptions (e.g. accumulation in abaxial leaf tissues and in obligatory shade plants) and accumulation is usually transient. Anthocyanin accumulation requires light and generally coincides with periods of high excitation pressure and increased potential for photo-oxidative damage due to an imbalance between light capture, CO_2 assimilation and carbohydrate utilization (e.g. greening of developing tissues, senescence and adverse environmental conditions). Light attenuation by anthocyanin may help to re-establish this balance and so reduce the risk of photo-oxidative damage. Although it has been suggested that anthocyanins may act as antioxidants, the association between anthocyanins and oxidative stress appears to relate to the ability of anthocyanins to reduce excitation pressure and, hence, the potential for oxidative damage. The various aspects of anthocyanin induction and pigmentation presented here are compatible with, and support, the proposed general role of anthocyanins as photoprotective light screens in vegetative tissues.

Introduction

The visual function of anthocyanins in reproductive organs as an aid in pollination and seed dispersal is generally accepted (Harborne, 1965). However, ascribing a function to the transient accumulation of anthocyanins in green, vegetative tissues has proven elusive. This may be due to the diversity of inducers and the various patterns of red pigmentation in vegetative tissues. Recently, Smillie and Hetherington

(1999) demonstrated that, by acting as visible light screens, anthocyanins may protect photosynthetic tissues against photoinhibition. Subsequently, they proposed that anthocyanins have a general function in photoprotection of vegetative tissues that are predisposed to photoinhibition.

Our objective with this review is to evaluate the merit of the proposed general photoprotective function for anthocyanins in vegetative tissues. Our intention is to determine if the photoprotective function is congruent with the histological, developmental and environmental aspects of anthocyanin induction and variation in pigmentation. We are also interested in evidence of any underlying physiological connection between the various inducers of red pigmentation. Initially we needed to establish whether there is other data to support this proposed role of anthocyanins in photoprotection.

Anthocyanins As Photoprotective Pigments

Photoinhibition and photoprotection

The harvesting of sunlight by green tissues is inherently hazardous. Energy capture occurs at a much faster rate than electron transport and dissipation, hence over-excitation of the photosynthetic apparatus is a constant threat. Over-excitation manifests as a repression of photosynthesis, a phenomenon called photoinhibition (Long *et al.*, 1994). Chronic photoinhibition can significantly reduce productivity and may have a negative effect on survival (Ball *et al.*, 1991). Photoinhibitory conditions may lead to the formation of reactive oxygen species, which in turn cause photodynamic bleaching and perturbation of cellular metabolism (Foyer *et al.*, 1994).

Plants employ multiple mechanisms to balance energy capture with energy consumption and dissipation, thereby preventing oxidative damage (Demmig-Adams & Adams III, 1992; Niyogi, 1999). These include tolerance mechanisms that regulate energy distribution and dissipation, repair mechanisms, and avoidance mechanisms that decrease the absorbance of light by green tissues. Avoidance mechanisms include alteration of whole-leaf light absorption by paraheliotropic leaf orientation and leaf folding, enhanced reflectance through pubescence, salt deposition, epicuticular wax layers, and, more permanent morphological adaptations, for example smaller

leaf size, thicker leaves and compact growth habit. Internal measures to reduce light absorption include chloroplast movements and the accumulation of screening compounds. It is as visible light screens that some non-photosynthetic pigments e.g. anthocyanins, betalains and rhodoxanthin may exert their function by reducing light levels incident on chlorophyllous tissues (Weger *et al.*, 1993; Smillie & Hetherington, 1999).

Reduction of light levels by anthocyanins

Anthocyanins significantly modify both the quantity and quality of light incident on chloroplasts (Krol *et al.*, 1995; Ntefidou & Manetas, 1996). The red anthocyanins present in vegetative tissues preferentially absorb green and ultraviolet (UV) light and show lower absorbance of blue light, while little red light is absorbed (McClure, 1975). Absorbance of blue-green light by anthocyanins reduces light available to chlorophyll (Pietrini & Massacci, 1998; Smillie & Hetherington, 1999) in proportion to the anthocyanin concentration (Neill & Gould, 1999). This presents a mechanism to modulate light absorption in accordance with environmental and developmental requirements (Pietrini & Massacci, 1998). A low level of absorbance, or complete lack of it in the blue and red spectra, possibly allows accumulation of pigments to high levels without interference with photoreceptors, for example phytochrome and chryptochrome (McClure, 1975). The absorbance maximum of anthocyanin in the green spectrum of visible light is probably related to the deeper penetration of this colour light into green tissues and its greater contribution to total solar energy levels compared with other wavebands (Merzlyak & Chivkunova, 2000). This may be the basis for the apparent evolutionary convergence for red nonphotosynthetic pigments.

Evolutionary convergence for red pigmentation

Anthocyanins in vegetative tissues are mostly red cyanidin glycosides that are generally simpler in structure than those found in reproductive organs (Harborne, 1965), where blue colour and UV-patterning are important for guiding or directing pollinators (Harborne, 1965; Harborne & Grayer, 1994). Although anthocyanins are characteristic of higher plants (Harborne, 1965), the ability to impart red colour to plants is not restricted to anthocyanins. Families within the order Centrospermae, including taxa like prickly-pear (*Opuntia* sp.) and paper flower (*Bougainvillea* sp.),

display transient red coloration in vegetative tissues. However, in nine of the 11 families comprising the order, red colour is imparted by nitrogenous betalains, unrelated to anthocyanins, though colourless flavonoid precursors of anthocyanin are still present (Mabry, 1980). Certain plants accumulate red carotenoids (e.g. rhodoxanthin) in patterns and under inductive conditions typically associated with anthocyanins, such as acclimation to low temperature (Diaz *et al.*, 1990; Weger *et al.*, 1993).

The evolutionary convergence for the ability to accumulate red pigments in vegetative tissues suggests that this provides an adaptive advantage (Stafford, 1994). The selectivity for either red anthocyanins or betalains in different plant species suggests that these pigments fulfil a similar function. Since this function is unrelated to the origin and chemical characteristics of the pigments, the purpose of anthocyanin accumulation in vegetative tissues may lie in its ability to absorb visible light as a red pigment.

Ability of anthocyanins to afford photoprotection

The difficulty in obtaining a contrast between tissues containing or lacking anthocyanin, but not differing in any other respect, has hindered the study of anthocyanin function. Circumstantial evidence for the ability of anthocyanins to provide photoprotection has been obtained from studies of crosses made between yellow chlorophyll-deficient and red anthocyanin-containing hazelnut varieties for horticultural purposes (Mehlenbacher & Thompson, 1991). Chlorophyll-deficient seedlings lacking anthocyanin died under field conditions while chlorophyll-deficient progeny containing anthocyanins survived.

Evidence for the participation of anthocyanins in photoprotection was obtained from studies on jack pine seedlings subjected to variable excitation pressures (Krol *et al.*, 1995). Seedlings acclimated at 5ºC accumulated anthocyanins in needles exposed to direct light (250 μmol m^{-2} s^{-1} over the waveband 250-750 nm). Needles from the same seedlings shaded from direct light did not accumulate anthocyanin and were more susceptible to photoinhibition at moderate irradiance (600 μmol m^{-2} s^{-1}). Control seedlings kept at 20ºC also did not accumulate anthocyanin and, upon exposure to

high irradiance (1200 μmol m^{-2} s^{-1}), were twice as susceptible to photoinhibition than seedlings acclimated at 5ºC. However, shaded needles of acclimated seedlings were more tolerant of photoinhibition than exposed needles of control seedlings, indicating that factors other than anthocyanin accumulation also participated in the acquisition of hardiness. Krol *et al.* (1995) attributed the increased tolerance of acclimated jack pine seedlings to photoinhibition to a combination of light attenuation by anthocyanin in the epidermis and an increased photosynthetic capacity that facilitates increased utilisation of absorbed light energy. Shading of conifer seedlings exposed to low temperatures and high irradiance had previously been found to reduce photoinhibition (Strand & Lundmark, 1987). Anthocyanin light screens may fulfil a similar role.

Smillie and Hetherington (1999) circumvented the problems associated with studies of anthocyanin function by using white, red or blue-green light to subject pods of red and green *Bauhinia variegata* phenotypes to photoinhibitory conditions. Red light of high irradiance, which is not absorbed by anthocyanin, induced a similar degree of photoinhibition in pods of both colours. The increased ability of red pods to tolerate high intensities of blue-green and white light compared to green pods was attributed to the presence of anthocyanin. This was first conclusive evidence supporting a photoprotective function for anthocyanins that was not obviously confounded by other photoprotective measures.

Since then Feild *et al.* (2001) has used the same method to demonstrate that anthocyanins reduced photodamage in red compared to yellow senescing leaves of red-osier dogwood. Further evidence for anthocyanin-mediated photoprotection was provided by a study using apple peel tissue (Merzlyak & Chivkunova, 2000). Peel tissue ranging in colour from green to red, was subjected to severe light stress (4600 μmol m^{-2} s^{-1} photosynthetic photon fluence rate (PPFR)). The presence of anthocyanin reduced the susceptibility of chlorophyll to photobleaching, ostensibly by absorption of green-orange light.

However, Burger and Edwards (1996) found no difference in photoinhibition between leaves of red and green *Coleus* varieties exposed to severe photoinhibitory treatment

(2 hours at 1800 μmol m^{-2} s^{-1} PPFR). On the other hand, screening of moderate irradiance by anthocyanin reduced the light use efficiency of photosynthesis, indicating that anthocyanin did, in fact, attenuate light. Krol *et al.* (1995) also found no difference in photoinhibition between control and acclimated seedlings at high irradiance (1200 μmol m^{-2} s^{-1} over the waveband 250-750 nm), even though anthocyanin was found to provide photoprotection at moderate irradiance. The failure to observe differences in photoinhibition at high irradiance leads us to believe that photoinhibition reaches a maximum at sub-saturating irradiance and is not a good indicator of additional photostress at super-saturating irradiance.

The extent to which anthocyanins reduce light capture by chlorophyll depends on the histological distribution of the pigment, that is whether it is located in single or multiple layers in the epidermis, mesophyll or both.

Anatomical Aspects Of Anthocyanin Function

Localization of anthocyanins in vegetative tissues

The distribution of anthocyanins within organs and tissues is genetically determined by tissue specific expression of regulatory genes. These genes control expression of structural genes in response to environmental and developmental cues (Mol *et al.*, 1996). Anthocyanin synthesis is a cell-autonomous response, meaning that colour development is controlled at the level of the individual cell (Nick *et al.*, 1993; Lancaster *et al.*, 1994). This allows local accumulation of anthocyanin resulting in a specific light screen, in contrast to other whole-leaf light avoidance measures. Cells without anthocyanin are found dispersed throughout red anthocyanin-rich apple peel (Lancaster *et al.* 1994). Heterogeneity in cell response to stimuli allows the gradual increase in pigmentation at whole-organ-level with increasing intensity of stimulation (Nick *et al.*, 1993).

As can be expected of light screens, anthocyanins generally accumulate in peripheral tissues exposed to direct light, such as the upper epidermis (McClure, 1975; Chalker-Scott, 1999). They also accumulate throughout the leaf in mesophyll tissue (McClure, 1975) and even in trichomes (Ntefidou & Manetas, 1996). In leaves of *Quintinia serrata*, varying sizes and frequencies of red areas occurred on the lamina as a result

of anthocyanin accumulation in mesophyll cells, both epidermal layers and/or vascular parenchyma at the midrib (Gould *et al.*, 2000). Generally, however, these red areas were more prevalent in leaves experiencing high light conditions.

Red pigmentation in the abaxial surfaces of expanding mango and cacao leaves (Lee *et al.*, 1987), mustard cotyledons (Drumm-Herrel & Mohr, 1985) and unfolding leaves of various fern species (unpublished observations) is, seemingly, incompatible with a photoprotective function. However, unfolding leaves and cotyledons are often orientated in such a way that, for a short period, abaxial surfaces are exposed to high irradiance while adaxial surfaces are shaded from direct light (Drumm-Herrel & Mohr, 1985; unpublished observations). Abaxial leaf surfaces are also more light sensitive than adaxial surfaces (Sun *et al.*, 1996).

Purpling in response to phosphorous (P) and nitrogen (N) deficiency develops first in abaxial leaf surfaces before spreading to the whole leaf (Cobbina & Miller, 1987; Awad *et al.*, 1990). This may be attributed to differential stress sensitivity of different leaf tissues (Kingston-Smith & Foyer, 2000). Vital organs or tissues may be preserved in favour of more expendable components (Baysdorfer *et al.*, 1988). Hence, the lower leaf surface may either be more sensitive to nutrient stress, or P and N partitioning may favour the palisade mesophyll within deficient leaves.

Anthocyanins in shade leaves

The presence of a permanently pigmented or coloured layer immediately below the palisade mesophyll is characteristic of many plants growing in light-limiting environments (Lee *et al.*, 1979; Lee & Graham, 1986). Selectivity for red pigmentation does not apply since the coloured layer in some forest understory plants may be an iridescent blue (Lee & Graham, 1986). Gould *et al.* (1995) found higher levels of photoinhibition in green-leaved compared with red-leaved individuals of the same two shade species under photoinhibitory conditions. They proposed that the anthocyanin layer protects these shade leaves from photoinhibition. However, the red-leaved individuals also displayed greater photosynthetic capacities, confounding the measured effect. Although shade plants are extremely vulnerable to high light stress because of the large capacity for energy capture required in their ecological

niche, the localization of anthocyanins within their leaves does not favour a photoprotective function. Any pigmentation pattern reducing light capture in extreme shade environments may, in fact, be a disadvantage. Attenuation of light by chlorophyll in upper leaf layers should impart considerable light protection to the lower layers (Sun *et al.*, 1996).

Lee *et al.* (1979) suggested that anthocyanins aid light capture in shade leaves through backscattering. This theory was not validated in a subsequent study (Lee & Graham, 1986). The very low light levels encountered in shade environments and the strong gradient of blue and red light attenuation (Vogelmann, 1993) ensures that mostly green light will reach the anthocyanin layer and be absorbed. For backscattering to occur, penetration and reflection of red light would be required.

Developmental Aspects Of Anthocyanin Function

Developmental patterns

Anthocyanin accumulation is associated with seasonal changes in growth conditions (Nozzolillo *et al.*, 1990; Krol *et al.*, 1995) and with greening and etiolation, for example early seedling growth, leaf expansion and senescence (Drumm-Herrel & Mohr, 1985; Krause *et al.*, 1995; Hoch *et al.*, 2001). Anthocyanins accumulate when either environmental or developmental changes render plants more sensitive to the environment. The ability to induce anthocyanin accumulation may sometimes be limited to the juvenile phase (Murray *et al.*, 1994), or is lost with increasing age and reduced sensitivity to environmental stress, as in many conifers (Nozzolillo *et al.*, 1990; Richter & Hoddinott, 1997). Developmental patterns of anthocyanin accumulation may also differ according to the developmental strategy of different plant species. For instance, *Craterostigma wilmsii*, a resurrection plant species that maintains high chlorophyll levels during dehydration, resumes photosynthesis and degrades anthocyanin as soon as water becomes available (Sherwin & Farrant, 1998). By contrast *Xerophyta viscosa*, which degrades chlorophyll during dehydration, maintains anthocyanin during rehydration and re-assembly of the photosynthetic apparatus.

Young, expanding leaves, as well as senescing leaves, are more susceptible to photoinhibition and photobleaching of photosynthetic pigments than mature, presenescent leaves (Kar *et al.*, 1993; Krause *et al.*, 1995; Hoch *et al.*, 2001). The increased sensitivity is mainly due to a lower ability to utilize absorbed light energy (Krause *et al.*, 1995; Bukhov, 1997). Also, environmental conditions in temperate regions may be more limiting during early leaf development than later when leaves are mature (Fryer *et al.*, 1998). An inability to export carbohydrate may impose a further 'feedback' limitation on photosynthesis in developing leaves (Barker *et al.*, 1997). Anthocyanin accumulation may also precede the accumulation of other photoprotective pigments, such as xanthophylls (Gamon & Surfus, 1999). Physical light barriers, such as wax layers, that will later protect mature leaves are absent in developing leaves (Barker *et al.*, 1997). This function may be accomplished by dense trichome layers which, together with anthocyanins, strongly attenuate light (Ntefidou & Manetas, 1996; Choinski & Wise, 1999). Recent molecular studies suggest that anthocyanin synthesis and trichome development are mutually regulated, at least in *Arabidopsis* (Payne *et al.*, 2000).

Hoch *et al.* (2001) proposed that anthocyanins reduce the potential for photo-oxidative damage to senescing leaf cells. Evidence was presented in a subsequent study (Feild *et al.*, 2001). While red senescing leaves were able to recover from a photoinhibitory treatment, yellow senescing leaves suffered photodamage. Red light caused a similar degree of photoinhibition in both red and yellow senescing leaves, but anthocyanins reduced photoinhibition in red leaves irradiated with blue-green light. The photoprotection afforded by anthocyanins is thought to increase the efficiency of nutrient retrieval from senescing leaves (Feild *et al.*, 2001; Hoch *et al.*, 2001).

Transient and permanent pigmentation

A feature of the developmentally-regulated accumulation of anthocyanin is its transient nature (Harborne, 1965). Seedlings and expanding leaves typically attain maximum pigmentation a few days after germination or sprouting, whereafter anthocyanins disappear rapidly, and apparently deliberately (Kubasek *et al.*, 1992). In developing leaves the disappearance of anthocyanins seems to coincide with the

transition from sink to source (Choinski & Wise, 1999). Chawla *et al.* (1999) found that the constitutive expression of anthocyanin regulatory genes in transgenic plants may be deleterious or lethal at certain developmental stages, probably by interfering with normal metabolism. Apparently, anthocyanin accumulation is normally suppressed at developmentally sensitive stages. Evidence of anthocyanin suppression through desensitisation of certain structural genes and/or negative regulation by other interrelated biosynthetic pathways has been reported (Nick *et al.*, 1993; Bowler *et al.*, 1994).

The prolonged presence of anthocyanin is usually restricted to tissues that do not have carbon assimilation as primary function, for example petioles, veins, stems and lower layers of shade leaves (Harborne, 1965; Lee *et al.*, 1979), or to inactive growth stages, for example dormancy (Sherwin & Farrant, 1998; Leng *et al.*, 2000). Taking into account that light is often limiting, especially on a whole plant level, permanent light screens are undesirable, except maybe in arid, high light habitats (Björkman & Demmig-Adams, 1995). Reduced photosynthesis due to reduced light capture in constitutively red plants may offset any potential benefit with regard to photoprotection (Burger & Edwards, 1996).

Anthocyanins are usually more permanent in horticultural plants because many constitutively red or variegated leaves or fruit of garden and crop plants have been selected for aesthetic reasons (Harborne, 1965). While mutations in genes of anthocyanin biosynthesis do not usually affect plant growth and development (Holton & Cornish, 1995), increased pigmentation is not necessarily an advantage. Although anthocyanin reduced photoinhibition in fruit of purple mango cultivars, these fruit were more susceptible to sunburn than fruit of green-fruited cultivars, presumably a result of higher heat-absorbing capacity of the darker peel (Schroeder, 1965; Hetherington, 1997). Red pear cultivars (selected bud mutations of green cultivars) are reported to be more difficult to grow, less vigorous and less productive than their parents. Martin *et al.* (1997) found that the mean maximum net photosynthetic rate and Rubisco activity in green, mature leaves of three red-fruited sports was 30-40% lower compared with their respective green-fruited parents. Photosynthesis in two of the red sports appeared to be saturated at lower light levels.

Accumulation and maintenance of anthocyanins carries an energy cost, may reduce light capture and ultimately carbon assimilation (Drumm-Herrel & Mohr, 1985; Burger & Edwards, 1996). Therefore, the transient accumulation of anthocyanin probably forms part of a short-term defence strategy to limit damage during developmental or environmental changes. Acclimation to new conditions entails the replacement of anthocyanins by more long-term physical, photosynthetic or metabolic adjustments that re-establish homeostasis between the plant and the environment as illustrated by the following three examples. First, postharvest synthesis of colourless flavonoids and anthocyanins in apples was reduced in proportion to previous light exposure (Lancaster *et al.*, 2000). Second, nutrient starved *Eucalyptus* seedlings contained high anthocyanin levels and were severely photoinhibited at planting, but photosynthetic efficiency recovered during winter while the anthocyanin content of such plants, unlike cold-stressed nutrient sufficient seedlings, did not increase (Close *et al.*, 2000). Third, exposure of lodgepole pine seedlings to conditions favouring acclimation (short daylengths and moderate temperatures) reduced subsequent anthocyanin synthesis in response to low temperatures (Camm *et al.*, 1993).

The metabolic cost of a transient presence of anthocyanin should be considerably lower than the cost attributable to damage incurred during rapidly changing environmental conditions or associated with other protection measures, for example permanent light screens and the down-regulation of the photosynthetic and assimilatory apparatus.

Environmental Aspects Of Anthocyanin Function

Light

Effect on anthocyanin accumulation. Consistent with a function in photoprotection, light exposure is a prerequisite for significant anthocyanin synthesis in vegetative tissues in response to both environmental (Franceschi & Grimes, 1991; Krol et al., 1995) and developmental factors (Mancinelli, 1983). Depending on the species and developmental stage, red, blue or UV light may effect synthesis through mediation by phytochrome, cryptochrome or the putative UV-receptor (Mancinelli, 1983; Mol *et al.*, 1996 for review on signal perception and transduction). Generally, induction of anthocyanin synthesis requires high light intensities, and anthocyanin levels in plants

and in individual leaves vary in relation to light exposure levels (Mancinelli, 1983; Krol *et al.*, 1995). Endogenous signals, developmental stage, environmental factors and previous light exposure modify the effect of light on anthocyanin synthesis (Mancinelli, 1983).

Physiological studies. Recently, molecular tools have broadened our understanding of the light regulation of anthocyanin synthesis during photomorphogenesis. The underlying molecular basis for the high light requirement for anthocyanin synthesis and the synchronization of anthocyanin accumulation with other photomorphogenic processes, such as greening, has been established in etiolated tomato seedlings (Bowler *et al.*, 1994).

Anthocyanin, PSI and PSII synthesis are regulated through the phytochrome–mediated activation of their respective signal transduction pathways (Bowler *et al.*, 1994). Negative reciprocity between the pathways ensures synthesis of anthocyanins and suppression of greening during early seedling growth when seedlings are most susceptible to light-induced stress (Drumm-Herrel & Mohr, 1985). Anthocyanin synthesis is suppressed as chlorophyll starts to accumulate and emphasis shifts to carbon assimilation. The signalling pathway leading to anthocyanin synthesis is less sensitive to the signalling compound shared by the pathways (Bowler *et al.*, 1994). The result is that stronger signals are required to trigger the anthocyanin pathway, which is the basis for the high light requirement for anthocyanin synthesis. Anthocyanin synthesis requires an investment of carbohydrate reserves before seedlings become self-sufficient (Drumm-Herrel & Mohr, 1985). The high light requirement and the strict regulation of synthesis ensure that anthocyanin will only accumulate to the concentrations required and only at specific times and locations (Drumm-Herrel & Mohr, 1985).

Recently, Iida *et al.* (2000) described a gene apparently involved in acclimation to visible light stress. This gene was rapidly induced in proportion to intensity and duration of irradiation stress. Over-expression of the gene resulted in constitutive high-light tolerance, anthocyanin accumulation and adaptive phenotypic changes, such as thicker leaves, usually associated with acclimation to high light, suggesting that anthocyanin accumulation is part of the general plant response to light stress.

Anthocyanins and UV-B protection. The UV-inducibility of anthocyanins and the ability of anthocyanins to absorb UV-B radiation have led to suggestions that these pigments protect plants from UV-B. High concentrations of anthocyanin can provide protection against UV-B radiation in cells and tissues where it is the major UV-absorbing compound (Takahashi *et al.*, 1991; Stapleton & Walbot, 1994; Burger & Edwards, 1996). However, a general UV-protective function for anthocyanins through the attenuation of UV-B radiation is unlikely.

Like the colourless flavonoids, the perfect UV-B screen should be permanent, ubiquitous in peripheral cell layers where most attenuation of UV-B occurs (DeLucia *et al.*, 1992) and should accumulate to high levels without any negative effect on photosynthetic yield (Teramura, 1983). However, anthocyanin accumulation is mostly transient, not confined to the epidermis (Gould *et al.*, 2000) and may reduce photosynthesis (Burger & Edwards, 1996). Furthermore, anthocyanins have a lower UV absorbance than colourless flavonoids and simpler phenolics (Caldwell et al., 1983; Teramura, 1983; Landry *et al.*, 1995) and, when present, often contribute little to total UV-B absorbance (Lee *et al.*, 1987; Woodall & Stewart, 1998). Increased UV-B radiation has been found to reduce anthocyanin levels, in some instances while UV-B absorbance increases due to accumulation of phenols and flavonoids (Moorthy & Kathiresan, 1997).

But why does anthocyanin accumulate in response to UV-B radiation if it does not have a general function in attenuation of UV-B radiation? UV-B radiation induces the down-regulation of photosynthesis primarily by damaging PSII (Teramura & Sullivan, 1994) and reducing the content and activity of Rubisco and other Calvin-cycle enzymes (Jordan et al., 1992; Allen *et al.*, 1998), thereby increasing susceptibility of plants to photoinhibition. It is conceivable that anthocyanins protect the photosynthetic apparatus against photodamage by reducing visible light under conditions when UV-radiation inhibits photosynthesis. However, high visible light levels alleviate many of the detrimental effects of UV-B radiation (Teramura, 1980; Caldwell *et al.*, 1994).

Experimental conditions in studies of plant responses to UV radiation were often unrealistic in the past and bore no resemblance to field conditions (Björn, 1996; Allen *et al.*, 1998). UV-B levels much higher than would occur naturally have often been combined with low visible light levels, exacerbating the effect of UV-B (Teramura, 1980). Realistic levels of UV-B irradiance together with corresponding levels of white light do not appear to have a significant influence on photosynthesis in many species (Allen *et al.*, 1998). Rather, UV-B induction of anthocyanin synthesis, down-regulation of photosynthesis, altered growth habit and changes in leaf morphology seem to form part of an adaptive rather than injurious general photomorphogenic response to UV-B (Björn, 1996). Under natural conditions, anthocyanin induction by UV-B may perform a photoprotective role similar to that which has been proposed for visible light induction of anthocyanin via phytochrome (Drumm-Herrel & Mohr, 1985; Bowler *et al.*, 1994).

Temperature

Suboptimal temperatures, experienced either as sudden, short-term cold spells or long-term seasonal reductions in temperature, induce anthocyanin synthesis (Nozzolillo *et al.*, 1990; Christie *et al.*, 1994; Leng *et al.*, 2000), while high temperatures reduce synthesis and are associated with net pigment loss (Oren-Shamir & Levi-Nissim, 1997; Haselgrove *et al.*, 2000). Anthocyanin accumulation often coincides with acclimation or deacclimation of overwintering tissues and, although it appears to be a general response to cold stress (Christie *et al.*, 1994), does not seem to be involved in the acquisition of hardiness (Steponkus & Lanphear, 1969; Leyva *et al.*, 1995). Evidence suggests that anthocyanin synthesis and hardening are linked at a regulatory or biochemical level (McKown *et al.*, 1996). Environmental cues other than temperature that participate in the acquisition of hardiness, for example photoperiod, may also induce anthocyanin accumulation (Howe *et al.*, 1995). Interestingly, four of seven *Arabidopsis* mutations showing reduced freezing tolerance, displayed reduced pigmentation (McKown *et al.*, 1996).

Maximal pigmentation usually requires low night temperatures (10°C) followed by mild day temperatures (25°C). Low temperatures enhance transcription of anthocyanin regulatory and structural genes, but post-transcriptional events leading

to anthocyanin synthesis require higher temperatures (Christie *et al.*, 1994). Temperatures that effectively induce synthesis vary between species, cultivars and tissues as well as developmental stage and growth conditions. For example, mature apple fruit will colour at night temperatures below 15°C (Curry, 1997) while maximum pigmentation in dormant apple shoots occurs at -20°C (Leng *et al.*, 2000). A heat treatment of short duration (3h at 30°C) was found to reduce the effect of preceding inductive low temperatures on anthocyanin accumulation (Reay, 1999), suggesting intervention of high temperatures at molecular level in preventing anthocyanin accumulation. Light and high temperatures have been found to increase anthocyanin and betacyanin degradation in solution, preserves and whole fruit (Attoe & Von Elbe, 1981; Marais *et al.*, 2001). Increased heat load and a reduction in carbon gain due to the presence of anthocyanin at high temperatures (Schroeder, 1965; Burger & Edwards, 1996), may be reasons for the negative relationship between temperature and anthocyanin.

Anthocyanins disappear with the resumption of growth or with increasing temperature, although it may persist with the continuation of cold conditions (Nozzolillo *et al.*, 1990; Oren-Shamir & Levi-Nissim, 1997). Conversely, anthocyanin synthesis may coincide with resumption of photosynthetic activity and increasing temperatures in cold environments. Starr and Oberbauer (2002) found that anthocyanin levels in three arctic evergreens increased as light intensity increased with melting of the snow cover. Environmental and growth conditions that predispose the photosynthetic apparatus to photoinhibition and photooxidation may increase the extent of anthocyanin accumulation in response to low temperature (Nozzolillo *et al*, 1990; Close *et al.*, 2000). For example, newly planted *Eucalyptus* seedlings displayed photoinhibition and anthocyanin accumulation during winter, while established saplings did not (Close *et al.*, 2000).

While light capture and O_2 evolution are temperature insensitive, enzymatic assimilatory reactions decrease with decreasing temperature (Huner *et al.*, 1998). Consequently, light levels required to saturate photosynthesis decrease while the probability of photoinhibition at a constant light level increases with decreasing temperature (Hetherington & Smillie, 1989; Falk *et al.*, 1990). Low temperatures may have an even greater effect on carbohydrate metabolism by limiting assimilate

utilization and decreasing sink strength (Azcón-Bieto, 1983; Paul *et al.*, 1992). Either shading or protecting conifer seedlings against frost reduced photoinhibition (Strand & Lundmark, 1987). Decreasing the source : sink ratio through shading may also relieve the low temperature-imposed sink-limitation on photosynthesis (Paul *et al.*, 1992). Similarly, attenuation of light by anthocyanins probably provides protection against photoinhibition in tissues exposed to a combination of low temperatures and light (Krol *et al.*, 1995; Starr & Oberbauer, 2002).

Nutrient deficiency

Anthocyanin accumulation is a distinctive symptom of P deficiency in many plants, though N deficiency may also induce purpling (Cobbina & Miller, 1987; Nozzolillo *et al.*, 1990; Close *et al.*, 2000). Highest anthocyanin yield in suspension cultures was obtained when N and/or P concentrations were low (Dedaldechamp *et al.*, 1995). Addition of N to cell suspension cultures reduced anthocyanin accumulation (Pirie & Mullins, 1976; Sakamoto *et al.*, 1994).

An *Arabidopsis* mutant deficient in the ability to maintain adequate internal P levels displayed at least a 100-fold greater anthocyanin content than the normal phenotype (Zakhleniuk *et al.*, 2001). Similarly, *Arabidopsis* mutants with diminished expression of two RNase genes usually induced by P starvation and thought to sequester P, displayed increased anthocyanin levels in P adequate and deficient growth medium (Bariola *et al.*, 1999). Anthocyanin synthesis in flooded (Andersen *et al.*, 1984) or cold soils (Cobbina & Miller, 1987) may be due to reduced P uptake experienced under these conditions (Engels *et al.*, 1992 Topa & Cheeseman, 1992). Salinity stress, reported to result in anthocyanin accumulation, induces P deficiency in leaves of tomato and increases the P requirement of young leaves (Awad *et al.*, 1990).

P and N deficiency results in growth reduction, carbohydrate accumulation, sugar-repression of photosynthesis, and increased susceptibility to photostress (Lauer *et al.*, 1989; Paul & Driscoll, 1997; Verhoeven *et al.*, 1997; Nielsen *et al.*, 1998). Very low P levels may eventually limit photosynthesis due to the insufficient regeneration of ribulose bisphosphate (Rao & Terry, 1995). Interestingly, low temperatures may predispose leaves to phosphate limitation by suppressing photorespiration and

therefore cycling of orthophosphate (P_i) (Leegood & Furbank, 1986) and by causing loss of the synchronization between activity of enzymes such as sucrose phosphate synthase and diurnal assimilatory activity of photosynthesis (Jones *et al.*, 1998). Cold tolerance in some, mainly herbaceous, plants is achieved through greater availability of P_i. This is brought about by a change in carbon sinks from exporting fixed carbon to support new growth to increased flux of fixed carbon to storage (Huner *et al.*, 1993). The reduced sensitivity to photoinhibition displayed by hardened rye leaves could partially be reproduced by feeding P to non-hardened leaves (Hurry *et al.*, 1993).

Gaume *et al.* (2001) attributed increased tolerance to P deficiency in maize to the accumulation of anthocyanins. Shading of N-deficient leaves prevented carbohydrate accumulation and the subsequent repression of photosynthesis (Paul & Driscoll, 1997) providing an indication of how anthocyanin light screens possibly protect nutrient deficient plants against light stress.

Wounding and pathogen attack

Plants often accumulate anthocyanin in response to wounding (Bopp, 1959) and pathogen infection (Hammerschmidt & Nicholson, 1977a; Hipskind *et al.*, 1996). Fungal elicitors enhance anthocyanin accumulation in cell cultures (Rajendran *et al.*, 1994; Fang *et al.*, 1999) although, in other cases, fungal inoculation or elicitors were found to reduce anthocyanin accumulation (Gläßgen *et al.*, 1998; Lo & Nicholson, 1998). This probably relates to regulation of the phenylpropanoid pathway ensuring allocation of resources from less essential metabolic activities to those of immediate concern for survival (Gläßgen *et al.*, 1998; Lo & Nicholson, 1998).

Environmental conditions may modify anthocyanin accumulation in response to pathogens and wounding. Sweet corn hybrids infected with barley yellow dwarf virus accumulated anthocyanin during a cool, but not during a warm season (Itnyre *et al.*, 1999). Anthocyanin accumulation in response to methyl jasmonate or jasmonic acid (JA) was greater in cooled than in uncooled tulip bulbs (Saniewski *et al.*, 1998). Sucrose has a synergistic effect on JA-induced gene expression in the light while P partially inhibits the JA effect (Berger *et al.*, 1995). Pests and pathogens damaging

and reducing root function may give rise to reddening probably by inducing P deficiency (Cobbina & Miller, 1987).

Exogenous JA application, acting at transcriptional level, induces anthocyanin accumulation in various tissues (Franceschi & Grimes, 1991; Tamari *et al.*, 1995; Saniewski *et al.*, 1998). The effect of JA could be reproduced by wounding (Tamari *et al.*, 1995). In at least some host-pathogen interactions, anthocyanin induction may proceed via the jasmonate-wounding pathway (Feys *et al.*, 1994). Coronatine, a supposed jasmonic acid mimic produced by some *Pseudomonas syringae* pathovars, and jasmonic acid both induced anthocyanin synthesis in *Arabidopsis* seedlings, while coronatine insensitive mutants did not accumulate anthocyanin and were resistant to the pathogen.

Increased anthocyanin accumulation is often indicative of resistance or hypersensitivity responses while anthocyanin accumulation is repressed in susceptible host-parasite combinations (Hammerschmidt & Nicholson, 1977b; Heim *et al.*, 1983; Hipskind *et al.*, 1996). Anthocyanin does not seem to play a direct role in the pathogen-host interaction, but accumulates in healthy uninfected epidermal cells surrounding restricted lesions only after fungal growth is repressed (Heim *et al.*, 1983; Hipskind *et al.*, 1996). Rather, anthocyanin synthesis may be related to the accumulation of carbohydrates, reduced photochemical quenching and local demise of the photosynthetic apparatus that are typical responses to pathogen infection (Balachandran *et al.*, 1997).

Water status

Although drought is said to increase pigmentation (Balakumar *et al.*, 1993; Yang *et al.*, 2000), no evidence of drought-induced anthocyanin synthesis could be found. Combinations of high UV-B radiation and water stress increased pigmentation in cowpea (Balakumar *et al.*, 1993) and cucumber (Yang *et al.*, 2000) seedlings, but not relative to UV alone. On its own, water stress had no significant effect on pigmentation. Additional, non-photosynthetic pigmentation could presumably increase the heat-load of tissues (Schroeder, 1965; Hetherington, 1997) and high leaf temperature has been found to aggravate photoinhibition in water-stressed plants

(Ludlow & Björkman, 1984). Many environmental stresses, including water stress, may induce leaf senescence (Gan & Amasino, 1997) and so bring about anthocyanin pigmentation.

The red carotenoid, rhodoxanthin, accumulates in *Aloe vera* in response to high light and drought stress and is thought to provide protection against the resultant photo-oxidative stress (Diaz *et al.*, 1990). Also, water stress predisposes leaves to photo-oxidative damage, which can be reduced or prevented through light avoidance mechanisms (see section above entitled 'Photoinhibition and photoprotection') (Ludlow & Björkman, 1984; Smirnoff, 1993).

Some resurrection plants accumulate anthocyanins in exposed surfaces in response to severe dehydration (Farrant, 2000). The anthocyanins are thought to reduce light stress and provide protection against oxidation. Anthocyanin accumulation in these plants should, however, be seen as part of a distinct developmental strategy analogous to the development of dormancy in response to low temperature, and not as a response to drought stress.

Biochemical Commonality Between Inducers Of Anthocyanin Synthesis

According to Foyer *et al.* (1997), plant response to changing environmental conditions involves changes in the expression of two sets of genes, those involved in antioxidative defence and those involved in carbohydrate metabolism. Photoinhibitory conditions and excess excitation increase the levels of reactive oxygen species, which may result in oxidative damage to cells (see section above entitled 'Photoinhibition and photoprotection'). Changes in carbohydrate metabolism comprise partitioning of resources for employment of defence mechanisms (Foyer *et al.*, 1997) and are required for the adjustment of source activity to reduced sink strength, a general effect of various stresses (Sheen, 1994).

Antioxidative defence

Tissues may experience increased oxidative stress at sensitive developmental stages, for example early leaf development (Fryer *et al.*, 1998). Many environmental

stresses including those associated with anthocyanin synthesis, for example low temperature (Prasad *et al.*, 1994), UV radiation (Landry *et al.*, 1995), wounding and pathogen infection (Grantz *et al.*, 1995; Lamb & Dixon, 1997), also increase the levels of oxidants and induce the expression of genes involved with protection against oxidative stress. Oxidative stresses, for example ozone (Foot *et al.*, 1996) and salt (NaCl & $CaCl_2$) stress (Kennedy & Filippis, 1999; Donahue *et al.*, 2000), were found to induce anthocyanin accumulation. Anthocyanins probably provide protection against oxidative metabolites produced during the expression of disease resistance (Hipskind *et al.*, 1996), dehydration of resurrection plants (Sherwin & Farrant, 1998) and P deficiency (Gaume *et al.*, 2001). Chromoplast-specific carotenoids accumulate in green tissues in response to oxidative stress where they effectively quench free radicals (Bouvier *et al.*, 1998). These carotenoids include rhodoxanthin, which is thought to play a photoprotective role similar to that of anthocyanin (Diaz *et al.*, 1990; Weger *et al.*, 1993).

Flavonoids, including anthocyanins, are potent antioxidants (Yamasaki, 1997; Yamasaki *et al.*, 1997), but are spatially separated from sites of oxidant generation in the chloroplast and mitochondria. Despite rigorous quenching in these organelles, H_2O_2 may leak to the vacuole during severe stress. Yamasaki (1997) suggested that the H_2O_2 is quenched by anthocyanin and other phenolics. However, the equal effectiveness of other colourless flavonoids and phenolics as antioxidants suggests that the putative photooxidative protection afforded by anthocyanins should be unrelated to their ability to quench oxidants. Rather, it is conceivable that anthocyanins protect plants against photooxidation through the attenuation of visible light and consequent reduction of excitation pressure (Smillie & Hetherington, 1999; Merzlyak & Chivkunova, 2000; Feild *et al.*, 2001).

Sink-source regulation

Carbohydrate accumulation, locally or at a whole plant level, is a common response to all the main environmental inducers of anthocyanin synthesis, for example low temperature (Strand *et al.*, 1997), nutrient deficiency (Paul & Stitt,), wounding and pathogen infection (Balachandran *et al.*, 1997), flooding (Topa & Cheeseman, 1992) and oxidative stress (Foot *et al.*, 1996). Exogenous sucrose and hexose sugars

strongly induce anthocyanin synthesis in suspension cultures, detached leaves and leaf disks (Murray *et al.*, 1994; Decendit & Mérillon, 1996; Larronde *et al.*, 1998). Anthocyanin synthesis in response to treatments that increase carbohydrate levels, such as girdling (Jeannette *et al.*, 2000), CO_2 enrichment (Tripp *et al.*, 1990; Stitt, 1991), sink removal (Hussey, 1963) and treatment with sulfonylurea herbicides (Hall & Devine, 1993; Nemat Alla & Younis, 1995) may be related to this. So may the reduction in pigmentation in response to treatments that reduce carbohydrate levels such as source removal (Hussey, 1963) and phenylurea herbicides (Downs *et al.*, 1965). Expression of chalcone synthase (CHS), a key enzyme in anthocyanin synthesis, has been found to be induced by sugar (Tsukaya *et al.*, 1991; Takeuchi *et al.*, 1994).

Apart from anthocyanin biosynthesis, sugar regulation of gene expression may also affect processes as diverse as photosynthesis, carbohydrate metabolism, oxidative stress defence and senescence (Sheen, 1994; Ehness *et al.*, 1997). Generally, sugar-mediated regulation of gene expression is thought to assist plants in balancing carbohydrate supply with demand in response to environmental change and the transition from heterotrophic to autotrophic growth (Sheen, 1994). The jasmonic acid-mediated accumulation of vegetative storage proteins, induction of anthocyanin synthesis and repression of the assembly of the photosynthetic apparatus in sink cells that have a low capacity to export or store carbon is thought to have a similar function by creating a carbon and nitrogen sink, releasing phosphate from sugar-phosphate pools and reducing light levels incident on chloroplasts (Sadka *et al.*, 1994; Creelman & Mullet, 1997; and also refer to section above entitled 'Nutrient deficiency').

P exercises a direct effect on anthocyanin synthesis by inhibiting sucrose-stimulated expression of CHS (Sadka *et al.*, 1994). Depletion of P induces expression of CHS even in the absence of sucrose (Sadka *et al.*, 1994). This is probably related to the role P plays in the regulation of carbohydrate metabolism and the balancing of source capacity with sink demand (Stitt, 1991; Marschner, 1995).

Anthocyanin accumulation and reduced expression of Calvin-cycle enzymes in response to sink limitation probably represents a mechanism to down-regulate

photosynthesis in order to restore the source to sink balance, and to prevent photoinhibition and subsequent photooxidative damage (Creelman & Mullet, 1997; Jeannette *et al.*, 2000).

Conclusion

Light may become toxic to green tissues under environmental stress as well as at certain stages during normal development. In this review a picture has emerged of anthocyanins as effective and flexible light screens allowing the sensitive modulation of light absorption, and so reducing photoinhibition in photosynthetic tissues. Currently, there is proof of the photoprotective role of anthocyanins in senescing leaves, but evidence also supports a photoprotective function in de-etiolating tissues and in plants experiencing environmental stress. Light attenuation may be especially beneficial under conditions that impose a sink limitation on plants and may help to re-establish a balance between light capture, CO_2 assimilation and carbohydrate utilization. Reduced light capture may also decrease the potential for photo-oxidative damage in cells experiencing high excitation pressure.

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OVERALL OBJECTIVE

Downgrading of fruit due to insufficient red colour has limited the profitability of the lucrative blushed pear cultivars 'Rosemarie', 'Flamingo' and 'Forelle' (Huysamer, 1998). These pears constitute a fifth of the South African pear industry in terms of hectarage (Deciduous Fruit Producers Trust, 2001). Poor fruit colour has been ascribed to the pre-harvest loss of red colour during periods of high temperature (Huysamer, 1998). However, the few studies conducted on pear colour development have all focused on fully red pear cultivars that maintain considerable red colour throughout fruit development (Dayton, 1966; Dussi et al., 1997). The regulation of colour development in pears has not been studied. Little is known about anthocyanin degradation in attached fruit (Lancaster, 1992).

In this study, we attempted to improve the understanding of the regulation of colour development in pears. To achieve this goal, we studied anthocyanin synthesis and degradation as influenced by developmental and environmental factors and used the results to interpret the pigmentation patterns of blushed and fully red pear cultivars. Evaporative cooling as measure to improve red colour in blushed pears was evaluated. We also assessed the photoprotective function of anthocyanin in pears as this might explain the significance of different pigmentation patterns.

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PAPER 1:

REGULATION OF PEAR COLOUR DEVELOPMENT IN RELATION TO ACTIVITY OF FLAVONOID ENZYMES.

Abstract. **The objective of this study was to establish the developmental and environmental regulation of colour development in red and blushed pear (*Pyrus communis* L.) cultivars produced in South Africa in relation to pigmentation patterns. We also assessed the activity of phenylalanine ammonia-lyase (PAL) and UDPGalactose: flavonoid-3-*o*-glycosyltransferase (UFGT) in response to cold fronts and during the development of pear fruit. Changes in enzyme activity were related to changes in red colour. Red and blushed pear cultivars displayed a similar general pigmentation pattern, which entailed the attainment of best red colour and maximum anthocyanin concentrations in immature fruit. Red colour generally faded towards harvest. UFGT activity increased over fruit development and was apparently not limiting to colour development. However, the fading of red colour and the decreasing level of phenolics towards harvest might relate to decreasing PAL activity. Skin colour and enzyme activity in the red cultivar, Bon Rouge, displayed little responsiveness to low temperatures. In contrast, low temperatures increased red colour and activity of both PAL and UFGT in the blushed cultivar, Rosemarie. Consistent with the general pigmentation pattern described above, the effect of temperature on enzyme activity was much greater early during fruit development than in the week prior to harvest.**

Downgrading due to insufficient red colour has limited the profitability of blushed pears in South Africa (Huysamer, 1998). These pears are sought after by consumers and fetch higher prices than green or full red fruit. Progress with the production of blushed pear cultivars is held back by a general lack of knowledge regarding the regulation of colour development in pears. Few studies have been conducted on pear colour development and these all focused on red cultivars where considerable red colour remains at harvest despite a gradual reduction during maturation (Dayton, 1966; Dussi et al., 1997).

Red and blushed pears acquire their red colour from anthocyanins present in their peel (Francis, 1970). The biosynthesis of anthocyanins is well established, with the exception of a few enzymatic steps (Macheix et al., 1990). The conversion of phenylalanine to *trans*-cinnamate, mediated by phenylalanine ammonia-lyase (PAL), is the first committed step in the synthesis of phenolic compounds. PAL activity increases concomitantly with the accumulation of anthocyanin and other phenolic compounds in many plants, including apple fruit (Lister et al., 1996; Macheix et al., 1990). In one of the final steps of anthocyanin biosynthesis, UDPGalactose: flavonoid-3-*o* –glycosyltransferase (UFGT) catalyses the attachment of a sugar to the anthocyanin aglycone, considerably increasing its stability. UFGT activity was strongly correlated with the accumulation of anthocyanin in maturing apples (Ju et al., 1995a, 1999; Lister et al., 1996) and grape berries (Boss et al., 1996).

Low temperatures induce red colour development in many crops e.g. apples (Curry, 1997) as well as in vegetative tissues (Christie et al., 1994). High temperatures, on the other hand, are generally associated with poor red colour of fruit (Reay, 1999; Haselgrove et al., 2000). PAL and other enzymes of anthocyanin biosynthesis were low temperature-inducible in different plant species (Christie et al., 1994; Leyva et al., 1995; Shvarts et al., 1997). Also, subjecting apples to low (6-10°C) compared to high temperatures (>20°C) increased PAL activity and increased anthocyanin synthesis (Faragher, 1983; Tan, 1980). The effect of temperature on colour development in pears has not been studied.

We conducted a study to establish the pigmentation patterns of red and blushed pear cultivars produced in South Africa together with the developmental and environmental regulation of colour development. Changes in red colour during fruit development and with the passing of cold fronts were assessed in a blushed and a red pear cultivar, and related to changes in the activity of PAL and UFGT.

Material and Methods

Pigmentation patterns during 2000/2001. Weekly changes in red colour, anthocyanin concentration and levels of phenolics in fruit of the blushed pear cultivars

'Rosemarie', 'Flamingo' and 'Forelle', and the red cultivars 'Bon Rouge' and 'Red d' Anjou' were assessed. Fully exposed fruit were randomly selected from the western side of north to south planted rows in each of up to three orchards (15 fruit per orchard) in up to three production regions namely Stellenbosch (latitude: 33°58'S, longitude: 18°50'E), Grabouw (latitude: 34°10'S, longitude: 19°03'E) and Ceres (latitude: 33°23'S, longitude: 19°19'E). All three regions are located in the Western Cape province (Mediterranean climate) of South Africa. In the case of 'Red d' Anjou' of which only one orchard was available, ten fruit were collected from each of three rows in the orchard. Although the orchards varied in age, all the trees were grafted on the vigorous BP1 or BP3 rootstocks. Data collection commenced on 19 October 2000, with the exception of 'Bon Rouge' where it started two weeks later due to later bloom, and continued until commercial harvest (11 January 2001 for 'Rosemarie' and 'Flamingo', 1 February for 'Bon Rouge', 8 February for 'Red d'Anjou' and 22 February for 'Forelle').

Fruit were placed in cooler boxes and brought to the laboratory where colour was measured with a chromameter (NR-3000; Nippon Denshoku, Tokyo, Japan) halfway between the calyx and stem ends. Although lightness, chroma and hue were recorded, usually only hue angle (h^o=arctangent [b/a]) is presented. Hue angle refers to the angle formed by a line from the origin to the intercept of the a (x-axis) and b (y-axis) coordinates, where 0° = red-purple, 90° = yellow, 180° = bluish-green and 270° = blue, and was the most appropriate means to express differences in colour in this study (McGuire, 1992). Whole fruit were peeled with a potato peeler (±1 mm thickness), the peel was frozen in liquid nitrogen and kept at -80°C until analysis. Linear regression analysis was used to describe changes in hue, anthocyanin concentration ($\mu g.g^{-1}$ fresh weight), flavonol glycosides (A 350 nm) and other phenolics (A 280 nm) that occurred during fruit development.

Pigmentation patterns and enzyme activity during 2001/2002. Changes in the hue of fifty 'Rosemarie', 'Forelle', 'Flamingo' and 'Bon Rouge' pears in orchards (one of each cultivar) grafted on BP1 rootstock and established in the Stellenbosch region were assessed over the course of fruit development. Fully exposed fruit on the western side of trees were tagged on 31 October 2001 and their colour measured weekly at the reddest position on the fruit.

Using tagged fruit as reference to colour, ten fully exposed fruit were picked weekly before 10:00 in the morning from the western side of each of three adjacent rows in both the 'Rosemarie' and 'Bon Rouge' orchards to quantify changes in PAL and UFGT activity over the course of fruit development. Collection of fruit started on 31 October 2001, 24 and 42 days after full bloom of 'Bon Rouge' and 'Rosemarie', respectively, and continued until 9 January 2002. Commercial harvest commenced during the following week. Fruit diameter was measured and colour assessed at the reddest position on the fruit. Whole fruit were peeled and peel kept at -80ºC until quantification of enzymes. Peel of all the fruit of a row was pooled to obtain three replicates.

Effect of temperature on colour development during 2001/2002. The colour of 'Rosemarie', 'Flamingo', 'Forelle' and 'Bon Rouge' pears was measured daily at the reddest position on the fruit and correlated with temperature data obtained for the Nietvoorbij automatic weather station (±4 km from the trial sites). To further establish the regulation of colour development in response to temperature, PAL and UFGT activities in the peel of 'Rosemarie' and 'Bon Rouge' fruit were assessed twice during fruit development with the passing of cold fronts. Using tagged fruit as reference, ten fully exposed fruit were picked before 10:00 in the morning from the western side of each of three adjacent rows in the 'Rosemarie' and 'Bon Rouge' orchard on five sample dates early during fruit development (19 until 23 November 2001) and again on four sample dates in the week prior to harvest (6 until 9 January 2002). Collection of fruit started before the onset of frontal weather and continued until temperatures returned to pre-frontal levels. Colour was measured at the reddest position whereafter whole fruit were peeled and peel stored as described. Enzyme activity was correlated with temperature data obtained for the Nietvoorbij automatic weather station.

Pigment analysis. Fruit were peeled into liquid nitrogen with a potato peeler removing approximately 2 mm of peel. Peel was kept frozen at -80ºC until pigment analysis. Pigments were extracted in 10 ml 100% acetone for one hour at 4ºC and the extract decanted after centrifugation for ten minutes at 10000 x *g*. The extract was rotary-evaporated and taken up in one to two ml (depending on the cultivar) of 5% (v/v)

formic acid in methanol. After filtration through 0.45 μm filters (Millex-HV; Millipore Corporation, Milford, MA), pigments were quantified via reverse-phase high performance liquid chromatography (HP 1100; Agilent Technologies, Palo Alto, CA). A C_{18} column (250 x 4.6 mm with 5 um ; Spherisorb; Phase Separations, Deeside, UK), with a 12.5 mm 5 micron guard column (Zorbax SB-C18; Agilent Technologies, Palo Alto, CA) was maintained at 30°C. The mobile phase consisted of 5% formic acid in water (A) and 5% formic acid in methanol (B) with a linear gradient of 25% to 65% during the first 18 min and from 65% to 100% during the last 3 minutes. The flow rate was 1.0 ml min^{-1}, with an injection volume of 10 or 15 μl, depending on the cultivar. Eluted anthocyanins were monitored at 530 nm. Chromatographs indicated the presence of two anthocyanin pigments in all the pear cultivars studied. The major pigment co-eluted with cyanidin 3-galactoside. The minor pigment is considered to be either cyanidin 3-arabinoside (Francis, 1970) or peonidin 3-galactoside (Dussi et al., 1995). Both anthocyanins were quantified using a standard curve obtained with idaein chloride (cyanidin 3-galactoside) (Carl Roth, Karlsruhe, Germany). Eluted phenolics were monitored at 280 and 350 nm. Chromatographs indicated the presence of several peaks, many of which were present at both 280 and 350 nm. Flavonol glycosides were assessed by adding up the absorbance units of peaks that only occurred or had their higher absorbance at 350 nm (Lister et al., 1994). Other phenolics were assessed by adding up the absorbance units of peaks that only occurred or had their higher absorbance at 280 nm.

Enzyme extraction and assay of activity. The method of Lister et al. (1996) was used for enzyme extraction. Samples were taken from storage at -80°C and ground in liquid nitrogen. A 20 g sample of ground tissue was mixed in 100 ml extraction buffer (50 mM Na_2HPO_4/KH_2PO_4 at pH 7.0 containing 50 mM ascorbic acid; 18 mM mercaptoethanol; 5% (w/v) PVP (MW 44000); 0.1% Triton X-100) for five minutes before centrifugation at 20000 x *g* for five minutes to remove particulate matter. Ammonium sulphate was added to the supernatant (35% saturation) to precipitate the protein. Samples were centrifuged for a further 20 minutes to remove PVP, then more ammonium sulphate was added to a final saturation of 80% before a final 20 minute centrifugation. The pellet were re-suspended with 2 ml dialysing buffer (extraction buffer without PVP and Triton X-100) and dialysed for 18 hours in the same buffer. The enzyme extract was frozen in liquid nitrogen and stored at -80°C

until the analysis was performed. All extractions were done on ice and buffers were pre-cooled to ~4°C. The protein concentration of the enzyme extract was determined by means of the dye-binding assay of Bradford (1976).

PAL activity in the enzyme extracts was assayed according to Lister et al. (1996) who in turn adapted the method of Zucker (1965). Crude enzyme extract (250 µl) was added to 875 µl of a 60 mM borate buffer. The reaction was started by adding 250 µl L-phenylalanine (10 mg ml^{-1}). After incubation for one hour at 30°C, the reaction was stopped by addition of 35% (w/v) trifluoroacetic acid (125 µl) followed by centrifugation for 5 minutes at 5000 x *g*. The absorbance was measured at 290 nm on a spectrophotometer (DU Series 64; Beckman, California) to estimate the yield of cinnamic acid. Duplicate assays were performed for each extract also including blanks containing no substrate to compensate for basal levels of absorbance in the absence of the reaction product. Enzyme activity was expressed as pkat mg^{-1} protein, where 1 kat is the amount of enzyme required to produce 1 $mol.s^{-1}$ *trans*-cinnamic acid under the assay conditions.

The procedure of Lister et al. (1996) was used to assay UFGT activity. The reaction was initiated by adding 200 µl crude enzyme extract, 30 µl quercetin (1mM) and 20 µl UDP-galactose (2.5 mM) to 200 µl bicine buffer (50 mM; pH 8.5). Reaction tubes were incubated for 30 minutes at 30°C and the reaction stopped by addition of 150 µl trichloroacetic acid (20%). After centrifugation for five minutes at 5000 x *g*, the supernatant was filtered through a 0.45 µm Millipore filter and the quercetin 3-galactose quantified by HPLC using the same methodology as described for anthocyanins, but with an injection volume of 5 µl. Quercetin-3-galactoside eluted at about 12.5 minutes and was detected at 350 nm. UFGT activity was expressed as pkat mg^{-1} protein.

Statistical analysis. The data were analysed with the General Linear Models (GLM), Linear Regression (REG) and Correlation (CORR) procedures of SAS (SAS release 6.12P; SAS Institute, 1996, Cary, NC).

Results and Discussion

Pigmentation patterns. The red and blushed pear cultivars included in our study varied considerably in anthocyanin concentration and red colour (Table 1; Fig. 1-3). Although changes in hue were gradual in most cultivars, 'Rosemarie' colour fluctuated from week to week (Fig. 1, 3). Despite this, all cultivars displayed a similar general pigmentation pattern, which entailed the attainment of best red colour and highest anthocyanin concentrations in immature fruit and a tendency of colour to fade towards harvest (Fig. 1-3). The similarity in pigmentation patterns was more evident during 2001/2002 when colour was repetitively measured on the reddest position on the same fruit (Fig. 3). Measuring colour halfway between the calyx and stem ends on a harvested sample of fruit during 2000/2001 (Fig. 1) increased variation and resulted in the underestimation of the colour of juvenile fruit. This is because the position of the reddest area on fruit shifted during fruit development from around the calyx to the side of the fruit as fruit orientation changed from an upright position after anthesis to the customary hanging position towards harvest (data not presented).

Though the pigmentation pattern described in this study is consistent with previous reports for various red pear cultivars (Dayton; 1966; Dussi et al., 1997), it is contrary to most other crop species where maximum pigmentation and colour is attained in ripe fruit (Macheix, 1990). Accumulation of anthocyanin towards maturity is thought to assist seed dispersal (Harborne, 1965). The function and ecological significance of the colouration pattern of pears is uncertain. However, it has to be considered that red and blushed cultivars are selected for aesthetic reasons, often from green parents, and that the increased pigmentation does not necessarily confer any adaptive advantage (Harborne, 1965). Dussi et al. (1997) described two 'Bartlett' mutations in which anthocyanin and red colour increased towards harvest. These two cultivars clearly emerged through a different mutation to that of 'Bon Rouge', which is also a 'Bartlett' mutation.

The red cultivars 'Bon Rouge' and 'Red d'Anjou' maintained higher anthocyanin concentrations during fruit development and were redder at harvest than the blushed cultivars 'Forelle', 'Flamingo' and 'Rosemarie' (Table 1). Among the blushed cultivars, 'Rosemarie' had the lowest anthocyanin concentration (Table 1). The standard

deviation in the hue of the different cultivars over fruit development seemed to decrease as the maximum anthocyanin concentration attained by the respective cultivars increased (Table 1). The greater ability to accumulate anthocyanin increased the stability of red colour over fruit development.

Developmental regulation of colour development. PAL activity decreased during fruit development in both 'Rosemarie' and 'Bon Rouge' (Fig. 4A). This is consistent with previous findings in various fruit kinds where PAL activity generally peaked in juvenile fruit whereafter it gradually decreased towards harvest (Macheix et al., 1990). PAL activity increases again in fruits that accumulate anthocyanin or other phenolics during maturation (Macheix et al., 1990). The fading of pear red colour towards harvest could possibly relate to the diminished activity of PAL towards harvest (Fig. 4). However, anthocyanin synthesis in mature apples only required PAL activity if there were insufficient phenolic or flavonoid precursors brought about by etiolation (Ju et al., 1995b). In contrast, PAL activity was required for the reddening of maturing strawberry fruit (Given et al., 1988). In apples, high PAL activity in juvenile fruit corresponded with the first peak of anthocyanin synthesis (Ju et al., 1995b). In contrast, 'Rosemarie' and 'Bon Rouge' fruit colour was relatively poor early during fruit development when PAL activity was highest (Fig. 4). High PAL activity in juvenile fruit is thought to facilitate the accumulation of large amounts of different phenolics and flavonoids required for protection against various stresses, e.g., UV-light, fungal infection and browsing (McClure, 1972). Therefore, although PAL activity is required for anthocyanin synthesis, it does not necessarily guarantee its synthesis since the cinnamic acid can be diverted into numerous other phenolic or flavonoid compounds (Lister et al., 1994).

Congruent with the position of PAL at the start of phenylpropanoid metabolism, levels of phenolic acids and flavonoids in fruit skin generally correlate with PAL activity (Lister et al., 1996; Macheix et al., 1990). Inhibition of PAL has been found to result in a comparable reduction in the phenolic content of 'Delicious' apples (Ju et al., 1995b). Since sub-samples kept for determining anthocyanins and phenolics were lost due to malfunction of the -80°C freezer, we could not directly correlate enzyme activity with levels of different phenolics in the same fruit. However, absorbance at 280 nm steadily declined towards harvest in all the pear cultivars studied during the

2000/2001 season (Fig. 5). It is likely that these changes in the levels of phenolics relate to the decreasing activity of PAL (Fig. 4A). Chlorogenic acid contributed most to absorbance at 280 nm (data not presented), hence, changes in absorbance primarily reflect changes in the concentration of this compound. Flavonol glycoside levels decreased towards harvest in 'Red d'Anjou', 'Bon Rouge' and 'Rosemarie' (Fig. 5B). In 'Forelle' and 'Flamingo' flavonol glycoside levels increased from low initial levels until the end of December whereafter it remained relatively constant until harvest. Evidently, a direct relationship between PAL activity and flavonoid glycoside levels seems less likely.

In contrast to PAL, UFGT activity increased during fruit development in both 'Rosemarie' and 'Bon Rouge' (Fig. 4) and was evidently not the rate-limiting step in synthesis or the reason for the fading of red colour towards harvest in 'Rosemarie' or 'Bon Rouge'. In contrast to our results, UFGT activity was strongly correlated with colour development in maturing apples (Ju et al., 1995a, 1999; Lister et al., 1996) and grape berries (Boss et al., 1996). High basal activity of UFGT in green apple peel has been attributed to its ability to also catalyse synthesis of flavonol glycosides (Ju et al., 1995a, 1999). The inverse patterns of PAL and UFGT activity during fruit development of 'Bon Rouge' and 'Rosemarie' (Fig. 4) was contrary to previous observations in apple where these enzymes showed similar patterns of activity, thought to be indicative of coordinated regulation of anthocyanin synthesis (Lister et al., 1996).

Despite the apparently poor correlation between PAL and UFGT activity and 'Rosemarie' and 'Bon Rouge' colour, flux through the phenylpropanoid pathway appeared to be greater in the red 'Bon Rouge' compared to the blushed 'Rosemarie' fruit. PAL activity in 'Bon Rouge' was at least 40% and UFGT activity 10-20% higher throughout fruit development than in 'Rosemarie' (Fig. 4). Levels of flavonol glycosides and phenolic acids were also higher in red compared to blushed pear cultivars (Table 2; Fig. 4, 5). Similarly, Lister et al. (1996) found that PAL and UFGT activity and synthesis of flavonoids were considerably higher in a red compared to a green apple cultivar.

The synthesis of anthocyanins and flavonol glycosides appeared to be separately regulated in the different pear cultivars. 'Rosemarie' and 'Flamingo', both selected from the progeny of crosses made between 'Bon Rouge' and 'Forelle' (ARC Infruitec-Nietvoorbij & SAPO, 1998), reached comparable hues and pigment concentrations at harvest (Table 1). However, immature 'Flamingo' fruit had a greater capacity to accumulate anthocyanin and did not display the weekly fluctuation in colour evident in 'Rosemarie' (Fig. 3). In this regard, 'Flamingo' resembled 'Bon Rouge' while 'Rosemarie' apparently inherited its pigmentation pattern from 'Forelle'. Conversely, with regard to the levels of flavonol glycosides, 'Flamingo' resembled 'Forelle' while 'Rosemarie' resembled 'Bon Rouge' (Fig. 5B).

Effect of temperature on colour development. 'Rosemarie' pears increased in redness with the passing of cold fronts while red colour faded during intermittent warmer periods (Fig. 6). In contrast, 'Bon Rouge' colour did not fluctuate in response to temperature. This difference in response was investigated in a separate paper (Paper 2). In the current study, we assessed PAL and UFGT activity with the passing of two of these cold fronts, the first during early fruit development and the second in the week before harvest (Fig. 6).

PAL and UFGT activity in 'Rosemarie' peel increased by 130% and 200%, respectively, while hue decreased by 19° in response to the first cold front (19-23 November) (Fig. 7A, B). Activities of both enzymes strongly correlated with the lower minimum temperatures (Table 3). There was also a weaker, but significant correlation with hue. Activities of both enzymes decreased after the passing of the cold front, though the activity of UFGT was still 134% higher than pre-frontal levels on 23 November. The cold front had no effect on 'Bon Rouge' colour, though PAL activity was 33% higher (P=0.1) on 21 November than on 19 November (Fig. 7C, D). UFGT levels increased by 28% up to 22 November, but this increase was not statistically significant (Fig. 7C, D). Enzyme activities in 'Bon Rouge' did not correlate with hue or temperature (Table 3).

The second cold front brought about a greater reduction in temperature than the first (Fig. 8B, D). 'Rosemarie' hue decreased by nearly 14° between 6 and 9 January (Fig. 8B). UFGT activity increased by 11% over this period, but the increase was not

significant (Fig. 8A). UFGT activity was poorly correlated with minimum temperature and hue (Table 3). PAL activity was unaffected by the cold front (Fig. 8A). 'Bon Rouge' hue was variable, fluctuating between 18° and 23° (Fig. 8D). PAL activity in 'Bon Rouge' did not respond to the cold front (Fig. 8C). The apparent reduction in UFGT activity in 'Bon Rouge' on 8 January is not readily explainable (Fig. 8C). However, enzyme activities in 'Bon Rouge' did not correlate with hue or temperature (Table 3).

The above results indicate that the activity of anthocyanin biosynthetic enzymes in 'Rosemarie' respond to temperature, at least early during fruit development. Anthocyanin structural and regulatory genes were previously found to be induced by low temperatures in various tissues of many different plants (Christie et al., 1994; Leyva et al., 1995; Shvarts et al., 1997). Faragher (1983) and Tan (1980) found much higher PAL activity and better red colour development in apples held at low (6-10°C) compared to higher temperatures (>20°C). Contrary to our results, anthocyanin synthesis benefited from low temperatures in all the different apple cultivars studied (Curry, 1997; Marais et al., 2001; Uota, 1951; Reay, 1999). Hence, the apparent lack of response of 'Bon Rouge' to low temperatures was rather surprising, although it could relate to its high anthocyanin concentration. Lancaster et al. (2000) found that apple fruit already containing high anthocyanin concentrations had a reduced ability to further accumulate anthocyanin at 10°C and 20°C.

Considering that lower minimum temperatures were experienced the week before harvest, the weaker response of 'Rosemarie' colour and enzyme activity (Table 3; Fig. 7, 8) suggests that the fading of red colour in maturing fruit cannot only be ascribed to the increasing temperatures typically experienced from anthesis in spring until harvest in mid-summer. It also provides further evidence that, despite the fluctuation in 'Rosemarie' colour during fruit development, the ability to synthesize anthocyanin also decreases towards maturity as was more evident in other cultivars (Fig. 3). Furthermore, the increase in redness in response to the second cold front without any significant increase in enzyme activity casts doubt on whether activity of these enzymes regulated anthocyanin synthesis in 'Rosemarie' at this stage of fruit development. The increase in UFGT activity during fruit development was probably sufficient to allow anthocyanin synthesis in response to low temperatures at later

stages of fruit development in 'Rosemarie' without requiring any further increase in activity. As in apples (Lister et al., 1996), colour development in 'Rosemarie' and 'Bon Rouge' pears was apparently regulated at an enzymatic step preceding UFGT. Since the activity of both PAL and UFGT increased with the passing of the first cold front, reddening at this stage of fruit development apparently entailed the coordinated up regulation and increased flux through the entire anthocyanin biosynthesis pathway. Reddening in response to the second cold front appeared to rely more on the precursor pool as previously found in maturing apple (Ju et al., 1995b).

Considering the good correlation between hue, enzyme activity and low temperatures early during fruit development (Table 3), it was rather surprising that the considerable fluctuation in 'Rosemarie' colour over fruit development, especially at early stages, was not reflected in PAL or UFGT activity (Fig. 4). It is possible, however, that enzyme activity might more rapidly return to pre-frontal levels than the anthocyanin concentration and red colour. Faragher and Chalmers (1977) found that PAL activity increased after a short lag period in whole apples transferred to light at 20°C and decreased again after 30 hours, but anthocyanin continued to accumulate for a further 100 hours. Subjecting apples, pre-cooled at 4°C, to 30°C for three hours nearly halved subsequent anthocyanin synthesis at 20°C (Reay, 1999). The same rapid inhibition of elicitor-induced PAL expression has been observed in parsley cell cultures transferred to 37°C (Walter; 1989). We did not follow enzyme activity for a long enough period after the passing of cold fronts to verify this.

In conclusion, the red and blushed pear cultivars grown in South Africa displayed a similar general pattern of red colour development, which entailed the fading of red colour or a reduced ability to synthesize anthocyanin towards harvest. Our results indicated that colour development and the fading of colour in both 'Rosemarie' and 'Bon Rouge' had an underlying developmental component. In 'Rosemarie', however, environmental regulation of colour development was superimposed on the developmental component so that red colour development depended on the passing of cold fronts. PAL and UFGT activity did not appear to regulate anthocyanin synthesis in pears, though PAL activity and red colour both decreased towards harvest.

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Table 1. Hue and anthocyanin concentration of red ('Red d'Anjou' and 'Bon Rouge') and blushed ('Forelle', 'Flamingo' and 'Rosemarie') pear cultivars as measured during the 2000/2001 season. Means were separated by LSD (5%). Statistical analysis for anthocyanin concentration was also done for blushed cultivars alone since the very high anthocyanin concentrations in red cultivars concealed differences between blushed cultivars when analysed together.

Cultivars	Hue (°)[z]				Anthocyanin concentration (μg.g^{-1} fr wt)		
	Lowest	Highest	Harvest	Standard deviation	Highest	Lowest	Harvest
Red cultivars							
Red d'Anjou	5.2 a	16.2 a	13.6 a	3.24 a	295.4 a	189.0 a	188.4 a
Bon Rouge	8.6 b	24.2 b	21.0 b	5.92 b	160.6 b	38.8 b	68.3 b
Blushed cultivars							
Forelle	23.6 a	44.9 a	34.5 a	11.1 a	18.4 a	3.2 b	10.5 a
Flamingo	19.7 a	52.5 a	52.5 b	15.1 b	14.6 ab	4.5 a	5.3 b
Rosemarie	42.6 b	81.5 b	52.8 b	16.9 b	9.3 b	2.1 b	5.6 b
Contrasts							
Within Red	0.0291	0.0084	0.0241	0.0028	0.0125	0.0001	0.0008
Within Blushed	0.0001	0.0001	0.0001	0.0009	0.0072	0.0031	0.0069
Red vs Blushed	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001

[z] 0° = red-purple, 90° = yellow, 180° = bluish-green and 270° = blue

Table 2. Phenolics and flavonol glycosides in peel of red ('Red d'Anjou' and 'Bon Rouge') and blushed ('Forelle', 'Flamingo' and 'Rosemarie') pear cultivars during the 2000/2001 season as inferred from absorbance readings at 280 nm and 350 nm, respectively. Means were separated by LSD (5%).

Cultivar	Absorbance at 280 nm (mAU g^{-1} fr wt)		Absorbance at 350 nm (mAU g^{-1} fr wt)	
	Highest x 1000	Harvest x 1000	Highest x 1000	Harvest x 1000
Red d'Anjou	156 a	74.7 a	76.5 a	41.1 a
Bon Rouge	110 b	54.9 b	61.3 b	33.7 b
Forelle	63 c	39.7 c	32.4 d	30.1 bc
Flamingo	55 c	32.2 d	33.0 d	26.4 c
Rosemarie	63 c	42.6 c	43.1 c	34.6 b

Table 3. Correlation of phenylalanine ammonia-lyase (PAL) and UDPGal:flavonoid-3-o-glycosyltransferase (UFGT) activity in 'Rosemarie' and 'Bon Rouge' pear peel with hue and daily minimum temperatures recorded during the passing of cold fronts at an early stage of fruit development (19-23 November 2001) and again in the week before harvest (6-9 January 2002).

	Rosemarie		Bon Rouge	
	PAL	UFGT	PAL	UFGT
COLD FRONT 1				
Daily minimum temperature	-0.79 ***	-0.72 **	-0.46	-0.37
Hue	-0.59 *	-0.58 *	0.18	-0.20
COLD FRONT 2				
Daily minimum temperature	0.21	-0.60 *	0.14	0.50
Hue	-0.11	-0.60 *	-0.32	-0.51

*, ** and *** denote Pearson correlation coefficients significant at P = 0.05, 0.01, 0.001, respectively, N = 15 CF1, N = 12 CF2.

Fig. 1. Hue angles for 'Red d'Anjou', 'Bon Rouge', 'Forelle', 'Flamingo' and 'Rosemarie' pears during the 2000/2001 seasons. Hue angles, measured halfway between the calyx and stem ends of fruit, fluctuate between 0° (red-purple) and 90° (yellow). Vertical bars indicate LSD (5%).

Fig. 2. Anthocyanin concentrations in red ('Red d'Anjou' and 'Bon Rouge') (A) and blushed ('Forelle', 'Flamingo' and 'Rosemarie') (B) pear peel during the 2000/2001 season. Vertical bars indicate LSD (5%).

Fig. 3. Hue angles for 'Bon Rouge', 'Forelle', 'Flamingo' and 'Rosemarie' pears during 2001/2002. Colour was repeatedly measured on 50 marked fruit of each cultivar. Hue angles, measured at the reddest position on fruit, fluctuate between 0° (red-purple) and 90° (yellow).

Fig. 4. Changes in the activity of phenylalanine ammonia-lyase (PAL), UDPGal:flavonoid-3-o-glycosyltransferase (UFGT) and hue in 'Rosemarie' (A) and 'Bon Rouge' (B) pears during 2001/2002. Hue angles, measured at the reddest position on fruit, fluctuate between 0° (red-purple) and 90° (yellow). Means were separated by LSD (5%). LSD values for PAL activity, UGGT activity and hue in 'Rosemarie' are 7.3, 18.1 and 8.5, respectively. The respective LSD values in 'Bon Rouge' are 23.0, 18.5 and 3.3.

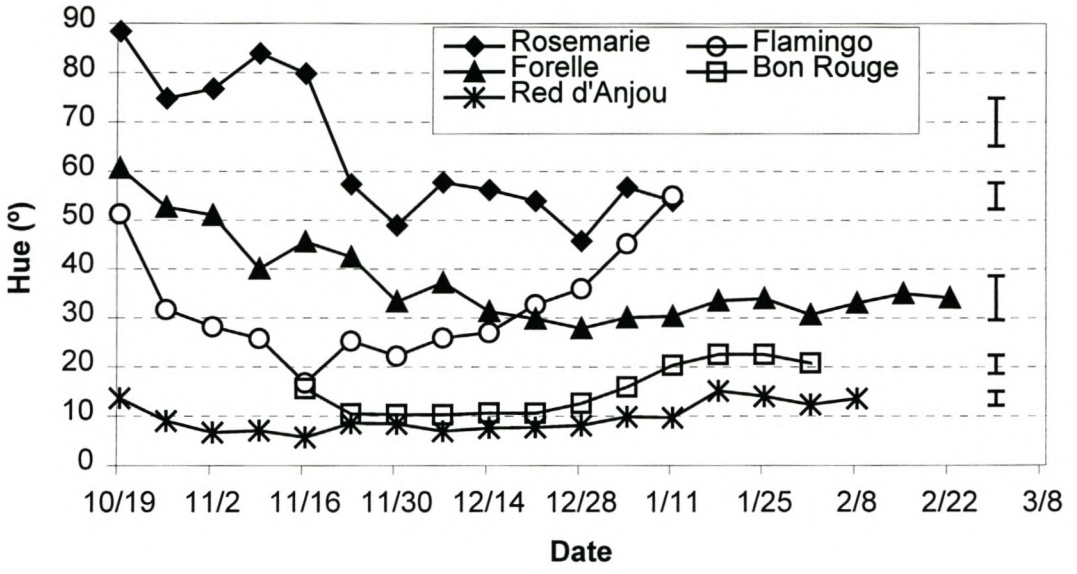
Fig. 5. Phenolics (A), and flavonol glycosides (B) in peel of 'Red d'Anjou', 'Bon Rouge', 'Forelle', 'Flamingo' and 'Rosemarie' pears during the 2000/2001 season as inferred from absorbance readings at 280 nm and 350 nm, respectively.

Fig. 6. (A) Daily changes in hue angle of 'Rosemarie' and 'Bon Rouge' pears during the 2001/2002 season. Hue angles, measured at the reddest position on fruit, fluctuate between 0° (red-purple) and 90° (yellow). Average daily temperatures are presented in (B). PAL and UFGT activities were assessed on the days indicated by open symbols.

Fig. 7. Phenylalanine ammonia-lyase (PAL) and UDPGal:flavonoid-3-*o*-glycosyltransferase (UFGT) activity in 'Rosemarie' (A) and 'Bon Rouge' (C) peel

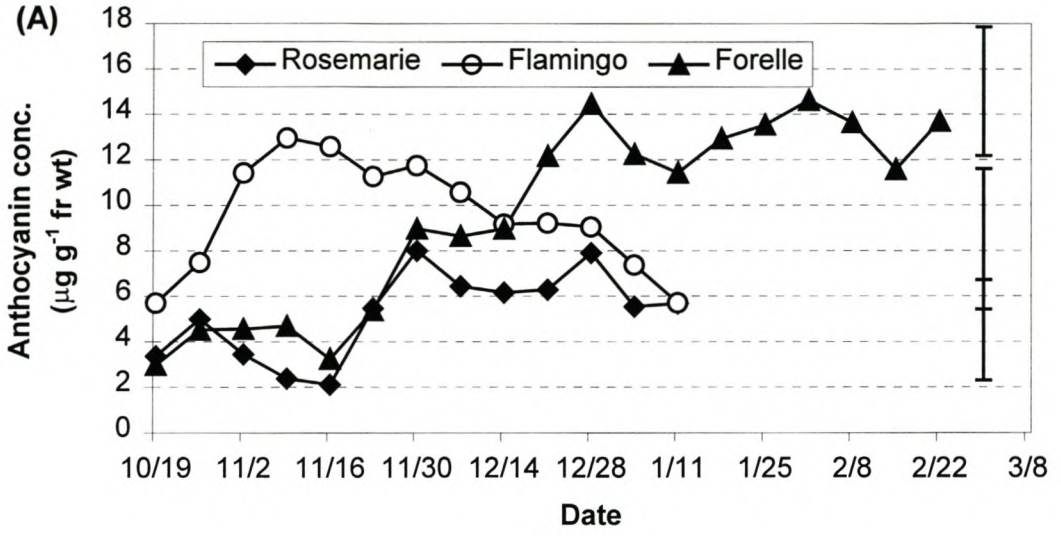
during the passing of a cold front (19-23 November 2001). Hue angles and minimum temperatures are also presented in (C) 'Rosemarie' and (D) 'Bon Rouge'. Hue angles, measured at the reddest position on fruit, fluctuate between 0° (red-purple) and 90° (yellow).

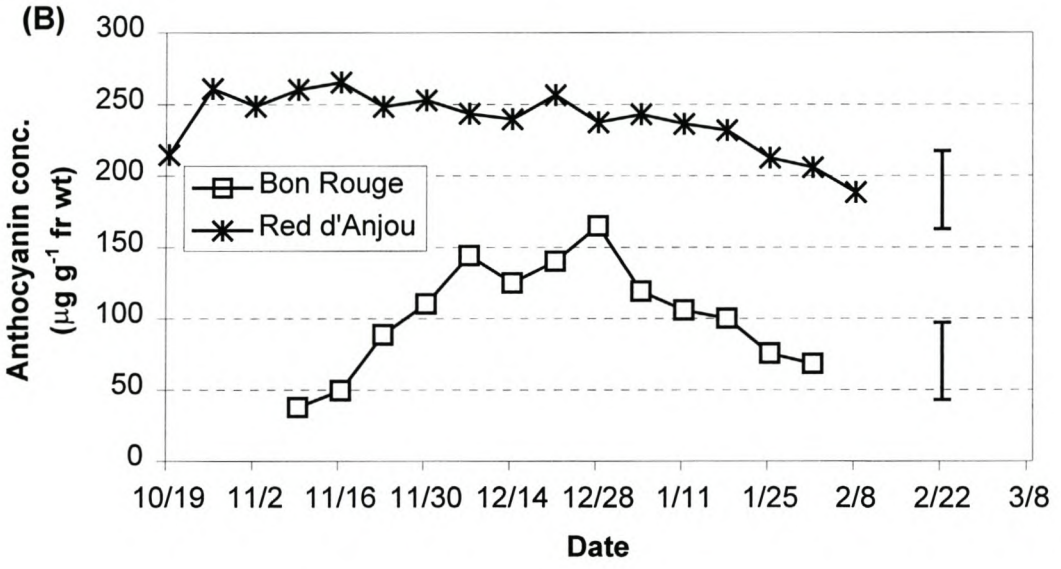
Fig. 8. Phenylalanine ammonia-lyase (PAL) and UDPGal:flavonoid-3-o-glycosyltransferase (UFGT) activity in 'Rosemarie' (A) and 'Bon Rouge' (C) peel during the passing of a cold front (6-9 January 2002). Hue angles and minimum temperatures are also presented in (C) 'Rosemarie' and (D) 'Bon Rouge'. Hue angles, measured at the reddest position on fruit, fluctuate between 0° (red-purple) and 90° (yellow).



	Contrasts	
	Linear	Quadratic
Red d'Anjou	0.0001	0.0001
Bon Rouge	0.0001	0.0001
Forelle	0.0001	0.0001
Flamingo	0.0001	0.0001
Rosemarie	0.0001	0.0017

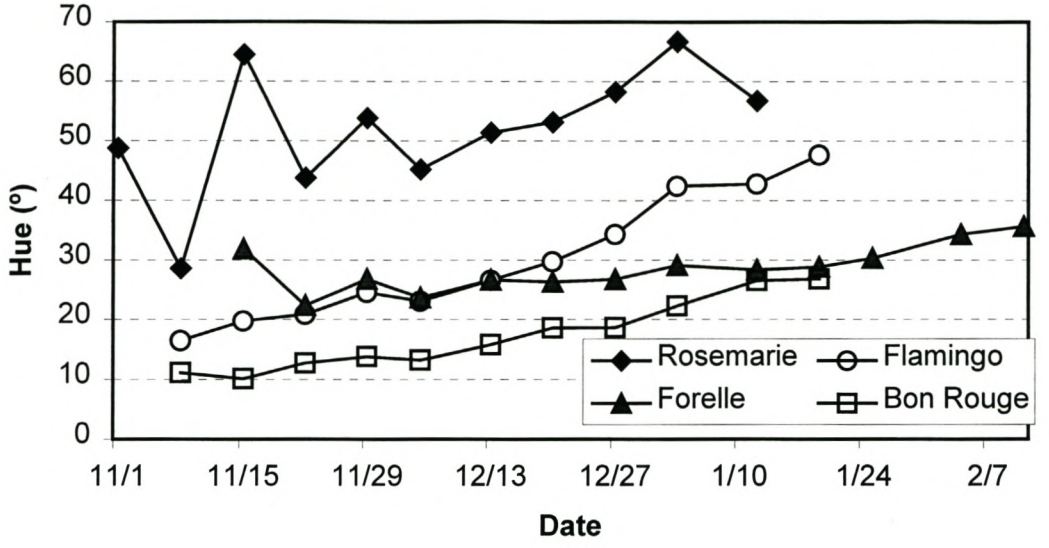
Fig. 1. Paper 1





	Contrasts		
	Linear	Quadratic	Cubic
Red d'Anjou	0.0068	0.0093	0.8182
Bon Rouge	0.1018	0.0001	0.4840
Forelle	0.0001	0.0262	0.0700
Flamingo	0.3744	0.0013	0.1691
Rosemarie	0.0001	0.4628	0.0008

Fig. 2. Paper 1



	$y = xb + a$	$y = cx^2 + bx + a$
Bon Rouge	0.93 ***	0.97 ***
Forelle	0.46 *	0.72 **
Flamingo	0.94 ***	0.98 ***
Rosemarie	0.29	0.30

*, ** and *** denote R^2 significant at $P = 0.05$, 0.01 and 0.001, respectively.

Fig. 3. Paper 1

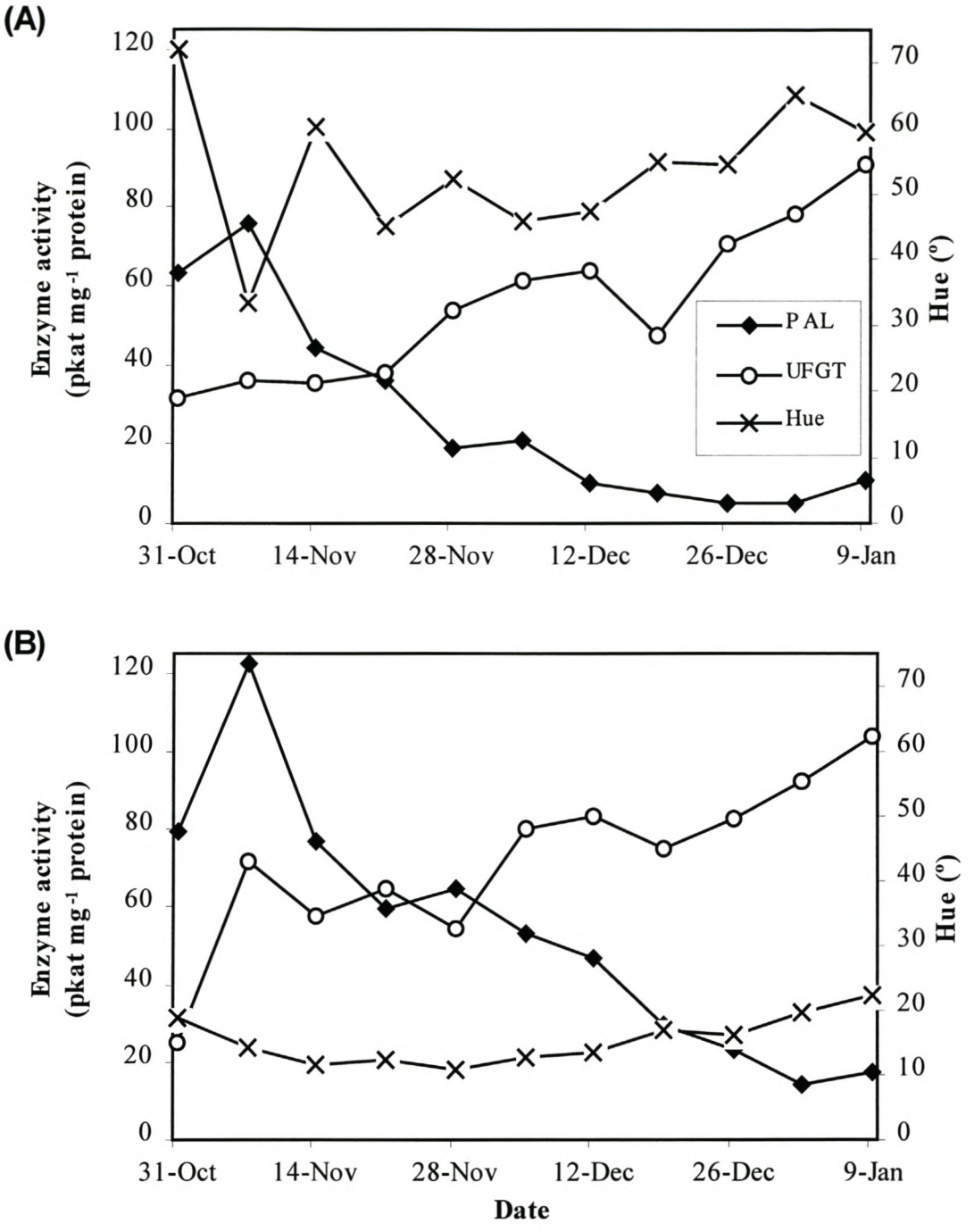
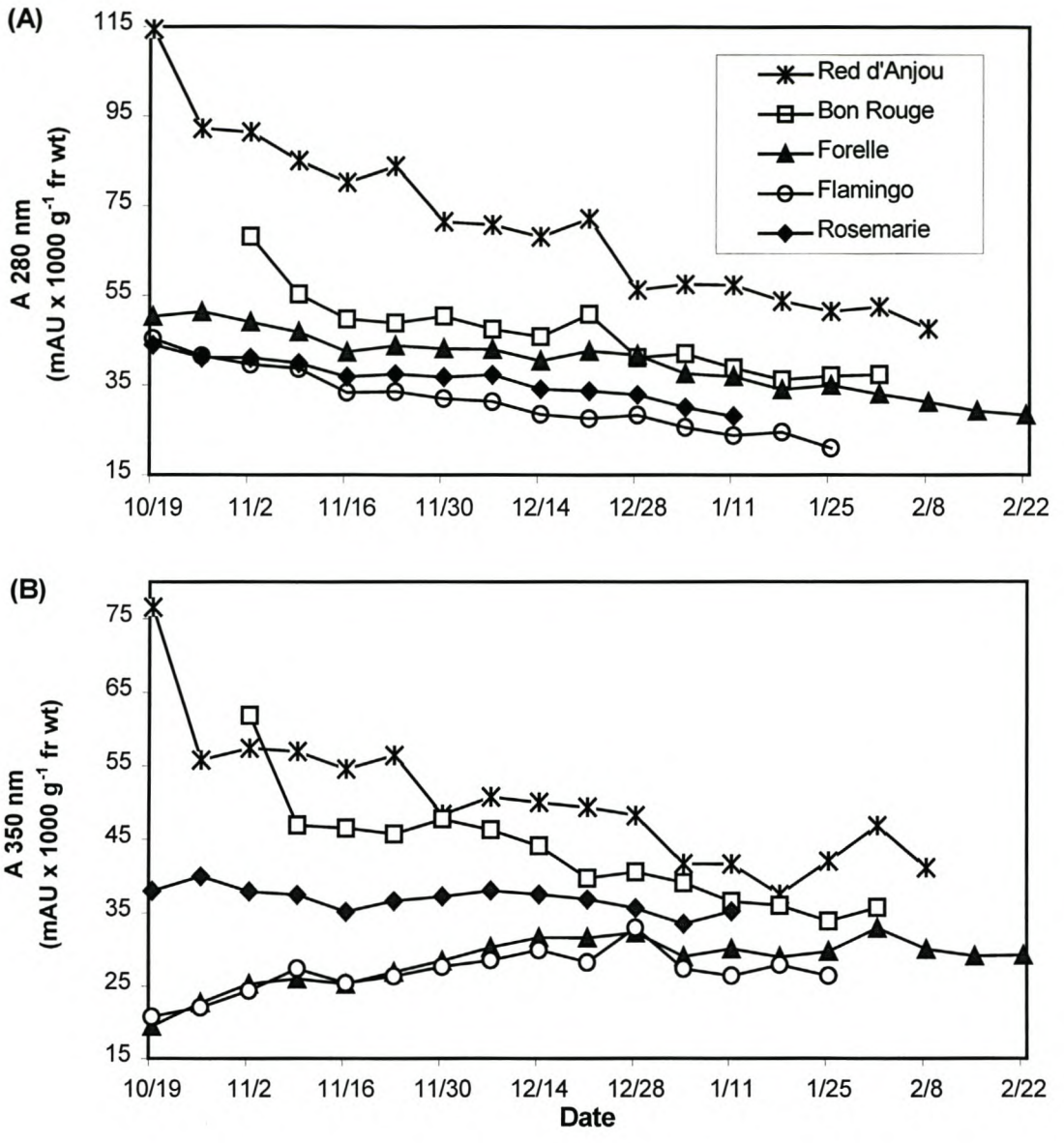

(A)
Enzyme activity (pkat mg-1 protein)
Hue (°)
PAL
UFGT
Hue
31-Oct
14-Nov
28-Nov
12-Dec
26-Dec
9-Jan
(B)
Enzyme activity (pkat mg-1 protein)
Hue (°)
Date

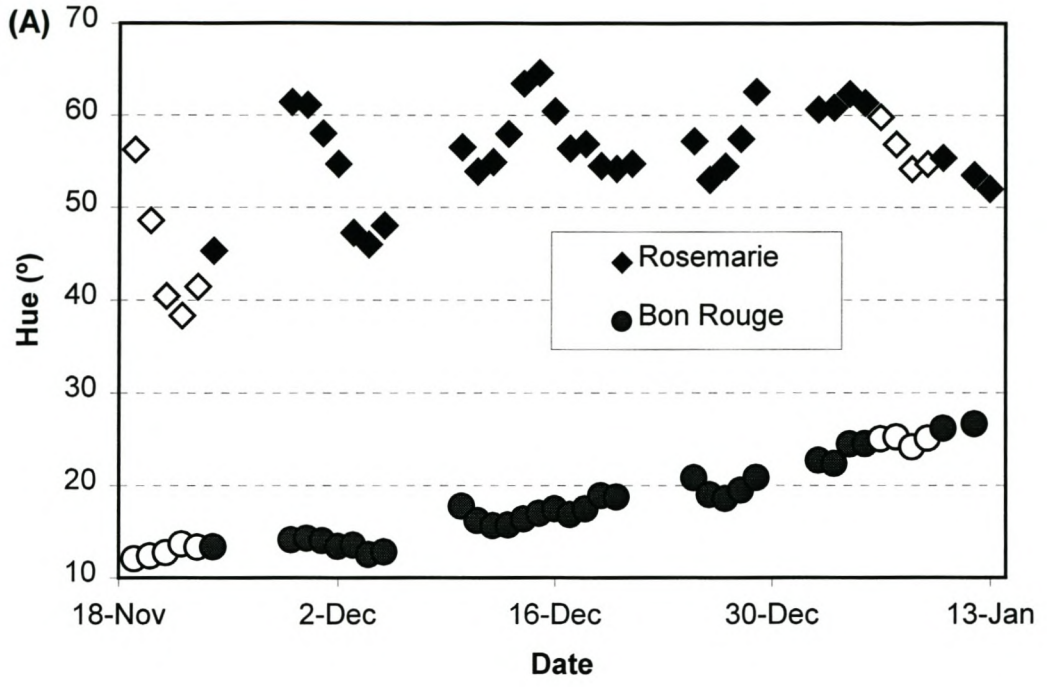
Fig. 4 Paper 1



	A 280	A 350	
	y = xb + a	y = xb + a	$y = cx^2 + bx + a$
Red d'Anjou	0.88 ***	0.73 ***	-
Bon Rouge	0.80 ***	0.80 ***	-
Forelle	0.87 ***	0.77 ***	0.91 ***
Flamingo	0.95 ***	0.53 **	0.76 ***
Rosemarie	0.95 ***	0.49 **	-

** and *** denote R^2 significant at $P = 0.01$ and 0.001, respectively.

Fig. 5. Paper 1


(A)
70
60
50
40
30
20
10
Hue (°)
Rosemarie
Bon Rouge
18-Nov
2-Dec
16-Dec
30-Dec
13-Jan
Date

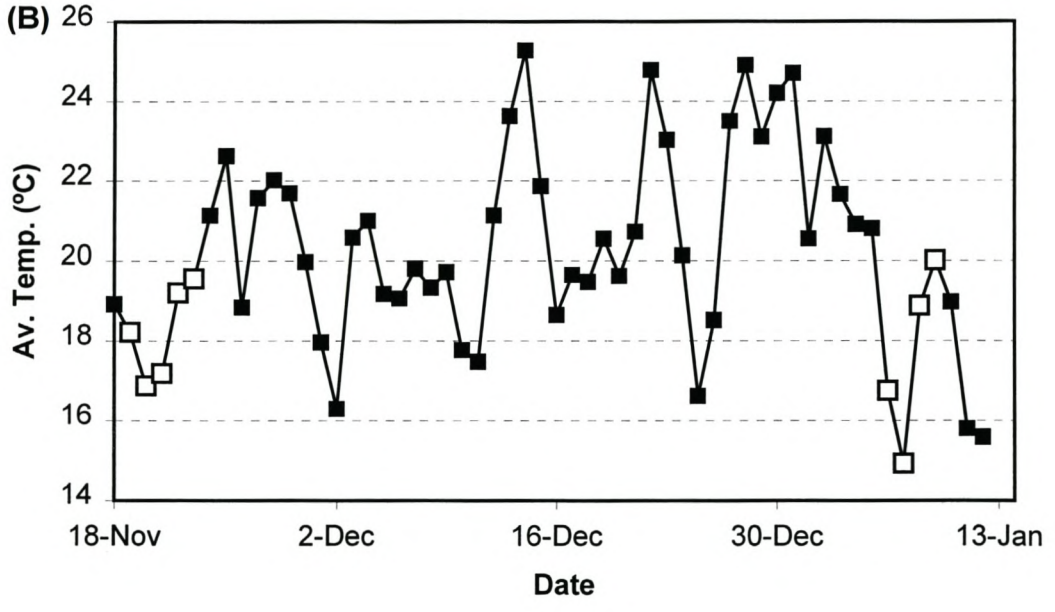

(B)
26
24
22
20
18
16
14
Av. Temp. (°C)
18-Nov
2-Dec
16-Dec
30-Dec
13-Jan
Date

Fig. 6 Paper 1

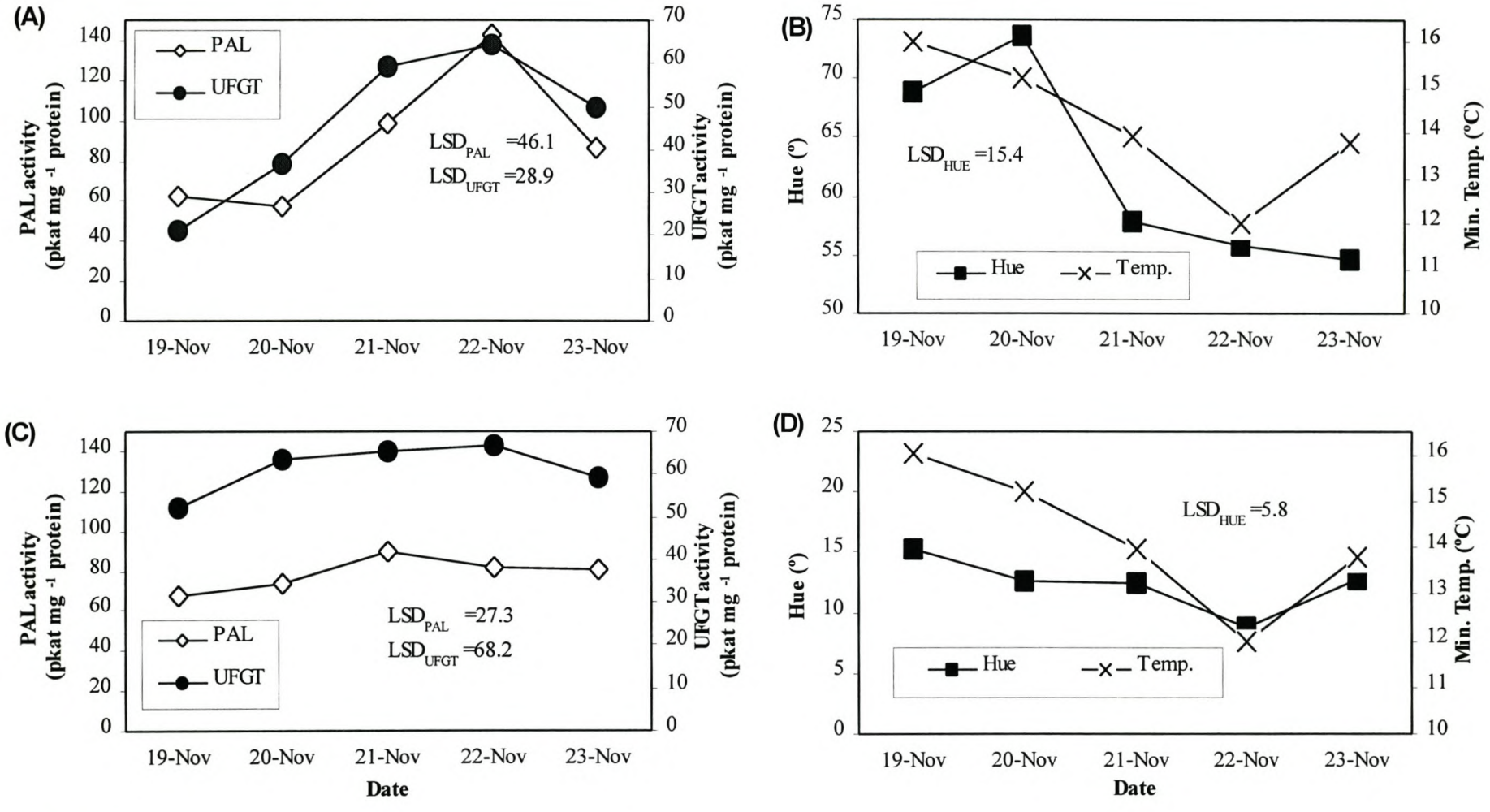


Fig. 7 Paper 1

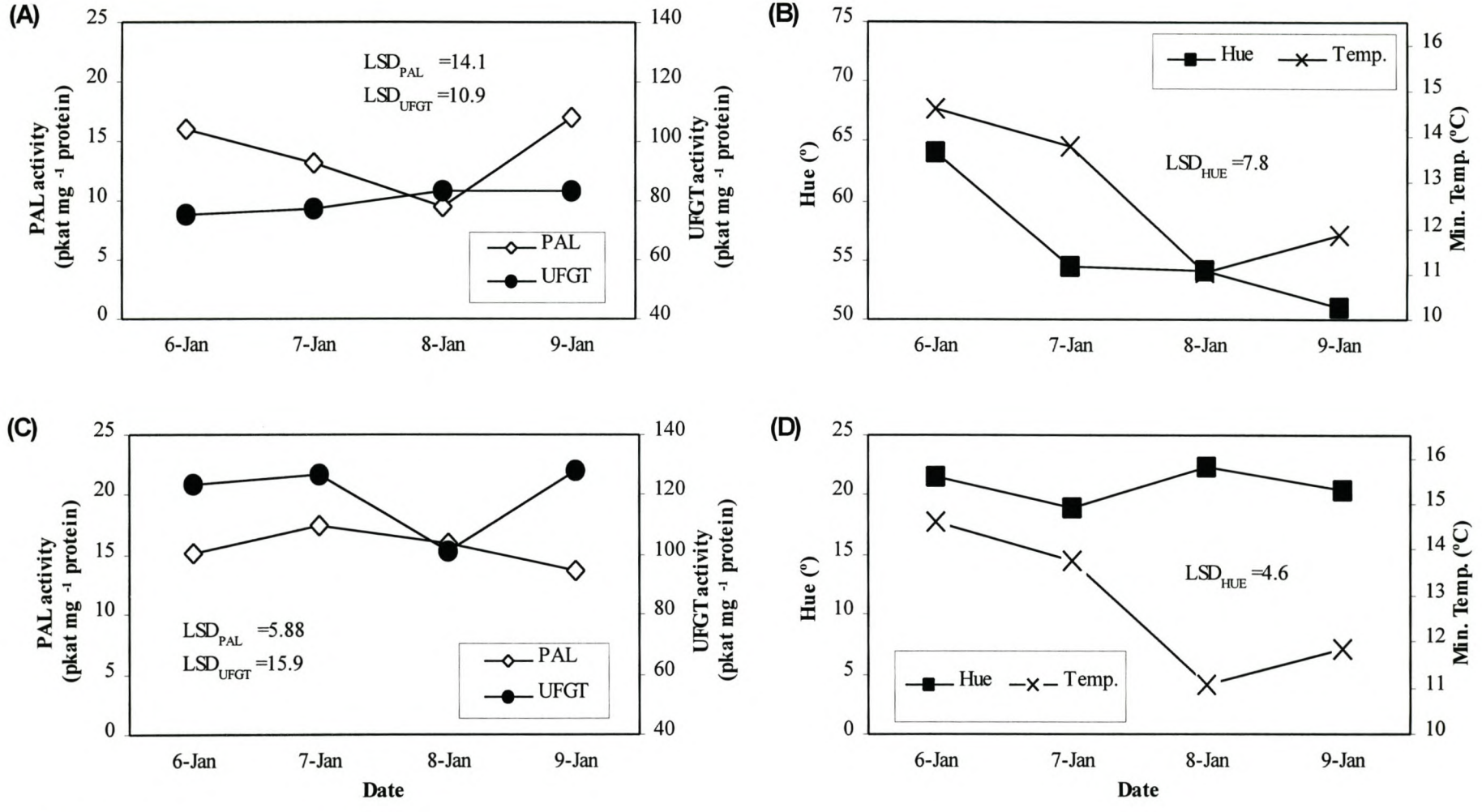


Fig. 8 Paper 1

PAPER 2:

ANTHOCYANIN DEGRADATION IN DETACHED POME FRUIT WITH REFERENCE TO PRE-HARVEST RED COLOUR LOSS AND PIGMENTATION PATTERNS OF BLUSHED AND FULLY RED PEAR CULTIVARS (*Pyrus communis* L.).

Abstract. **Exposed fruit of the blushed pear cultivar, 'Rosemarie' (*Pyrus communis* L.), displayed considerable daily fluctuations in colour in response to temperature while colour was more stable in other blushed and fully red cultivars. 'Rosemarie' pears increased in redness with the passing of cold fronts, but rapidly lost red colour during intermittent warmer periods. Detached pome fruit was used to study the effect of temperature and light on anthocyanin degradation and fruit colour and to assess the modifying effect of synthesis and anthocyanin concentration on colour loss. We hypothesised that pre-harvest red colour loss is due to net anthocyanin degradation at high temperatures and that susceptibility to colour loss is dependent on the ability of fruit to accumulate anthocyanin. The latter hypothesis was based on the exponential relationship that we found between anthocyanin concentration and hue at high pigment levels and the linear relationship at lower pigment levels in 'Forelle' pear peel. Anthocyanin degradation and red colour loss increased linearly between 10°C and 30° in detached fruit. Irradiation increased the rate of degradation and colour loss. Congruent with our hypothesis, fruit containing little anthocyanin (41 $\mu g\ g^{-1}$ fr wt) lost red colour nearly 13 times faster than fruit containing four times as much anthocyanin (163 $\mu g\ g^{-1}$ fr wt). Anthocyanin synthesis prevented colour loss at mild temperatures (20°C). In the absence of anthocyanin synthesis, pear fruit enclosed in light-impermeable bags rapidly lost anthocyanin. Colour loss proceeded at a much faster rate than could be attributed to dilution due to fruit growth. Red colour loss and net anthocyanin degradation in attached 'Rosemarie' pears corresponded with a warm period during fruit development. These results are discussed with reference to pear pigmentation patterns and pre-harvest red colour loss.**

The lucrative blushed pear cultivars 'Rosemarie', 'Flamingo' and 'Forelle' constitute a fifth of the South African pear industry in terms of hectarage (Deciduous Fruit Producers Trust, 2001). Unfortunately, downgrading due to insufficient red colour has limited the profitability of these pears (Huysamer, 1998). Poor fruit colour has been ascribed to the loss of red colour prior to harvest during periods of high temperature (Huysamer, 1998).

In a previous study in our laboratory, Marais et al. (2001a) found that exposure to light for 144 hours at 37°C reduced the anthocyanin content of 'Cripps' Pink' apples by more than half, but anthocyanin levels remained unchanged at the same temperature if fruit were shaded. Partial shading of 'Red Bartlett' pears for a month prior to harvest reduced the decrease in anthocyanins and red colour that occurred towards harvest in exposed fruit (Dussi et al., 1995). Light-mediated red colour loss was also reported in pomerac (*Syzygium malaccense*) fruit stored at 5°C (Sankat et al., 2000). Red colour loss is quite common in vegetative tissues and a causal relationship with high temperatures has been observed (Nozzolillo et al., 1990; Oren-Shamir & Levi-Nissim, 1997).

The ready degradation of anthocyanins in food products in response to heat and light has received much research attention (Francis, 1989). However, little is known about the contribution of environmental conditions to anthocyanin degradation in attached fruit (Lancaster, 1992). Few studies have been conducted on pear colour development and these have all focused on fully red pear cultivars that maintain considerable red colour throughout fruit development (Dayton, 1966; Dussi et al., 1997). Colour development in blushed cultivars, characterised by a red blush on an otherwise green background, has not been studied.

We studied anthocyanin degradation in detached fruit in relation to synthesis and pigment concentration and used the data to interpret the pigmentation patterns of blushed and red pears. We aimed to quantitatively confirm the occurrence of pre-harvest red colour loss in attached fruit and establish its relation with high temperatures. Daily changes in the hue of different pear cultivars were recorded *in situ* and correlated with temperature parameters during the course of fruit development. A number of laboratory experiments were conducted to establish the

relationship between hue and anthocyanin concentration, the effect of temperature on anthocyanin degradation and the effect of synthesis and anthocyanin concentration on the rate of colour loss. Apples were used in some of these experiments since both anthocyanin synthesis and degradation are readily induced under laboratory conditions (Marais et al., 2001a). A previous attempt to induce anthocyanin synthesis in detached pears has failed (Marais et al., 2001b). To determine whether red colour loss in attached fruit is not merely due to dilution as fruit grow, 'Rosemarie' and 'Forelle' pears were enclosed in light-impermeable bags and changes in anthocyanin concentration and red colour were measured in the absence of anthocyanin synthesis. To confirm the occurrence of anthocyanin degradation and red colour loss, changes in the hue and anthocyanin pigmentation of 'Rosemarie' pears were assessed during fruit development.

Materials and Methods

Plant material. Field trials were conducted and fruit for laboratory trials obtained from farms located in the Stellenbosch district (latitude: 33º58'S, longitude: 18º50'E) of the Western Cape region in South Africa. This region has a Mediterranean climate with cold fronts associated with cyclonic weather systems responsible for most of the precipitation in winter. Fully exposed fruit from the western side of north to south orientated rows were used. Pear cultivars studied included three blushed cultivars; 'Rosemarie', 'Flamingo' and 'Forelle' and two fully red cultivars; 'Bon Rouge' and 'Red d'Anjou'. 'Rosemarie' and 'Flamingo' are locally developed selections from the progeny of crosses made between 'Forelle' and 'Bon Rouge' (ARC Infruitec-Nietvoorbij and SAPO, 1998). 'Bon Rouge' and 'Red d'Anjou' are the respective bud sports of 'Bon Chretien' and 'D'Anjou' (ARC Infruitec-Nietvoorbij and SAPO, 1998). 'Kieffer', a hybrid between Asian and European pears, was included in one of the trials. 'Royal Gala' and 'Cripps' Pink' apples were used to test some of the hypotheses.

Colour (hue) measurement and pigment analysis. External colour was measured with a chromameter (NR-3000; Nippon Denshoku, Tokyo, Japan) and anthocyanins assessed by reverse-phase high performance liquid chromatography (HP 1100; Agilent Technologies, Palo Alto, CA) as described in Paper 1. Chlorophyll a and b

were quantified by measuring absorption at 470, 645 and 662 nm on a spectrophotometer (DU Series 64; Beckman, California) and making use of the extinction coefficients of Lichtenthaler (1987). Extraction of anthocyanin and chlorophyll was performed and samples prepared as described in Paper 1.

Pigmentation patterns in different pear cultivars. The hue of 30 'Rosemarie', 'Flamingo', 'Forelle' and 'Bon Rouge' pears was measured daily before 10:00 in the morning at the reddest position on the fruit during the 2001/2002 season in orchards in the Stellenbosch region. Trees were grafted on BP1 rootstock with the exception of 'Forelle', which was grafted on Quince A. Measurements were taken intermittently with the passing of cold fronts, from 19 November 2001 (29 November for 'Flamingo') until 12 January 2002. Commercial harvesting commenced during the following week with the exception of 'Forelle', which was only harvested towards the middle of February. Temperature data were obtained for the Nietvoorbij automatic weather station (within 4 km from all trial sites) and correlated with daily changes in hue.

Effect of pigment concentration on colour expression. To better understand the relationship between anthocyanin concentration and colour (hue), the hues of 'Forelle' peel disks varying in colour from dark red to completely green were plotted against their respective anthocyanin concentrations. The pears were harvested on 14 February 2001 from an orchard established during 1998 on the Welgevallen experimental farm in Stellenbosch. Homogeneously coloured peel disks were removed with a cork borer (2 cm diameter) and scraped so that only pigmented layers remained (1 mm in thickness). Colour (hue) was measured, making use of a hue neutral background, and used to divide peel disks into 18 hue groups each of 5° range, from 10-15° (dark red) to 100-105° (yellow-green). Each group contained at least 50 disks. Anthocyanin concentrations ($\mu g\ g^{-1}$ fresh weight) were determined and plotted against hue.

Anthocyanin degradation in detached fruit. To determine the effect of temperature and light on the rate of anthocyanin degradation and hue, 'Forelle' pears and 'Royal Gala' apples were subjected to temperatures of 10°C, 20°C and 30°C with or without light. The pears and apples were taken from cold storage at 4°C (1 and 30 March 2000, respectively). Circles were marked on the reddest side of fruit, halfway

between the calyx and stem ends and colour was measured within these circles. Marked sides of fruit of one group of 24 fruit were peeled to assess initial pigment levels. Half of the remaining fruit was placed in light-impermeable two-layered 'Fuji' wrapping bags (Kobayashi Bag Mfg., Nagano, Japan) and four 6 fruit replicates of covered and uncovered fruit were randomly placed in each of three growth cabinets at 10°C, 20°C or 30°C ±2°C, with the marked sides facing upwards. Exposed fruit were subjected to irradiation of 500 μmol m^{-2} s^{-1} PPFD measured with a quantum meter (LI-189; Li-Cor, Lincoln, Nebraska, USA) provided by a single 400 W HPS light (SON-T; Osram Mgbh, Munich, Germany) placed on top of the cabinets with an acrylic (Perspex) layer between the lights and fruit. After 72 h, fruit were removed from the growth cabinets, colour was measured and whole pears or the marked sides of apples peeled for pigment analysis. The initial hue of fruit placed in growth cabinets was used as covariant to compensate for differences in colour at the onset of the experiment.

Effect of pigment concentration on the rate of colour loss. To test our hypothesis that high pigment concentrations buffer fruit against colour loss, the rate of colour loss was assessed in apple and pear fruit of varying red colour. 'Royal Gala' apples were taken from cold storage at 4°C (6 April 2000) and divided, according to the colour of their reddest side, into poor, medium and well-coloured groups making use of the 'Royal Gala' colour chart (Set A.42; Deciduous Fruit Board, South Africa). Each group was subdivided into four replicates of 12 fruit, of which six fruit were peeled to determine the initial anthocyanin concentration. Colour of the remaining fruit was measured within circles marked halfway between the calyx and stem ends before randomising them within a growth cabinet set at 30°C. Fruit were placed so that marked sides faced upwards. Fruit were subjected to irradiation of 500 μmol m^{-2} s^{-1} PPFD supplied by one 400 W HPS light set up as described previously. After 96 hours, fruit were removed, colour (hue) was measured and marked sides were peeled to assess anthocyanin concentrations.

The rate of colour loss was also studied in five pear cultivars differing in hue. Six uniformly coloured 'Bon Rouge', 'Forelle', 'Flamingo', 'Rosemarie' and 'Kieffer' pears were harvested on 10 January 2002. Fruit were halved, circles were marked halfway between the calyx and stem ends on the reddest side and colour was measured

within these circles. Halved fruit were randomly placed within a container of water (0.5 cm deep) within a growth cabinet kept at 40°C ±2°C. Irradiation of 1200 μmol m^{-2} s^{-1} PPFD was provided by two 400 W HPS lights placed on top of the cabinet with an acrylic (Perspex) layer between the lights and fruit. Colour was measured after 5, 10, 20 and 30 hours.

Degradation and synthesis in detached fruit. To assess the effect of the interaction between anthocyanin synthesis and degradation on red colour, initially red or green 'Cripps' Pink' apples were subjected to temperatures of 10°C, 20°C and 30°C in light. Apples were used for this experiment because a previous attempt at inducing anthocyanin synthesis in detached pears has failed (Marais et al., 2001b). An equal number of green and red fruit was harvested on 12 April 2000, approximately one week prior to commercial harvest, from exposed and shaded positions in an orchard established during 1998 on M793 rootstock at the Welgevallen experimental farm in Stellenbosch. Fruit were stored at 4°C until 12 June 2000 when the experiment started. Red and green fruit were randomly divided into four groups each. These groups were further subdivided into five 4-fruit replicates. Circles were marked halfway between the calyx and stem ends on the reddest side of red fruit and the least red side of green fruit and colour was measured within these circles. The marked sides of one group of each of the red and green fruit were peeled to assess initial anthocyanin concentrations (μg g^{-1} fresh weight). Replicates of the remaining six groups were randomised within three growth cabinets set up as described above and kept at 10°C, 20°C or 30°C ±2°C, respectively. Each growth cabinet contained one red and one green group with marked sides of fruit facing upwards. Colour was measured every 24 hours. After 168 hours, fruit were removed from the growth cabinets, marked sides were peeled and the anthocyanin concentration determined.

Anthocyanin degradation in attached fruit. The contribution of anthocyanin degradation to red colour loss in attached fruit, in addition to dilution, was assessed in the absence of synthesis achieved by keeping 'Rosemarie' and 'Forelle' fruit enclosed in light-impermeable, two-layered 'Fuji' wrapping bags. 'Rosemarie' pears were enclosed weekly in these bags from five weeks prior to commercial harvest on 9 January 2001. 'Forelle' pears were enclosed at fortnightly intervals from ten weeks prior to harvest on 13 February 2001. Fruit stayed enclosed until harvest. At the onset

of the trial, 24 control fruit of each cultivar were tagged as a reference. Fruit colour was measured at enclosure and again at harvest, always at the reddest position on the fruit. After harvest, fruit were peeled and the concentration of anthocyanin, chlorophyll and total carotenoids assessed. Results were expressed as $\mu g\ g^{-1}$ fresh weight of peel. The trial was conducted at the Welgevallen experimental farm in Stellenbosch where both orchards had been established on Quince A rootstock during 1998.

To determine whether anthocyanin degradation occurs in attached 'Rosemarie' pears and to establish the relationship with temperature, changes in the anthocyanin content of fruit were determined and related to preceding climatic conditions. Pears from an orchard established during 1991 on BP1 rootstock at the Welgevallen experimental farm in Stellenbosch were marked and collected at more or less weekly intervals. Collection started 49 days after full bloom (19 November 1999) and continued until commercial harvest on 13 January 2000. Until 25 November, three samples of 20 fruit each were collected, whereafter the sample size was reduced to 15 fruit. Colour was measured halfway between the calyx and stem ends on the light-exposed sides of fruit. Whole fruit were peeled, the peel was weighed and was used to determine the anthocyanin concentration ($\mu g\ g^{-1}$ fresh weight) and anthocyanin content (μg) per fruit. Temperature data were obtained for the Nietvoorbij automatic weather station (±4 km from the trial site) and the number of hours above 28°C and below 14°C experienced during the week preceding each sampling date calculated and presented as indication of climatic conditions experienced during fruit development.

Statistical analysis. The data were analysed with the General Linear Models (GLM), Correlation (CORR) and Linear Regression (REG) procedures of SAS (SAS release 6.12P; SAS Institute, 1996, Cary, NC). The STATISTICA data analysis software system (Version 6; StatSoft Inc., Tulsa, OK) was used to fit non-linear curves to data presented in Fig. 6.

Results

Pigmentation patterns in different pear cultivars. 'Rosemarie' displayed the most variation in hue (2.7° per day) of the four pear cultivars studied during the 2001/2002 season (Fig. 1A). The daily variance in the hue of 'Forelle', 'Flamingo' and 'Bon Rouge' was 1.4°, 1.1° and 0.7°, respectively, and these cultivars maintained a lower hue than 'Rosemarie' throughout fruit development (Fig. 1A). Daily changes in the hue of 'Rosemarie' strongly correlated with average daily temperatures (r^2 = 0.74) (Table 1). This is also evident from Fig. 1B where 'Rosemarie' hue can be seen to decrease in association with cold fronts and to rapidly increase again during intermittent, warmer periods. Correlations between daily changes in the hue of 'Bon Rouge', 'Forelle' and 'Flamingo' pears and temperature parameters were generally weak (Table 1). The rate at which the hue of 'Rosemarie' pears decreased in response to low temperatures decreased over fruit development averaging 6.0° per day between 19 and 22 November, 3.0° per day between 29 November and 4 December, 2.1° per day between 15 and 20 December, 2.0° per day between 4 and 8 January and 1.1° per day between 10 and 13 January (Fig. 1A, B). The rate at which 'Rosemarie' hue increased during warm periods remained constant over fruit development averaging 3.3° per day between 22 and 29 November and 3.2° per day between 26 and 29 December. The hue of 'Rosemarie' fruit on two occasions increased by more than five degrees within a single day (Fig. 1B). The hue of 'Flamingo' and 'Bon Rouge' fruit gradually increased over the experimental period, at a rate of 0.5 and 0.3° per day, respectively (Fig. 1A).

Effect of pigment concentration on colour expression. The relationship between the anthocyanin concentration and the hue of 'Forelle' peel disks was exponential at high anthocyanin concentrations (>97 $\mu g\ g^{-1}$) and low hue values (<40°) (Fig. 2). The relationship became linear at lower pigment concentrations and higher hue-values. The chlorophyll concentration of 'Forelle' peel disks was constant over the hue range studied (data not presented).

Anthocyanin degradation in detached fruit. Anthocyanin degradation increased linearly between 10 to 30°C in both 'Forelle' pears and 'Royal Gala' apples (Fig. 3, 4). Light increased the rate of anthocyanin degradation in 'Royal Gala', but not in

'Forelle' (Fig. 3, 4). Red colour loss, as evidenced by an increase in hue, increased linearly with increasing temperature in 'Forelle' and in irradiated, but not in shaded 'Royal Gala' (Fig. 3B). Though considerable anthocyanin degradation took place over the experimental period, changes in hue were small. The anthocyanin concentration of 'Forelle' and 'Royal Gala' fruit kept at 30°C decreased by 62% and 40%, respectively, compared to the control group of fruit peeled at the onset of the experiment. However, hue increased by only 6.4° and 4.2°, respectively.

Effect of anthocyanin concentration on the rate of colour loss. Hue increased nearly 13 times faster in 'Royal Gala' fruit of poor red colour and low anthocyanin concentration than in well-coloured fruit with a high anthocyanin concentration when irradiated for 96 hours at 30°C (Table 2; Fig. 5). However, the absolute reduction in the anthocyanin concentration was much greater in the well-coloured fruit (64 compared to 25 $\mu g\ g^{-1}$) (Table 2).

The rate of red colour loss in different pear cultivars irradiated for 30 h at 40°C was related to their initial hue with the dark red 'Bon Rouge' affected less than the comparably less red cultivars, 'Kieffer', 'Rosemarie' and 'Forelle' (Fig. 6). The rate of hue increase in 'Flamingo' did not differ from that of 'Bon Rouge' or the other cultivars.

Degradation and synthesis in detached fruit. Green 'Cripps' Pink' fruit contained little anthocyanin (1.5 $\mu g\ g^{-1}$ fr wt) at the onset of the experiment while red fruit were rich in anthocyanin (58 $\mu g\ g^{-1}$) (Fig. 7B). The hue of green fruit kept at 10 and 30°C slowly decreased from 114 to 95° over the 168 h experimental period, but the anthocyanin concentration of fruit did not increase significantly (Fig. 7A, B). However, the hue of green fruit kept at 20°C decreased to 58° as a result of anthocyanin synthesis. As found with green fruit, the anthocyanin concentration of red fruit kept at 20°C also increased, but the increase was smaller (17 $\mu g\ g^{-1}$ compared to 43 $\mu g\ g^{-1}$) and hue remained unchanged at approximately 45° (Fig. 7A). The hue and anthocyanin concentration of red fruit kept at 10°C did not change significantly. The hue of red fruit kept at 30°C increased by 20.4° and this was accompanied by a 74% (43 $\mu g\ g^{-1}$) reduction in the anthocyanin concentration of these fruit (Fig. 7A, B).

Anthocyanin degradation in attached fruit. The anthocyanin concentration and red colour of 'Rosemarie' and 'Forelle' fruit enclosed in light-impermeable 'Fuji' wrapping bags rapidly decreased as the duration of enclosure increased (Fig. 8A, B). The chlorophyll concentration also decreased, but at a much slower rate than the anthocyanin concentration. The total carotenoid concentration decreased in 'Rosemarie' (Fig. 8A), but not in 'Forelle' (Fig. 8B). While the peel mass of 'Rosemarie' fruit more or less doubled during the study (data not presented), the anthocyanin concentration decreased nearly 20 times. It took about twice as long for red colour to disappear completely from 'Forelle' compared to 'Rosemarie' peel (Fig. 8A, B).

The anthocyanin concentration and red colour of 'Rosemarie' pears, which were generally poor throughout fruit development, transiently increased between 19 and 26 November when the passing of a cold front brought about a respite from the exceptionally hot weather experienced throughout fruit development during the 1999/2000 season (Fig. 9A, B). Fruit accumulated approximately 30 μg anthocyanin resulting in an increase in anthocyanin concentration from about four to just below 11 μg g^{-1} and a decrease in hue from 72.5° to just below 50° (Fig. 9A). After the cold front had passed, the anthocyanin content of fruit rapidly decreased so that after a fortnight fruit contained 54% less anthocyanin. During this same period, hue increased at an average rate of 1.6° per day while the anthocyanin concentration decreased at a rate of 0.45 μg g^{-1} per day. A dilution rate of 0.24 μg g^{-1} anthocyanin per day was calculated from the increase in the fresh mass of fruit peel over this period. Hereafter, the anthocyanin content and the hue of fruit remained constant until the week prior to harvest while temperatures remained high over this period. The anthocyanin content of fruit decreased and the hue increased in the week before harvest though temperatures were mild and fewer hours above 28°C were accumulated.

Discussion

Anthocyanin degradation. In a previous study in our laboratory, Marais et al. (2001a) found that irradiation for 144 hours at 37°C reduced the anthocyanin content of

detached 'Cripps' Pink' apples by more than half, resulting in red colour loss. Results of the current study on 'Forelle' pears and 'Royal Gala' apples confirmed that high temperatures (30°C) accelerate the degradation of anthocyanin and the fading of red colour in detached fruit (Fig. 3, 4). In contrast to the results of Marais et al. (2001a), light was not a prerequisite for anthocyanin degradation (Fig. 3, 4), though it increased the rate of anthocyanin degradation and colour loss in 'Royal Gala' apples (Fig. 4). Though little is known about the mechanism of anthocyanin degradation in fruit (Lancaster, 1992), colour changes in food products have received much research attention.

Due to their reactive structure, anthocyanins are readily degraded in food products in response to heat and light (Francis, 1989). Degradation may be non-enzymatic, but may also be mediated by common enzyme groups, i.e. the glycosidases, polyphenoloxidases and peroxidases, (Francis, 1989; Macheix et al., 1990; Piffaut et al., 1994). Piffaut et al. (1994), found that anthocyanin degradation mediated by ß-glycosidases or induced by high temperature proceeded via the same pathway. Radiant heating might contribute significantly to anthocyanin degradation by increasing fruit peel temperature by up to 15°C at high ambient temperatures (Smart and Sinclair, 1976). Evaporative cooling by pulsed overhead irrigation, which is known to act by negating radiant heating of the fruit surface (Unrath, 1972), reduced the fading of 'Rosemarie' pear colour during warm days (Paper 3). However, light-mediated red colour loss in pomerac fruit (*Syzygium malaccense*) was observed at 5°C (Sankat et al., 2000), suggesting that light might have a direct effect on degradation. Since we took care to prevent radiant heating, the light-mediated degradation of anthocyanin in 'Royal Gala' apples is probably a direct effect of light (Fig. 4). Attoe and Von Elbe (1981) suggested that light increases the reactivity of the anthocyanin molecule and so contributes to its degradation.

The involvement of light in degradation seems to be at odds with its requirement for anthocyanin synthesis. However, light-mediated degradation of anthocyanin at mild temperatures is probably insignificant compared to the stimulating effect of light on anthocyanin synthesis.

Degradation and synthesis. Our results indicated that fruit colour is determined by the interaction between anthocyanin synthesis and degradation at different temperatures. The optimum temperature for anthocyanin synthesis in mature fruit of different apple cultivars vary between 20 and 25°C, with synthesis decreasing at lower and higher temperatures Curry (1997). On the other hand, anthocyanin degradation increased linearly between 10 and 30°C in both 'Royal Gala' apples and 'Forelle' pears (Fig. 3, 4). Hence, the anthocyanin concentration of mature, red 'Cripps' Pink' apples was stable at 10°C where both synthesis and degradation were low, increased at 20°C where synthesis exceeded degradation and decreased at 30°C where degradation was greater than synthesis (Fig. 7). Degradation and colour loss in detached 'Forelle' and 'Royal Gala' also occurred at 20°C, albeit at a slower rate (Fig. 3, 4). Net anthocyanin degradation at 20°C in these fruit is probably explained by the inability of mature pears to accumulate anthocyanin from the tree (Marais et al., 2001b) and the reduction of synthesis in post-climacteric apples (Curry, 1997).

It follows that pigmentation can be expected to fluctuate in response to temperature in attached fruit, increasing when mild temperature favours synthesis and decreasing when the rate of anthocyanin degradation exceeds synthesis at high temperatures. Of course the endogenous regulation of anthocyanin synthesis during fruit development would determine the general pigmentation pattern of fruit. Factors that reduce the rate of anthocyanin synthesis, such as substrate limitation, would decrease the temperature at which net anthocyanin degradation and colour loss occur. Expression of anthocyanin pigmentation in vegetative tissues (Christie et al., 1994) as well as in many crops e.g. apples (Curry, 1997) generally requires induction at low temperatures. High temperatures have been found to inhibit the induction of anthocyanin synthesis (Reay, 1999). The optimal day temperature for anthocyanin synthesis does not change in the absence of induction, but the rate of anthocyanin synthesis is reduced (Curry, 1997). Consequently, high temperatures could contribute to red colour loss by preventing or inhibiting the induction of anthocyanin synthesis and by reducing the rate of anthocyanin synthesis when induced.

High anthocyanin concentrations reduced the ability of red compared to green 'Cripps' Pink' fruit to further accumulate anthocyanin (Fig. 7). Lancaster et al. (2000) found a similar reduction in the ability of apple cultivars or fruit with high anthocyanin

concentrations to accumulate anthocyanin at 10°C and 20°C and attributed this effect to either previous light exposure or the genetic background of fruit. Because of the reduction in the rate of anthocyanin synthesis in red fruit, net anthocyanin degradation should occur at a lower temperature compared to less red fruit of the same cultivar, but this was not investigated. The manner in which high anthocyanin concentrations limits further accumulation, whether it is by reducing light levels, desensitisation of the signalling pathway or feedback inhibition, remains to be determined. Anthocyanin levels are tightly regulated to modulate light absorption in accordance with environmental and developmental requirements (Pietrini & Massacci, 1998).

Effect of pigment concentration on colour expression. The extent of colour change in response to anthocyanin synthesis or degradation was found to depend on the anthocyanin concentration of peel. If comparable rates of anthocyanin degradation are assumed, fruit containing little anthocyanin should lose red colour ahead of fruit containing more anthocyanin. However, due to the exponential relationship between hue and anthocyanin concentration at high concentrations (Fig. 2), much greater changes in pigment concentration are required to induce comparable changes in the hue of red compared to less red fruit. Put differently, high anthocyanin concentrations buffer red skin colour against fluctuations in anthocyanin concentration while low pigment concentrations allow greater fluctuation in colour. This principle was illustrated in 'Royal Gala' apples where red colour loss at 30°C occurred much faster in poorly coloured apples containing little anthocyanin than in well-coloured fruit containing large amounts of anthocyanin (Fig. 5) even though the well-coloured fruit lost more anthocyanin (Table 1). Red colour also decreased at a slower rate at 40°C in the dark red pear cultivar 'Bon Rouge' than in other less red cultivars (Fig. 6). Similarly, the hue of initially red 'Cripps' Pink' apples did not change while, in contrast, the hue of initially green 'Cripps' Pink' apples decreased by 56° during accumulation of anthocyanin at 20°C (Fig. 7). The effect of pigment concentration on the rate of colour loss also explains the relatively small increases in the hue of 'Royal Gala' and 'Forelle' fruit in response to the considerable degradation of anthocyanin (Fig. 3, 4).

Degradation in attached fruit. Rapid degradation of anthocyanin in attached 'Rosemarie' and 'Forelle' pears was revealed by enclosing fruit in light-impermeable bags, which prevented anthocyanin synthesis (Fig. 8). Fully exposed 'Rosemarie' pears also underwent red colour loss during a warm fortnight in early December, about a month prior to harvest during the 1999/2000 season (Fig. 9B). Though dilution contributed to the reduction in the anthocyanin concentration of the pears, the amount of anthocyanin per fruit decreased by more than 50% indicating the contribution of net degradation of anthocyanin to colour loss (Fig. 9A). Red colour loss and net anthocyanin degradation also occurred in the fortnight before the harvest of 'Rosemarie' pears even though temperatures were milder than earlier during fruit development (Fig. 9A, B). The reason for this further loss of colour is uncertain. Colour loss appeared to be distinct from poor colour development due to insufficient light exposure since it occurred in fruit exposed to full sunlight. The involvement of light in colour loss was not investigated. However, partial shading of 'Red Bartlett' pears reduced the fading of red colour that occurred during the month prior to harvest (Dussi et al., 1995).

The association of anthocyanin degradation and colour loss in 'Rosemarie' with high temperatures (Fig. 9B) is consistent with grower reports (Huysamer, 1998) and with our observations of anthocyanin degradation and colour loss in detached fruit (Fig. 3, 4 and 7). The fading of red colour in immature 'Jonathan' apples was also associated with high temperatures, but since total anthocyanin per fruit was not presented, it is impossible to determine whether colour loss was due to dilution or degradation (Faragher, 1983). A causal relationship with high temperatures has been observed in vegetative tissues where red colour loss is quite common (Dussi et al., 1995; Nozzolillo et al., 1990; Oren-Shamir & Levi-Nissim, 1997). However, in at least some plants, anthocyanin degradation seems to have a definite developmental component with little involvement of temperature and light. In the resurrection plant *Craterostigma wilmsii*, for example, anthocyanins decreased to levels prior to dehydration within 24 hours after re-hydration (Sherwin & Farrant, 1998). Anthocyanins also disappeared within a day during the normal development of *Arabidopsis thaliana* seedlings (Kubasek et al., 1992). Since increases in the hue of 'Rosemarie' pears in response to high temperatures were comparable at different

stages of fruit development (Fig. 1B), anthocyanin degradation does not seem to be under developmental control.

Pigmentation patterns in different pear cultivars. 'Rosemarie' red colour fluctuated considerably during fruit development in response to temperature while the colour of 'Bon Rouge', 'Flamingo' and 'Forelle' was more stable and less responsive to temperature (Table 1; Fig. 1). These differences can be interpreted according to the foregoing discussion of anthocyanin degradation in relation to synthesis and pigment concentration.

Anthocyanin synthesis in 'Rosemarie' requires low temperatures and fruit gain red colour with the passing of cold fronts (Table 1; Fig. 1B; Paper 1). However, during intermittent warmer periods, red colour fades due to reduced anthocyanin synthesis, increased anthocyanin degradation and, to a lesser extent, dilution. Evidently, even a single hot day could bring about considerable red colour loss (Fig. 1B). Rosemarie' possesses the lowest capacity for anthocyanin accumulation of all the red and blushed pear cultivars grown in South Africa (Paper 1), making it more susceptible to large fluctuations in colour and red colour loss.

The stability of red colour in 'Bon Rouge', 'Flamingo' and 'Forelle' could be due to continuation of anthocyanin synthesis regardless of temperature. In support, analysis of enzyme activity indicated that anthocyanin synthesis in 'Bon Rouge' did not require low temperatures (Paper 1). However, in light of the high pigment concentrations accumulated in these cultivars (Paper 1), even considerable anthocyanin synthesis or degradation might have little effect on red colour. The faster rate of colour fading towards harvest in 'Flamingo' compared to 'Bon Rouge' (Fig. 1A) is probably due to the lower capacity for anthocyanin synthesis in 'Flamingo' (Paper 1).

Conclusion. The occurrence of pre-harvest red colour loss in 'Rosemarie' pears was confirmed and found to be due to net anthocyanin degradation in response to high temperatures. The colour of 'Rosemarie' pears fluctuated considerably due to the low capacity of this cultivar to accumulate anthocyanin. Fruit increased in redness due to anthocyanin synthesis at low temperatures while red colour faded due to net degradation at high temperatures. Blushed and red pear cultivars that accumulate

more anthocyanin with lesser dependence on climatic conditions were less susceptible to fluctuation in colour.

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Table 1. Correlation of daily changes in hue of 'Rosemarie', 'Flamingo', 'Forelle' and 'Bon Rouge' pears as repetitively measured at the reddest position on the same fruit between 19 November 2001 and 12 January 2002 with daily maximum, minimum and average temperatures. Daily changes in hue were determined by subtracting hue values of the previous day from hue values of the current day. A decrease in hue indicates an increase in redness from one day to the next while an increase in hue indicates a decrease in redness.

Cultivar	Correlation coefficient (r)[z]		
	Daily Max. Temp. (ºC)	Daily Min. Temp. (ºC)	Av. Daily Temp. (ºC)
Rosemarie	0.65 ***	0.54 ***	0.74 ***
Flamingo	0.48 *	0.57 **	0.55 **
Forelle	0.30	0.37 *	0.42 *
Bon Rouge	0.13	0.45 **	0.19

[z] Pearson Correlation Coefficients / Prob > |R| under Ho: Rho=0 / N where N = 36, 35, 34 and 26 for 'Rosemarie', 'Forelle', 'Bon Rouge' and 'Flamingo', respectively.

*, ** and *** denotes correlation coefficient significant at P = 0.05, 0.01 and 0.001, respectively.

Table 2. Change in the hue and anthocyanin concentration in peel of 'Royal Gala' apples, subdivided according to initial red colour into good, medium and poorly coloured groups, irradiated (500 μmol m^{-2} s^{-1}) for 96 hours at 30ºC. Means in columns were separated by LSD (5%).

Fruit colour	Initial hue (º)	Final hue (º)	Increase in hue (º)	Initial anthocyanin conc. (μg g^{-1} fr wt)	Final anthocyanin conc. (μg g^{-1} fr wt)	Change in anthocyanin conc. (μg g^{-1} fr wt)
Good	20.7 a	21.9 a	1.3 c	163.2 a	99.6 a	63.6 a
Medium	31.8 b	44.0 b	12.2 b	76.3 b	36.5 b	39.8 b
Poor	55.9 c	75.1 c	19.2 a	41.2 c	16.0 c	25.3 b
Pr > F	0.0001	0.0001	0.0001	0.0001	0.0001	0.0035

Fig. 1. Changes in the hue of 'Rosemarie', 'Flamingo', 'Forelle' and 'Bon Rouge' pears measured daily from 19 November 2001 until 13 January 2002 (A). Daily changes in the hue of 'Rosemarie' and average daily temperatures are presented in (B). Hue angles reported fluctuate between 0° (red-purple) and 90° (yellow).

Fig. 2. Relationship between hue, anthocyanin and chlorophyll concentration in 'Forelle' peel disks grouped into 18 hue groups each of 5° ranging from 10-15° (red) to 100-105° (yellow-green).

Fig. 3. Changes in the hue and anthocyanin concentration of 'Forelle' pear peel exposed for 72 h to moderate HPS light (500 μmol m^{-2} s^{-1}) at 10, 20 or 30°C. Since irradiation had no effect on anthocyanin concentrations, only the temperature effect is presented. Increasing hue angles denote a reduction in redness. Means, separated by LSD (5%), are adjusted for hue at 0 h.

Fig. 4. Changes in the hue (A) and anthocyanin concentration (B) of 'Royal Gala' apple peel exposed for 72 h to moderate HPS light (500 μmol m^{-2} s^{-1}) at 10, 20 or 30°C. Increasing hue angles denote a reduction in redness. Means, separated by LSD (5%), are adjusted for hue at 0 h.

Fig. 5. Changes in the hue of good, medium and poorly coloured groups of 'Royal Gala' apples subjected for 96 hours to moderate HPS light (500 μmol m^{-2} s^{-1}) at 30°C. Increasing hue angles denote a reduction in redness. Means were separated by LSD (5%).

Fig. 6. Changes in the hue of different red and blushed pear cultivars subjected for 30 hours to strong HPS light (1200 μmol m^{-2} s^{-1}) at 40°C. Increasing hue angles denote a reduction in redness.

Fig. 7. Changes in the hue and anthocyanin concentration of initially red or green 'Cripps' Pink' apple peel exposed for 168 h to moderate HPS light (500 μmol m^{-2} s^{-1}) at 10, 20 or 30°C. Increasing hue angles denote a reduction in redness. Means were separated by LSD (5%).

Fig. 8. Changes in the hue and the anthocyanin, chlorophyll and total carotenoid concentration of 'Rosemarie' (A) and 'Forelle' (B) pears enclosed in light impermeable bags from five or ten weeks prior to harvest, respectively. Increasing hue angles denote a reduction in redness.

Fig. 9. Changes in the anthocyanin concentration and content of 'Rosemarie' pears during 1999/2000 from 49 days after full bloom (19 Nov. 1999) until commercial harvest (13 Jan. 2000) are presented in (A). Changes in hue and the number of hours below 10°C or above 30°C experienced over the six days prior to sampling of fruit are presented in (B). Hue angles reported fluctuate between 0° (red-purple) and 90° (yellow).

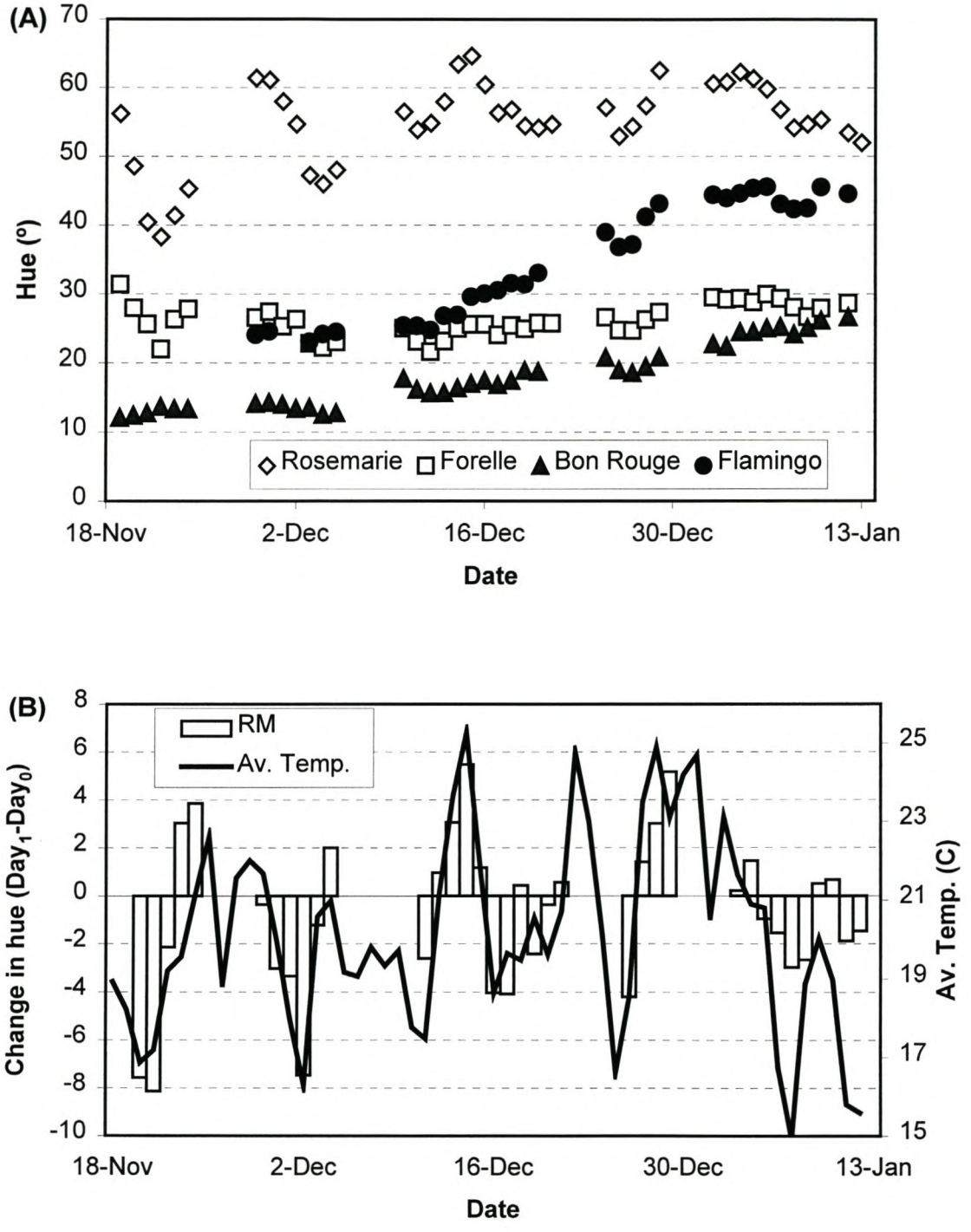

(A)
Hue (°)
70
60
50
40
30
20
10
0
Rosemarie
Forelle
Bon Rouge
Flamingo
18-Nov
2-Dec
16-Dec
30-Dec
13-Jan
Date
(B)
Change in hue (Day1-Day0)
8
6
4
2
0
-2
-4
-6
-8
-10
RM
Av. Temp.
25
23
21
19
17
15
Av. Temp. (C)
18-Nov
2-Dec
16-Dec
30-Dec
13-Jan
Date

Fig. 1 Paper 2

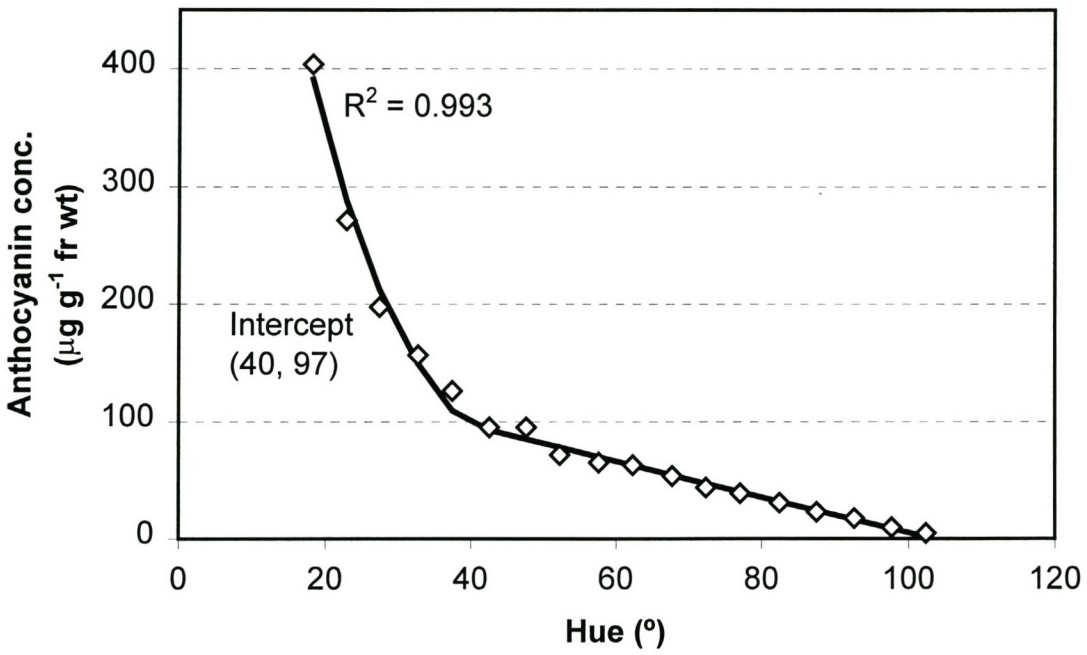
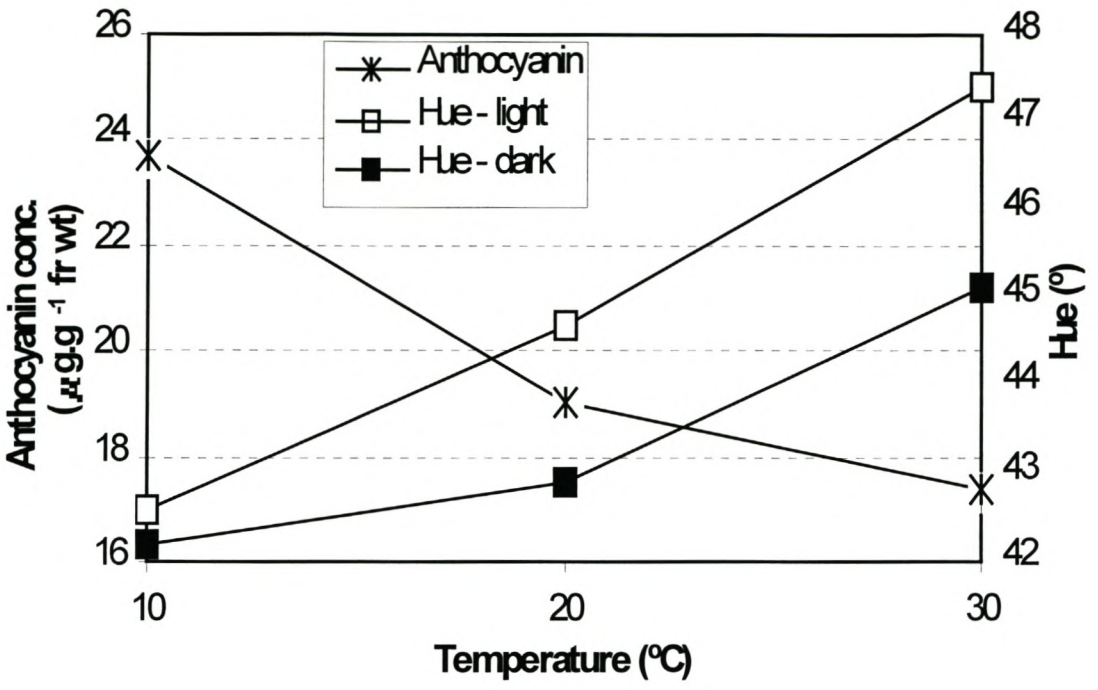
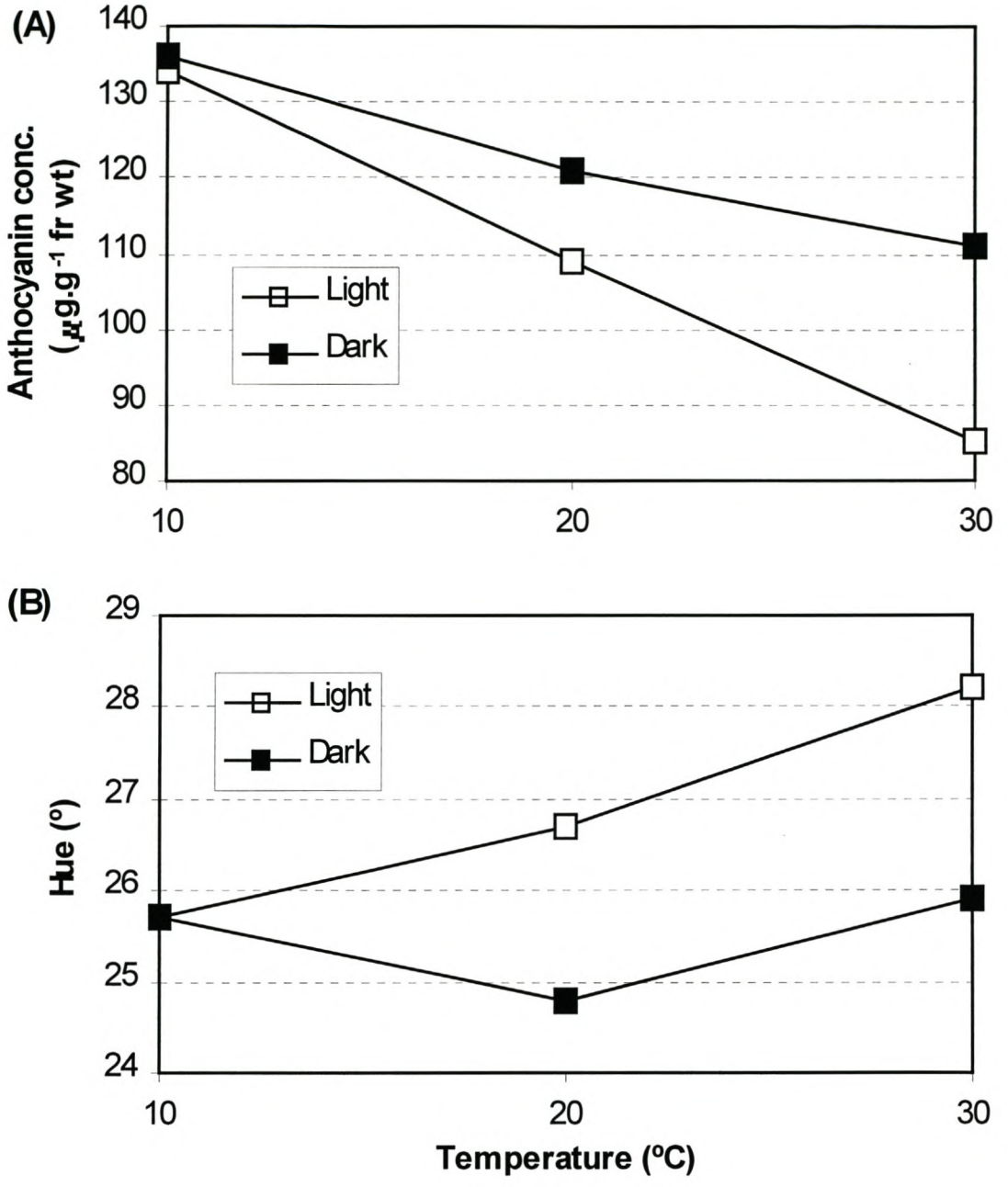


Fig. 2. Paper 2



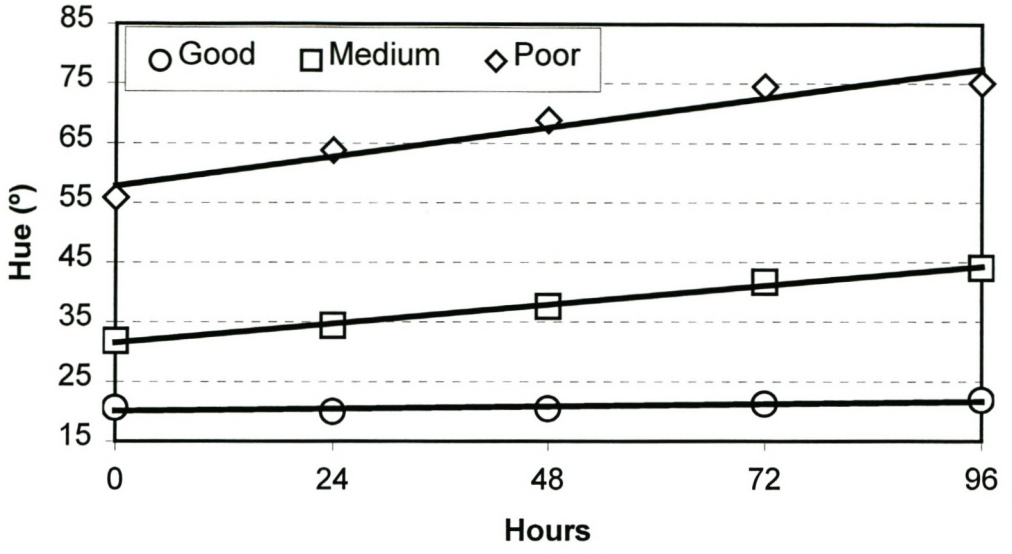
Contrasts	Hue (°)	Anth. conc. (µg g ^{1} fr wt)
Covariant (Initial hue)	0.0026	<0.0001
Light	0.0775	0.1380
Temperature (Temp) Linear	0.0010	0.0024
Temp Quadratic	0.5252	0.3377
Temp Linear * Light	0.3284	0.7011
Temp Quadratic * Light	0.7784	0.2061

Fig. 3. Paper 2



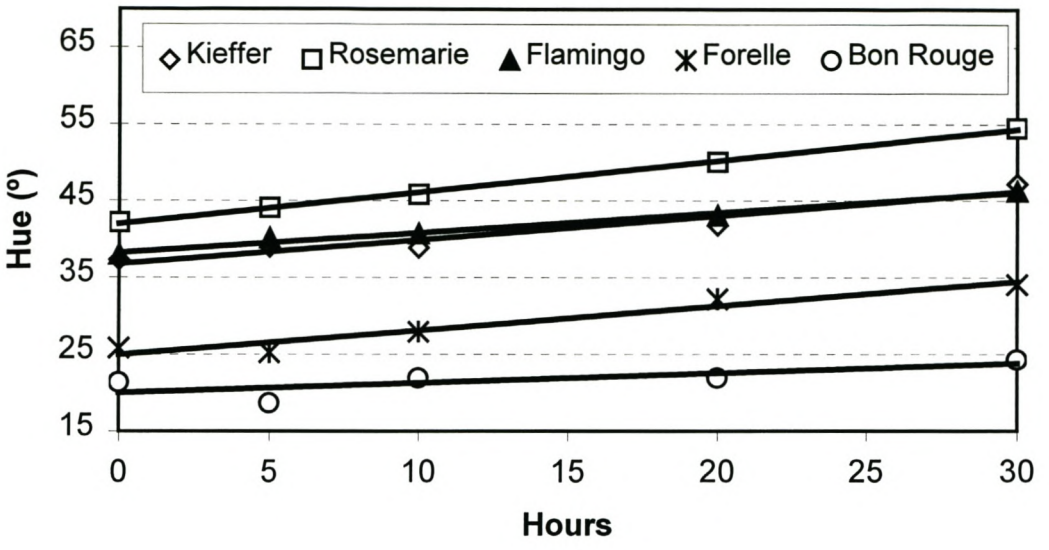
Contrasts	Hue (°)	Anth. conc. (μg g^{1} fr wt)
Covariant (Initial hue)	0.0078	0.0008
Light	0.0008	0.0288
Temperature (Temp) Linear	0.0063	0.0001
Temp Quadratic	0.1039	0.8559
Temp Linear * Light	0.0136	0.1028
Temp Quadratic * Light	0.2928	0.8260

Fig. 4. Paper 2



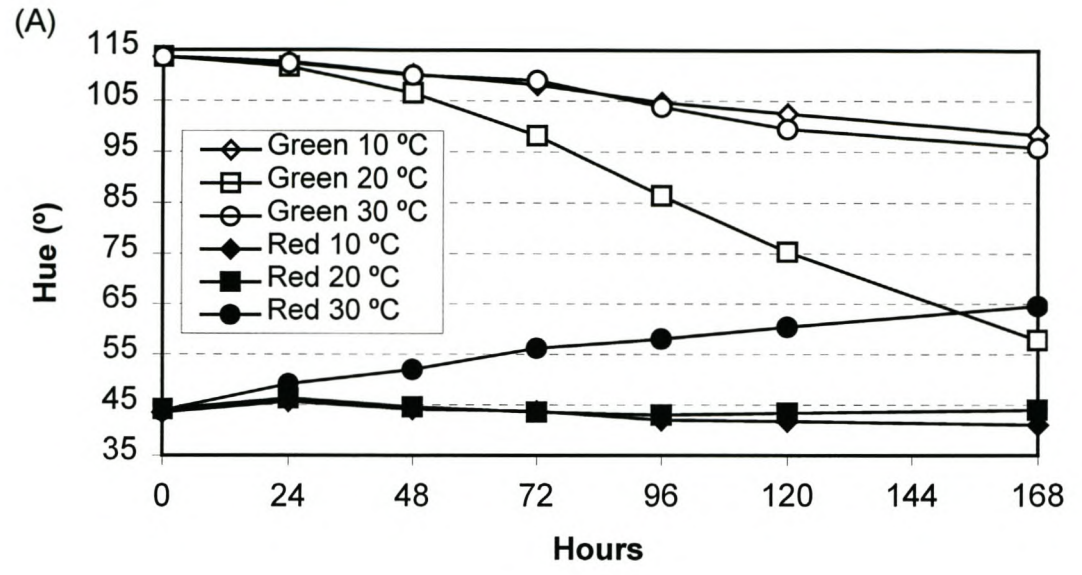
Colour Group	Gradient (° h^{-1})	Y-Intercept (°)
Good	0.016 c	20.10 c
Medium	0.132 b	31.60 b
Poor	0.205 a	57.80 a
Pr > F	0.0001	0.0001

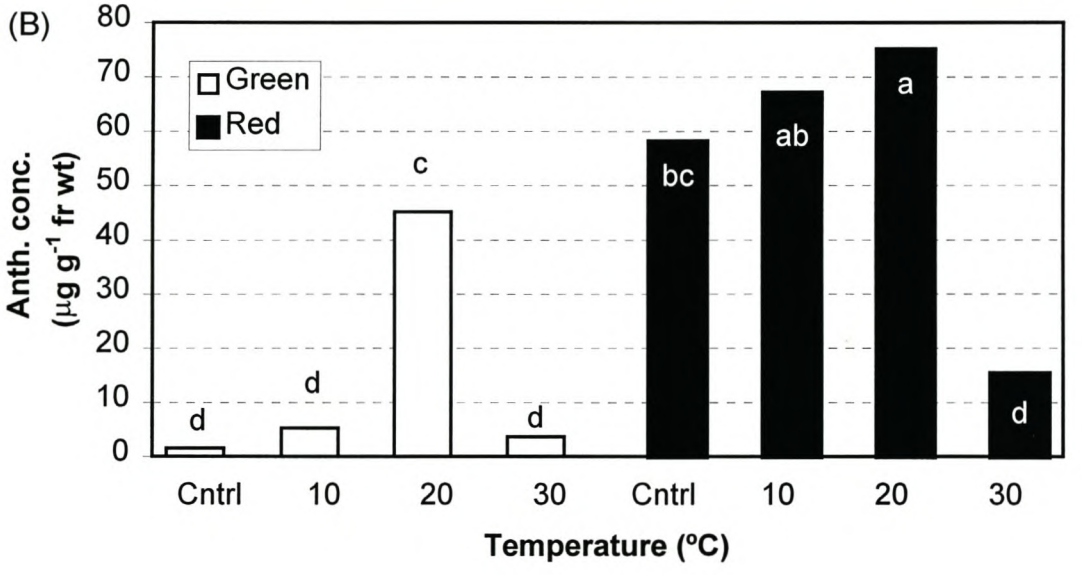
Fig. 5. Paper 2



Cultivar	Gradient (° h^{-1})	Y-Intercept
'Bon Rouge'	0.13 b	20.0 d
'Forelle'	0.31 a	25.9 c
'Flamingo'	0.26 ab	38.2 ab
'Kieffer'	0.34 a	36.2 b
'Rosemarie'	0.41 a	42.0 a
LSD	*0.134*	*4.68*
Pr > F	0.0245	0.0001

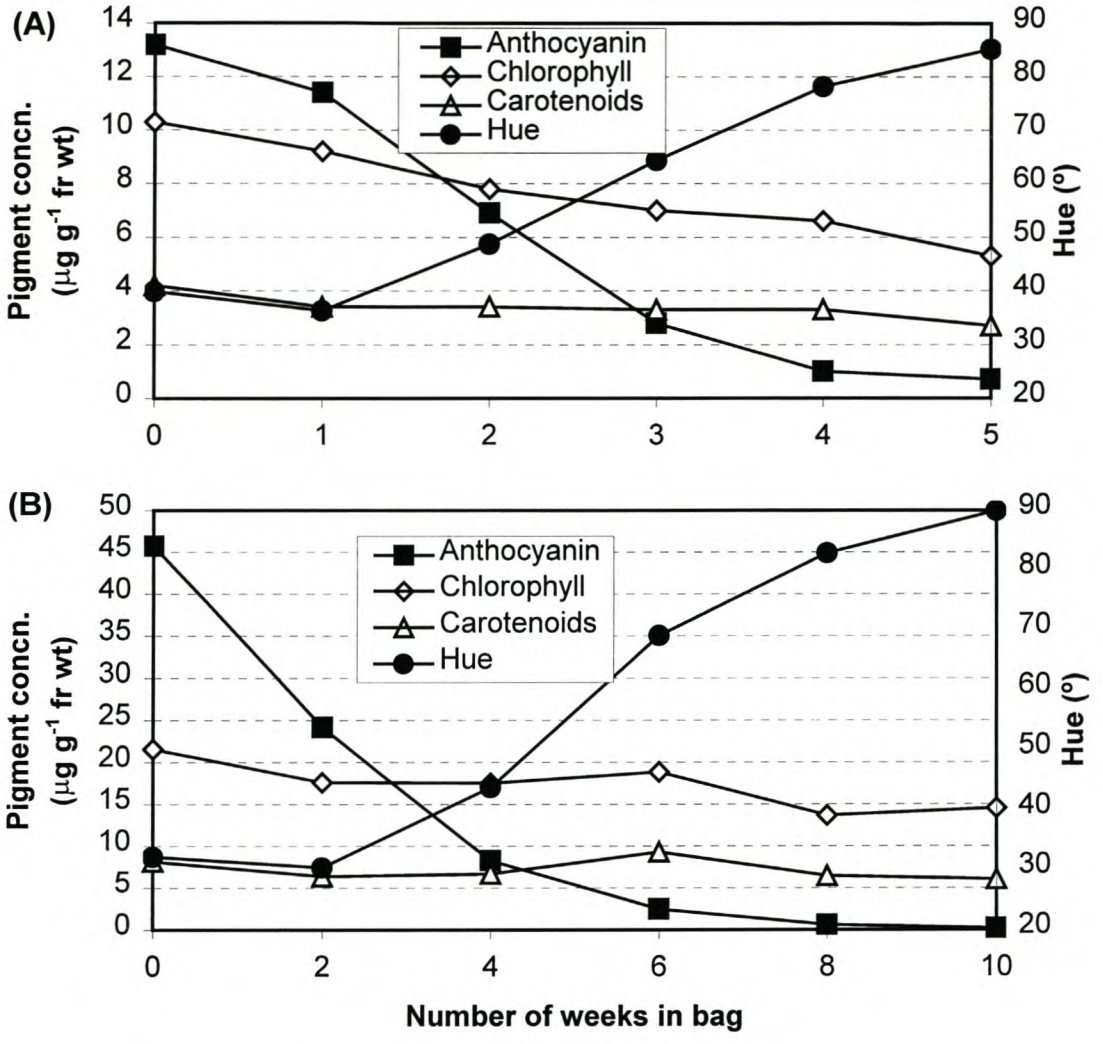
Fig. 6. Paper 2





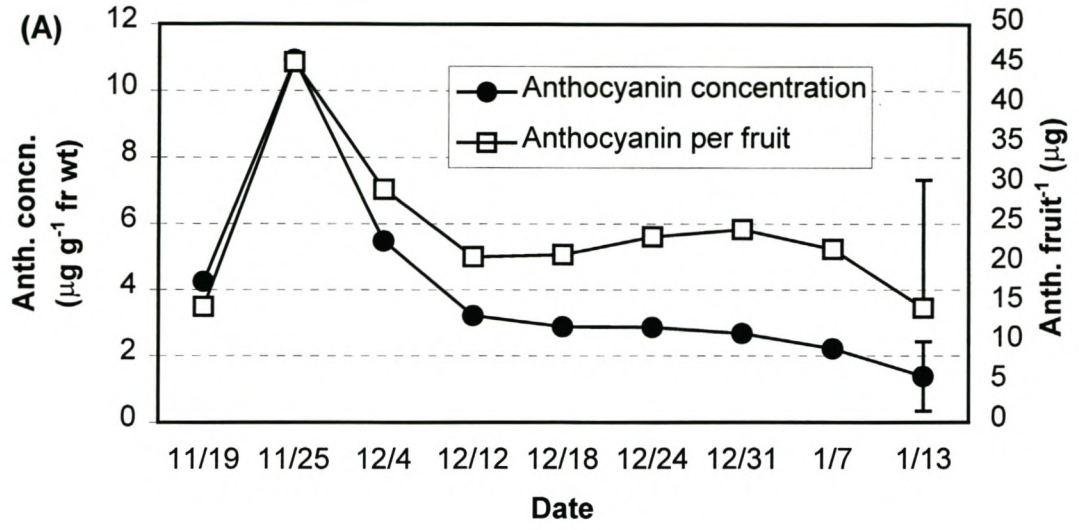
Contrasts	Hue (°)	Anth. conc. (µg g^{-1} fr wt)
Colour	0.0001	0.0001
Temperature linear (TL)	0.0001	0.0001
Temperature quadratic (TQ)	0.0001	0.0001
Colour * TL	0.0001	0.0113
Colour * TQ	0.0521	0.0009

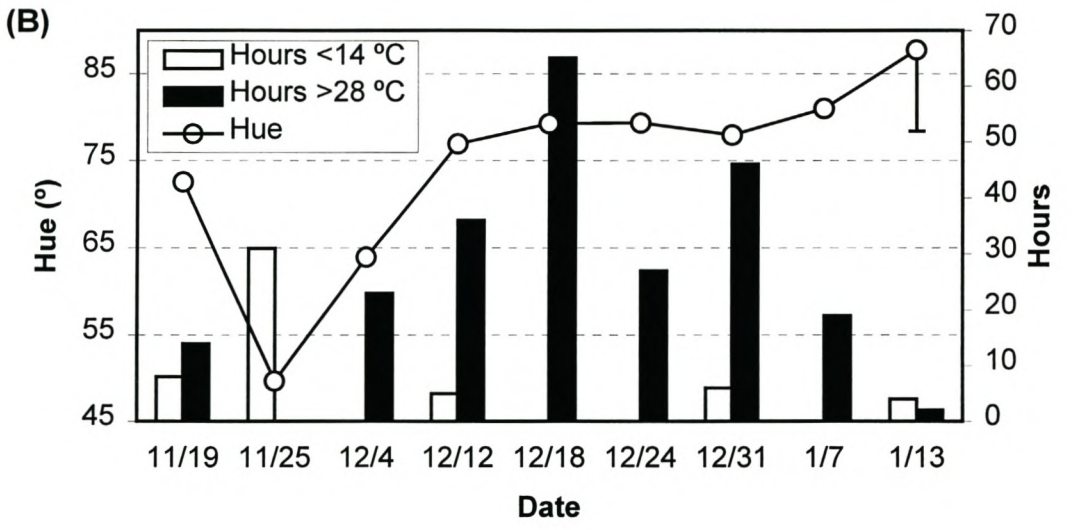
Fig. 7 Paper 2



Contrasts	Hue (°)	Anthocyanin	Chlorophyll	Carotenoids
			($\mu g\ g^{-1}$ fr wt)	
'Rosemarie'				
Linear	0.0001	0.0001	0.0014	0.0403
Quadratic	0.0190	0.0332	0.7489	0.7891
Cubic	0.0016	0.0385	0.7536	0.3266
'Forelle'				
Linear	0.0001	0.0001	0.0129	0.8893
Quadratic	0.0001	0.0078	0.8612	0.9555
Cubic	0.0284	0.9016	0.0585	0.0373

Fig. 8 Paper 2


(A)
Anth. concn. (µg g-1 fr wt)
Anth. fruit-1 (µg)
Anthocyanin concentration
Anthocyanin per fruit
0
2
4
6
8
10
12
0
5
10
15
20
25
30
35
40
45
50
11/19
11/25
12/4
12/12
12/18
12/24
12/31
1/7
1/13
Date


(B)
Hours <14 ºC
Hours >28 ºC
Hue
Hue (º)
Hours
45
55
65
75
85
0
10
20
30
40
50
60
70
11/19
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12/31
1/7
1/13
Date

Fig. 9 Paper 2

PAPER 3

COLOUR IMPROVEMENT OF BLUSHED PEARS WITH OVERHEAD EVAPORATIVE COOLING.

Abstract. **The efficacy of pulsed applications of overhead evaporative cooling (EC) to improve the red colour of blushed pears (*Pyrus communis* L.) was evaluated in the Western Cape region of South Africa over two seasons. Preliminary, semi-commercial trials conducted during 2000/2001 indicated that EC could be used to improve the red colour of the blushed cultivar Rosemarie, but that the beneficial effects were limited to a short period after the initiation of the treatment. During 2001/2002 the application of EC early or late during fruit development of 'Rosemarie' and 'Forelle' pears was compared statistically. EC was activated above 28°C between 09h00 and 18h00 and above 20°C between 18h00 and 21h00. EC did not improve the colour of 'Forelle' fruit, but again increased the redness of 'Rosemarie' fruit when activated two weeks before harvest. Fruit of both cultivars lost red colour before harvest compared to other treatments when cooled from early during fruit development. The lack of an effect of EC on 'Forelle' colour was attributed to the high anthocyanin concentration and colour stability of this cultivar. Colour improvement in 'Rosemarie' was attributed to a reduction in red colour loss at high temperatures. Our results indicate that EC could be used to improve the colour of 'Rosemarie' pears in warm production areas. However, the production of 'Rosemarie' in more suitable climatic regions should have an even greater beneficial effect on fruit colour.**

Environmental conditions greatly affect the profitability of blushed pears by influencing skin colour. Downgrading due to insufficient red colour has limited the profitability of blushed pears in the warm production areas of the Western Cape region of South Africa (Huysamer, 1998). 'Rosemarie', a locally bred cultivar, appears to be especially susceptible to colour loss and red colour may disappear entirely before harvest. The loss of red colour was attributed to the net degradation of anthocyanin in response to high temperatures (Paper 2).

The extent of red pigmentation in fruit peel is primarily determined by light and temperature. Anthocyanin accumulation in vegetative tissues and fruits generally requires low night and moderate day temperatures (Christie et al., 1994; Curry, 1997) while poor fruit colour is associated with high temperatures (Haselgrove et al., 2000). High temperatures inhibit the induction and reduce the synthesis of anthocyanin (Curry, 1997; Reay, 1999). They also increase the rate of anthocyanin degradation (Paper 2; Marais et al., 2001). The necessity to expose fruit to maximal sunlight for colour development can sometimes contribute to poor colour by increasing fruit temperature through radiant heating (Haselgrove et al., 2000; Smart and Sinclair, 1976).

Overhead evaporative cooling (EC) is used commercially to counteract the adverse effects of high temperature on fruit quality in warm production regions. Evaporative cooling acts by negating the radiant heating of the fruit surface thereby reducing peel temperature by up to 8ºC in apple peel (Parchomchuk and Meheriuk, 1996; Unrath, 1972). Improvement in the red colour of apple fruit in response to evaporative cooling has been reported from several studies in different production regions (Evans et al., 1995; Iglesias et al., 2002; Unrath and Sneed, 1974). Evaporative cooling also reduced the fading of red colour towards harvest in the red pear cultivar, Sensation Red Bartlett (Dussi., et al 1997). However, the only previous assessment of evaporative cooling in the Western Cape showed a contradictory reduction in red colour development in 'Starking' apples (Kotzé et al., 1988). We hypothesised that evaporative cooling would improve red colour in blushed pears.

Materials and Methods

2000/2001. Preliminary, semi-commercial experiments were conducted to assess the effect of evaporative cooling on 'Rosemarie' pear fruit colour. The experiments were conducted at the Welgevallen experimental farm in the Stellenbosch district (latitude: 33º58'S, longitude: 18º50'E) and at Langrivier and Lindeshof farms in the Ceres district (latitude: 33º23'S, longitude: 19º19'E). Both production areas fall within the Western Cape region of South Africa, characterised by a Mediterranean-type climate

with winter rainfall and warm, dry summers where day temperatures commonly exceed 30°C.

The orchard at the Welgevallen experimental farm was established during 1991 on BP1 rootstock at a spacing of 4.5 m x 2 m. The EC system consisted of sprinklers installed 8 m apart on 3.8 m high poles in three alternate rows. It provided 2 mm h^{-1} irrigation at 200 kPa in a test plot six rows wide and 10 trees long. The EC system was operated from 16 December until commercial harvest on 11 January 2001. It was activated manually at air temperatures above 25°C and continued at cycles of 30 minutes on, 30 minutes off until air temperature decreased below the set point. The system was also activated for 30 minutes during the late afternoon (18h00 - 19h00). The orchard received normal undertree micro-irrigation scheduled according to neutron probe assessments of soil water content in the part of the orchard that did not receive EC.

The 'Rosemarie' orchard at Lindeshof was established during 1990 on BP1 rootstock at a spacing of 4 m x 1.25 m in two blocks that are separately irrigated. Overhead sprinklers, providing 3.1 mm h^{-1} at approximately 200 kPa, were installed at 8 m intervals along alternate rows in one of these irrigation blocks. The other irrigation block was used as control. The system was activated manually from the beginning of December at an air temperature of 28°C and run continuously until air temperature decreased below the set value. Normal undertree irrigation was scheduled independently in the control and treatment blocks making use of neutron probe readings.

The 'Rosemarie' orchard at Langrivier was established during 1995 on BP3 rootstock at a spacing of 4.5 m x 1.5 m. EC was supplied by microsprinklers of the normal undertree irrigation system, which were extended and secured to a wire at a height of 3 m aboveground above every second tree. The application rate was 2.7 mm h^{-1}. The system was activated automatically from the beginning of December at an air temperature of 28°C and continued at a cycle of 8 minutes on and 20 minutes off until air temperature decreased below the set temperature. Treatment rows did not receive supplemental undertree irrigation.

Experiments were not replicated because of financial limitations. Fruit were harvested from the western sides of ten trees in each of three rows that received evaporative cooling and from ten trees in each of three adjacent control rows that only received normal undertree micro-irrigation. Ten fruit per row were collected weekly from 14 December at Lindeshof and Langrivier and a larger sample of 50 fruit per row on 11 January, the last collection date prior to commercial harvest. Collection of fruit at Welgevallen started on 28 December. Initially, ten fruit were collected per row, but at harvest on 11 January a larger sample of 75 fruit per row were collected. The hue of fruit was measured at the reddest position making use of a chromameter (NR-3000; Nippon Denshoku, Tokyo, Japan). Hue refers to the angle formed by a line from the origin to the intercept of the a (x-axis) and b (y-axis) coordinates, where 0° = red-purple, 90° = yellow, 180° = bluish-green and 270° = blue. Treatment means and their standard errors are presented.

2001/2002. Based on results of the preliminary trials, the effect of early-initiated and late-initiated (from two to three weeks before harvest) application of EC on the colour of 'Rosemarie' and 'Forelle' pears was studied in statistical trials conducted at Welgevallen experimental farm. The same 'Rosemarie' orchard used during 2000/2001 was used, as well as a 'Forelle' orchard on Quince A rootstock established in 1998 at a spacing of 4 m x 1.25 m. Pressure compensated microsprinklers with a 28 L h^{-1} discharge rate and a wetted radius of 1.5 m were installed in every row in both orchards at a spacing of 2.5 m along a suspended pipe at the top of the tree canopy 3.5 m aboveground. The irrigation rate was ±4 mm h^{-1} at 200 kPa. Overhead cooling was activated automatically at a set air temperature and continued at a cycle of 10 minutes on, 20 minutes off until the air temperature decreased below the set value. The activation temperature was set at 28°C from 06h00 to 18h00 and at 20°C from 18h00 to 21h00. A temperature sensor was positioned approximately 1.5 m from the ground in a Gill radiation shield positioned between adjacent trees in the adjacent apple orchard. Normal undertree micro-irrigation was scheduled according to neutron probe readings outside the EC area and was not adjusted for the supplemental water supplied by the EC system.

Treatments consisted of a control without EC, an early EC treatment and a late EC treatment. The starting dates of EC treatments as well as the harvest dates of

'Rosemarie' and 'Forelle' pears are presented in Table 1. The control and late EC treatments were achieved by plugging sprinklers with stoppers, which were subsequently removed for the late EC treatment. Treatments were randomised within eight or ten 3-row blocks in the 'Rosemarie' and 'Forelle' orchards, respectively. Only the middle row was used for data collection and adjacent treatments within a row were separated by at least two buffer trees. Five exposed fruit were marked on the western side of three trees per treatment per block. Colour was measured weekly at the reddest position of two fruit per tree making use of a chromameter (NR-3000; Nippon Denshoku, Tokyo, Japan). All marked fruit were harvested at commercial harvest, their hue was measured and the extent and intensity of red colour visually assessed making use of the 'Rosemarie' (Set P.26; Unifruco Ltd., Belville South Africa) and 'Forelle' blush colour charts (P.16; Deciduous Fruit Board, Belville South Africa). Fruit were peeled and the peel stored at -80ºC until analysis (Paper 1). Temperature data were obtained from the Nietvoorbij automatic weather station approximately 4 km from Welgevallen. The data were analysed with the General Linear Models (GLM) procedure of SAS (SAS release 6.12P; SAS Institute, 1996, Cary, NC). Initial hue values were used as covariant in the analysis of hue data.

Results and Discussion

Effect of EC on 'Rosemarie' colour during the 2000/2001 season. Results of the preliminary, semi-commercial trials conducted during 2000/2001 suggested that EC might be used to improve the colour of 'Rosemarie' pears (Table 2). However, the beneficial effect on fruit colour seemed to be limited to a short period after the onset of EC. At Lindeshof and Langrivier, where EC was operated from the beginning of December, the hue of cooled fruit was lower (redder) than that of control fruit on 14 December, the first collection date (Table 2). Thereafter, the benefit was lost and there was no difference in the hue of treatments from 21 December onwards. EC at Welgevallen only started on 16 December. The hue of cooled fruit was lower than control fruit on 4 January and at harvest on 11 January (Table 2).

Effect of EC on 'Rosemarie' colour during the 2001/2002 season. The hue of 'Rosemarie' fruit fluctuated considerably during the 2001/2002 season (Fig. 1A). Comparing hue values (Fig. 1A) with temperature data (Fig. 1B) indicate that

'Rosemarie' fruit was less red after hot days and redder after cold nights. This is in agreement with previous findings (Paper 2). Cooled fruit displayed the same fluctuation in colour as control fruit in response to temperature, but appeared to lose less red colour during hot periods and were, therefore, generally redder than control fruit (Fig. 1).

Dussi et al. (1997) found that EC application above a set environmental temperature of 29ºC reduced the fading of red colour of 'Sensation Red Bartlett' pears in the month before harvest. We previously found that red colour loss in 'Rosemarie' was due to the degradation of anthocyanin and that degradation increased with increasing temperature (Paper 2). EC reduced the peel temperature of fruit by up to 8ºC compared to control fruit (Parchomchuk and Meheriuk, 1996; Unrath, 1972). A similar EC-induced reduction of up to 7ºC in the peel temperature of 'Forelle' pears was measured in this trial (data not presented). Hence, it is fair to assume that the beneficial effect of EC on the colour of 'Rosemarie' fruit was due to a reduction in fruit temperature during warm days that resulted in a decrease in anthocyanin degradation.

Fruit from the early EC treatment seemed to lose red colour during the week before harvest while other fruit became redder in response to cold nights (Fig. 1A, B). As a consequence, the initial benefit of early EC on colour was lost and at harvest, cooled and control fruit did not differ with regard to hue (Fig. 1A), blush colour or anthocyanin concentration (Table 3). Fruit from the late EC treatment had a 6º lower hue, better blush colour and 39% higher anthocyanin concentration than control fruit at harvest (Table 3; Fig. 1A). As a result, 90% of late EC fruit were sufficiently blushed compared to 77% of the control fruit (Table 3).

This result, together with the consistency of the results obtained over the two seasons of this study, suggests that the application of EC from three to two weeks before harvest could be used to improve the colour of 'Rosemarie' pears. However, the beneficial effect of EC was relatively small compared to the weekly fluctuation in 'Rosemarie' colour in response to temperature (Fig. 1A, B), indicating that prevailing climatic conditions have a larger effect on colour. This suggests that the production of

'Rosemarie' in more suitable climatic regions should have a greater beneficial effect on fruit colour than EC.

Effect of EC on 'Forelle' colour during 2001/2002: 'Forelle' fruit were very red initially, but colour gradually faded from about 14 December towards harvest (Fig. 2). Hue showed little response to temperature (data not presented) or EC (Fig. 2). Neither the early nor the late application of EC had any significant beneficial effect on the colour of 'Forelle' pears at harvest (Table 3; Fig. 2). Fruit from the early EC treatment appeared to be slightly redder than control fruit until 4 January whereafter early EC seemed to increase the fading of red colour towards harvest relative to other treatments (Table 3; Fig. 2). The hues of these fruit were 5.4° and 9° higher at harvest than the hue of control and late EC fruit, respectively (Fig. 2), though only the difference between the two EC treatments was significant. Differences in the anthocyanin concentrations at harvest were not significant (Table 3).

The lack of a response of 'Forelle' fruit to late EC could be due to the short duration (11 days) of the treatment. The generally poor responsiveness of 'Forelle' to EC compared to 'Rosemarie' might also be attributed to the higher anthocyanin concentration and red colour that fruit of this cultivar maintains throughout development (Table 3, Fig. 2; Paper 2). As discussed in Paper 2, high anthocyanin concentrations have a buffering effect on the hue of fruit, reducing or preventing colour change in response to temperature. The stability of 'Forelle' hue in response to high temperature lowers the potential for EC to improve colour. Parchomchuk and Meheriuk (1996) used a similar argument to explain why EC had no effect on the colour of 'Jonagold' apples in a region where night temperatures are generally conducive for anthocyanin synthesis.

The degeneration of the initial beneficial or neutral effect of early EC on the colour of fruit was evident in both years of the study and in both cultivars. The negative effect of prolonged EC on fruit colour was more pronounced in 'Forelle', probably due to the longer duration of the early EC treatment (Fig. 2). However, the loss of colour of early EC 'Rosemarie' fruit in the week before harvest also suggests a negative effect in this cultivar (Fig. 1A). Kotzé et al., (1988) previously reported a severe depression of colour development in 'Starking' apples in response to EC. They attributed the poor

colour development to a 25% increase in total shoot length in response to the EC. Our measurement of hue on exposed fruit ruled out inter-tree shading as the cause of colour loss. Waterlogged conditions may also have a negative effect on fruit colour (Walter, 1967). Kotzé et al. (1988) did not account for the additional water provided by EC when scheduling undertree irrigation. However, in our trials, irrigation at Lindeshof was adjusted for EC while at Welgevallen trees displayed no evidence of waterlogging. The accumulation of anthocyanins in response to low temperatures has been found to protect green tissues against photoinhibition (Krol et al., 1995). Fruit might be able to acclimate to the milder conditions brought about by the EC, thereby reducing the requirement for photoprotection and anthocyanin during cold periods. Further research in trials where soil water content is controlled to prevent waterlogging is required to test this suggestion.

In conclusion, our results indicate that EC could be used to improve the red colour of 'Rosemarie', but not 'Forelle' pears, when applied from about two to three weeks prior to harvest. EC should not be initiated early during fruit development in the light of the possible unfavourable effect of the prolonged application of EC on fruit colour. The reason for long-term detrimental effect of EC on fruit colour requires further research. Since the improvement in 'Rosemarie' red colour seemed to be due to a reduction in red colour loss in response to high temperatures, the effect of EC should be greatest in hot production regions. However, EC will be of little benefit if 'Rosemarie' colour is already diminished prior to the start of the treatment. Furthermore, the beneficial effect of EC was relatively small compared to the weekly fluctuation in 'Rosemarie' colour in response to temperature. Hence, the production of 'Rosemarie' in more suitable climatic regions should have a greater beneficial effect on fruit colour than EC.

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Table 1. Harvest dates and starting dates of early- and late-season overhead evaporative cooling (EC) treatments on 'Rosemarie' and 'Forelle' pears at Welgevallen experimental farm in Stellenbosch during the 2001/2002 season.

Cultivar	Early EC	Late EC	Harvest date
Rosemarie	23 November	28 December	11 January
Forelle	4 December	4 February	15 February

Table 2. Effect of overhead evaporative cooling (EC) on the hue (°) of 'Rosemarie' pears at different trial sites during the 2000/2001 season. Values are means with standard errors.

Trial site	Treatment	14 Dec.	21 Dec.	28 Dec.	4 Jan	11 Jan
Welgevallen	Control	-	-	49.3 ±1.46	72.4 ±2.07	67.7 ±1.01
	EC	-	-	49.8 ±0.18	63.1 ±3.53	60.6 ±1.88
Lindeshof	Control	62.1 ±1.75	54.5 ±3.80	49.5 ±2.59	50.5 ±2.67	51.7 ±2.26
	EC	55.9 ±0.39	50.0 ±3.82	49.4 ±2.13	49.3 ±2.58	52.8 ±1.24
Langrivier	Control	81.6 ±3.02	51.2 ±0.91	52.9 ±1.60	50.1 ±1.01	48.0 ±2.80
	EC	61.8 ±1.29	51.7 ±1.39	53.4 ±1.54	51.2 ±1.66	49.3 ±2.51

Table 3. Effect of overhead evaporative cooling during 2001/2002 on the blush colour and anthocyanin concentration of 'Rosemarie' and 'Forelle' pears at harvest. See Table 2 for treatment times. Means in columns were separated by LSD (5%).

Treatment	Rosemarie			Forelle	
	Anth. conc. ($\mu g\ g^{-1}$ fr wt)	Colour chart value[z]	% fruit with insufficient blush[y]	Anth. conc. ($\mu g\ g^{-1}$ fr wt)	Colour chart value[z]
Control	6.4 b	7.1 b	23 b	28.9 a	3.2 a
Early EC	6.4 b	7.4 b	25 b	31.2 a	3.9 b
Late EC	8.9 a	5.8 a	10 a	30.3 a	3.2 a
Pr > F	0.0167	0.0046	0.0087	0.7053	0.0616

[z] Blush colour chart values where 1 = completely red and 12 = green.

[y] Blush colour chart values 10.

Fig. 1. (A) Effect of overhead evaporative cooling (EC) on the hue of 'Rosemarie' pears during the 2001/2002 season. Fruit were cooled from 23 November (Early EC) or 28 December (Late EC). Treatment means at harvest were adjusted for initial hue values and were separated by LSD (5%). Means with different letters differ significantly. (B) The number of hours above 28°C and below 14°C experienced daily during fruit development of 'Rosemarie' pears during the 2001/2002 season.

Fig. 2. Effect of overhead evaporative cooling (EC) on the hue of 'Forelle' pears during the 2001/2002 season. Fruit were cooled from 4 December (Early EC) or 4 February (Late EC). Treatment means at harvest were adjusted for the initial hue values and were separated by LSD (5%). Means with different letters differ significantly.

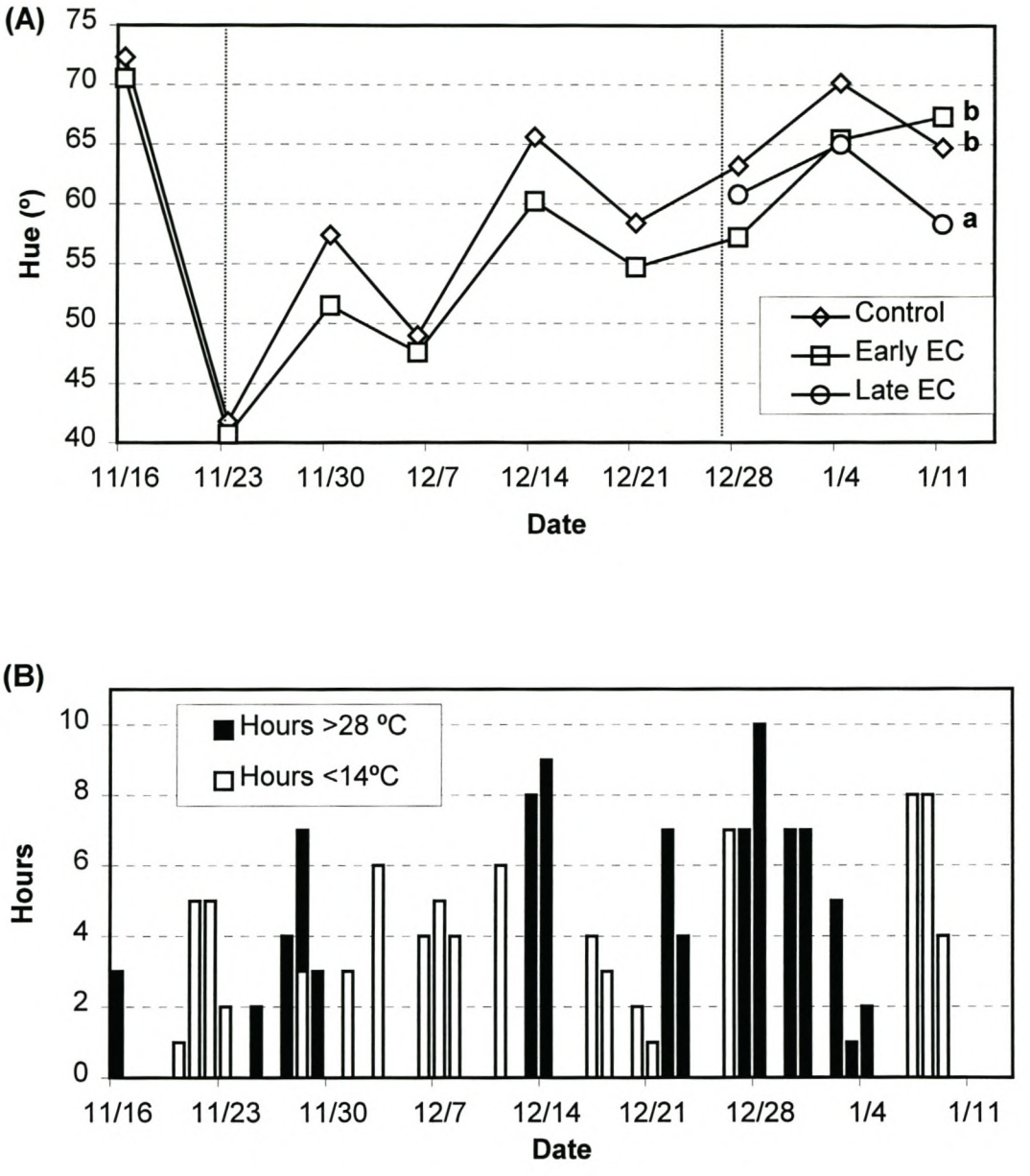

Fig. 1 Paper 3

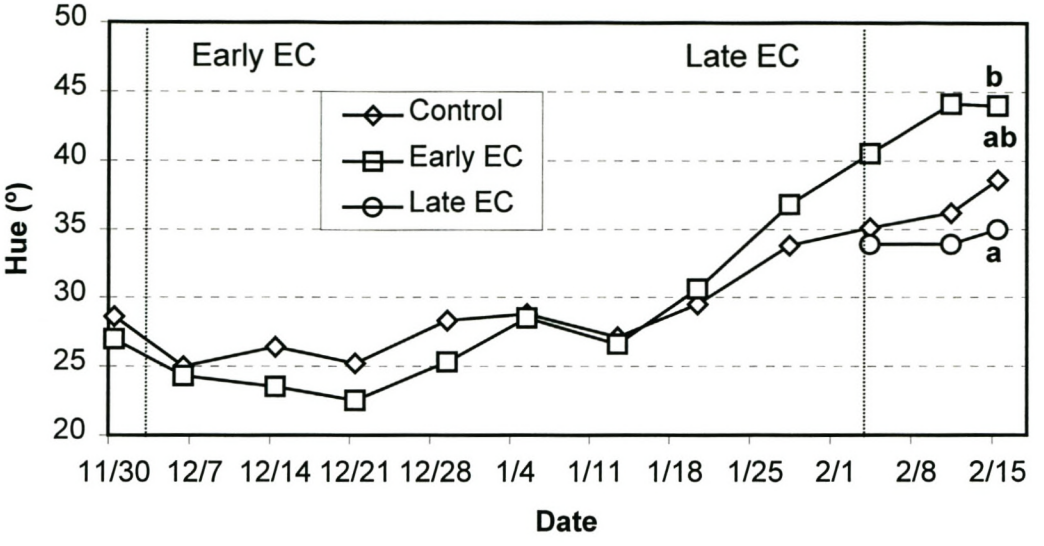

Fig. 2 Paper 3

PAPER 4:

EVIDENCE OF INCREASED RESISTANCE TO PHOTOINHIBITION WITH INCREASING REDNESS OF PEAR PEEL.

Abstract. **The study was conducted to establish the possible involvement of anthocyanins in the protection of peel of blushed and fully red pear cultivars against photoinhibition under natural conditions in the orchard as well as under stressful conditions. Chlorophyll a fluorescence was monitored in response to photoinhibitory treatments. Exposed pear peel incurred a slight degree of photoinhibition under natural conditions in the orchard. The extent of photoinhibition increased with decreasing redness of peel. Differences in susceptibility to photoinhibition were maintained after photoinhibitory treatment at 10°C and 40°C. However, we argued against the ability of anthocyanins to afford photoprotection at high temperatures as a general function. Although increased resistance to photoinhibition was associated with red skin colour, there were indications that anthocyanin was not responsible for photoprotection. Firstly, differences between cultivars in the extent of photoinhibition incurred during three hours at high light (1200 μmol m^{-2} s^{-1} PPFD) in green shaded peel appeared to reflect differences in photoinhibition in exposed peel. Also, the presence of anthocyanin did not reduce the bleaching of chlorophyll under severe photoinhibitory conditions in red compared to green skin. Photosystem II efficiencies and levels of photochemical quenching reflected the increased susceptibility to photoinhibition of shaded relative to exposed surfaces of fruit, but did not provide evidence of anthocyanin-associated photoprotection. In conclusion, the inherent ability of different cultivars to tolerate high light seemed to increase with their ability to accumulate anthocyanin, but increased resistance to photoinhibition did not appear to be due to anthocyanin.**

The presence of anthocyanins in reproductive organs is generally considered to aid pollination and seed dispersal (Harborne, 1965; Harborne and Grayer, 1994). Congruent with such a function, changes in colour and accumulation of anthocyanins

in many fruit kinds typically coincide with ripening (Macheix et al., 1990). However, in apples for instance, reddening also occurs shortly after anthesis (Lancaster, 1992) while the maximum anthocyanin concentration in fully red and blushed pears is attained in immature fruit and red colour gradually decreases towards harvest (Dayton, 1966; Dussi et al., 1997; Paper 1). The accumulation of anthocyanin at these earlier stages of fruit development is not as readily explained by the pollination/dispersal function (Lancaster, 1992).

It has been proposed that anthocyanins in immature organs afford protection against UV-light and possible oxidative damage (Lancaster, 1992; Yamasaki, 1997). However, the colourless phenolics and flavonoids that accumulate to very high levels early during fruit development (Macheix et al., 1990) are for various reasons more effective than anthocyanin in providing UV-protection and can also dispose of oxidants (Steyn et al., 2002). Anthocyanins, on the other hand, may protect green tissues from oxidative damage by reducing light levels incident on chlorophyll.

Light in excess of that required for photosynthesis results in the suppression of photosynthesis, better known as photoinhibition (Long et al., 1994). Photoinhibition reduces productivity and may eventually have a negative effect on survival (Ball et al., 1991). Even moderate light levels can become excessive when combined with other stresses that restrict the utilisation of light energy, in particular low temperatures (Haldimann et al., 1996). Severe photoinhibitory conditions can result in oxidative damage to the photosynthetic apparatus and the disruption of cellular metabolism (Foyer et al., 1994). Smillie and Hetherington (1999) demonstrated the photoprotective ability of anthocyanin in *Bauhinia variegata* pods and, subsequently, proposed that anthocyanins have a general function in protecting photosynthesising tissues from excess visible light. Recently, Merzlyak and Chivkunova (2000) found that interception of light by anthocyanin reduced photobleaching of chlorophyll in apple peel at extreme irradiation levels (4000 μmol m^{-2} s^{-1}) in the laboratory as well as under natural conditions on the tree. Congruent with a photoprotective function, anthocyanin synthesis in pears requires or, at least, benefits from light and red colour increases with increasing light intensity (Dussi et al., 1997).

We conducted a study to establish whether the anthocyanins that accumulate in the exposed peel of blushed and fully red pear cultivars protect these fruit from photoinhibition. This was done by monitoring photosynthetic activities in the shaded and exposed faces of pear cultivars before and after photoinhibitory treatments using chlorophyll fluorescence techniques. By measuring the maximal efficiency of photosystem II photochemistry (F_v/F_m), the photoprotective ability of anthocyanins has been demonstrated in cold-stressed jack pine needles (Krol et al., 1995), pods of *Bauhinia variegata* phenotypes (Smillie & Hetherington, 1999) and senescing leaves (Feild et al., 2001). A reduction in the value of F_v/F_m is a reliable indicator of photoinhibition or damage to photosynthetic processes (DeEll et al., 1999; Maxwell and Johnson, 2000).

Cultivars used in this study differed in their ability to accumulate anthocyanin in exposed peel and, consequently, ranged in colour from dark red to completely green on their exposed faces. Measurements were also taken on green shaded peel of the different cultivars to discern whether differences in photoinhibition in exposed peel, if present, were due to the presence of anthocyanin or to the inherent capacity of different cultivars to withstand high light. The ability of anthocyanin to reduce or prevent photobleaching of chlorophyll was also assessed.

Materials and Methods

Plant material. Fully exposed fruit from the western periphery of trees in north to south planted rows in orchards located in the Stellenbosch district (latitude: 33º58'S, longitude: 18º50'E) of the Western Cape region in South Africa were used. Cultivars included in the study varied in their ability to accumulate anthocyanin in exposed peel with redness decreasing from dark red to green in the following order; 'Bon Rouge', 'Forelle', 'Flamingo', 'Rosemarie' 'Kieffer', 'Early Bon Chretien' and 'Packham's Triumph'. 'Kieffer' and 'Packham's Triumph' were grafted on Quince A rootstock. All other orchards were grafted on BP1 rootstock.

Chlorophyll fluorescence. Chlorophyll fluorescence measurements were made using a pulse modulated fluorometer (FMS2; Hansatech Instruments Ltd., Norfolk, England). Detached fruit were dark-adapted for at least 30 minutes at 20ºC prior to

measurement of fluorescence. The intrinsic or maximal photon yield of photosystem II photochemistry was measured as F_v/F_m. To determine F_v/F_m, the minimal yield of fluorescence, F_o, was measured in the absence of photosynthetically active radiation. A saturating light pulse (10800 μmol m^{-2} s^{-1} photosynthetic photon flux density [PPFD] for 0.7 seconds) was then used to attain maximal yield of fluorescence, F_m. Variable fluorescence, F_v, was calculated as F_m - F_o. The actual efficiency (photon yield) of PS II photochemistry (ϕ_{PSII}) was measured as (F_m'-F)/F_m' (Genty et al., 1989) at stepwise increasing actinic radiation levels to a maximum of 885 μmol m^{-2} s^{-1} PPFD. Once a steady state level of fluorescence (F) was reached at each light level, F was recorded followed by measurements of F_m' and F_0'. F_m' was obtained by briefly saturating PS II reaction centres with a strong light pulse (10800 μmol m^{-2} s^{-1} for 0.7 seconds). Transient interruption of actinic radiation followed by irradiation with far-red radiation for 5 seconds was used to obtain F_0'. The photochemical chlorophyll fluorescence quenching coefficient, q_p, was determined as (F_m' - F)/(F_m' - F_0') and the nonphotochemical chlorophyll fluorescence quenching coefficient as (F_m - F_m')/(F_m - F_0').

Colour measurement and pigment analysis. External colour was measured with a chromameter (NR-3000; Nippon Denshoku, Tokyo, Japan). Hue angle and lightness (L) are presented to express differences in peel pigmentation (McGuire, 1992). Hue angles range between 0° = red-purple, 90° = yellow, 180° = bluish-green and 270° = blue, and provide an appropriate means to express differences in the redness of pear peel. L values range from black = 0 to white = 100 and gives an indication of the total light absorbance by peel pigments. In the absence of anthocyanins in shaded pear peel, differences in L values should relate to differences in the levels of plastid pigments.

Anthocyanins were assessed by reverse-phase high performance liquid chromatography (HP 1100; Agilent Technologies, Palo Alto, CA) as described in Paper 1. Chlorophyll a and b were quantified by measuring absorption at 470, 645 and 662 nm on a spectrophotometer (DU Series 64; Beckman, California) and making use of the extinction coefficients of Lichtenthaler (1987). Extraction of

anthocyanin and chlorophyll was performed and samples prepared as described in Paper 1.

Photoinhibition studies. Susceptibility to photoinhibition of exposed or shaded faces of pear fruit was measured after exposure to irradiance of ~500 μmol m^{-2} s^{-1} PPFD at 10ºC or ~1200 μmol m^{-2} s^{-1} PPFD at 10ºC and 40ºC (see Table 1 for detailed layout of experiments). PPFD was measured at fruit level with a quantum meter (LI-189; Li-Cor, Lincoln, Nebraska, USA). Experiments were conducted within growth cabinets maintained at the desired temperatures. The lower PPFD was provided by a single 400 W HPS light (SON-T; Osram Mgbh, Munich, Germany) placed on top of the cabinets with an acrylic (Perspex) layer between the lights and fruit. The higher light intensity was provided by two 400 W HPS lights set up as above. Replicates were randomised within the growth cabinets to allow for any spatial variation in light intensity. A 40 cm fan was used to remove radiant heat from the surface of the Perspex. Prior to the experiments, peel temperatures of sample fruit placed in the growth cabinets were measured with thermocouples and the set temperatures of growth cabinets adjusted to obtain the desired peel temperatures. Fruit samples were placed within a plastic container of water (0.5 cm deep) to reduce water loss.

Experiment 1: Photoinhibition at low temperature and moderate light. Six 'Bon Rouge', 'Forelle', 'Flamingo', 'Kieffer', 'Rosemarie', 'Early Bon Chretien' and 'Packham's Triumph' fruit were collected before 8:00 in the morning on 21 December 2001 and brought to the laboratory. The collection dates of fruit relative to blossom periods and harvest dates of the respective cultivars are presented in Table 1. Fruit were divided into three groups, each containing two fruit of each cultivar. Two peel disks (~5 cm diameter), one from the exposed side and the other from the opposite shaded side, were removed from each fruit. Peel from exposed and shaded sides of fruit are from here on referred to as exposed and shaded peel. Disks were randomised within three replicates within a container filled with 0.5 cm water and fluorescence and colour were measured in the centre of disks after dark-adaptation for 30 minutes. The container with disks was subsequently transferred to a growth cabinet set at 10ºC. After three hours at irradiation of 500 μmol m^{-2} s^{-1} PPFD, disks

were removed from the cabinet and fluorescence and colour measured after dark-adaptation for 30 minutes at 20°C.

Experiment 2: Photoinhibition at low temperature and high light. Fruit were collected on 28 December 2001 and the experiment conducted as in Experiment 1, with the exception that irradiation of 1200 μmol m^{-2} s^{-1} was used to induce photoinhibition and that F_v/F_m was again measured after a 24-hour recovery period at 20°C in darkness.

Experiment 3: Photoinhibition at low and high temperature and pigment bleaching. 'Forelle', 'Kieffer' and 'Packham's Triumph' fruit were collected from the western side of trees on 29 January 2002. Fruit were divided into three groups each containing 12 fruit of each cultivar. Groups were further subdivided into three replicates containing four fruit of each cultivar. Fruit were halved between the exposed and shaded faces of fruit and colour was measured at the centre of each half. Fruit halves of one group were peeled into liquid nitrogen to assess initial concentrations of anthocyanin, chlorophyll and carotenoids (μg g^{-1} fr wt). After dark-adaptation and measurement of fluorescence, the second group of fruit was randomised within a growth chamber set at 10 ±2°C. The third group was kept in darkness at 20°C with cut surfaces immersed in 0.5 cm water. These fruit were transferred to the growth cabinet set at 40 ±2°C after 24 hours when fruit of the second group were removed. Fluorescence and colour were measured after removal from the growth chamber and dark-adaptation. F_v/F_m was again measured after a 24-hour recovery period in dark at 20°C whereafter fruit halves were peeled to assess changes in pigment concentrations.

Fluorescence light response curves and quenching. 'Bon Rouge', 'Forelle', 'Rosemarie' and 'Packham's Triumph' pears were collected weekly from 25 November 2000 until 9 January 2001 before 07:00 in the morning. After dark-adaptation at 20°C, ϕ_{PSII}, q_p and q_{np}, were measured at stepwise increasing actinic light levels. Fluorescence was measured on the equator of both exposed and shaded surfaces of three fruit of each cultivar on every date.

Statistical analysis. The data were analysed with the General Linear Models (GLM) and Correlation (CORR) procedures of SAS (SAS release 6.12P; SAS Institute, 1996, Cary, NC).

Results

Variation in colour and photoinhibition under natural conditions. The pear cultivars included in our study differed in their ability to accumulate anthocyanin. Exposed peel ranged in colour from dark red to completely green in the following order: 'Bon Rouge', 'Forelle', 'Flamingo', 'Rosemarie' 'Kieffer', 'Early Bon Chretien' and 'Packham's Triumph' (Table 2, 3). In contrast, shaded peel was homogenously green, with the exception of 'Bon Rouge', which displayed some red pigmentation (Table 2, 3). L values of exposed peel related to hue angles, being lowest in 'Bon Rouge' and highest in 'Packham's Triumph' (Table 2, 3). Shaded peel was, generally, lighter in colour than exposed peel. L values in shaded peel were comparable between cultivars, with the exceptions of 'Bon Rouge', which was darker in colour due to the presence of anthocyanin, and 'Rosemarie', which was consistently lighter in colour than other cultivars (Table 2, 3).

Differences in F_v/F_m between cultivars and between shaded and exposed peel were evident before peel were subjected to photoinhibitory treatment. In general, values of F_v/F_m in shaded peel were similar to the average value of 0.83 found in healthy leaves (Table. 2, 3) (Björkman and Demmig, 1987). 'Packham's Triumph' showed slightly lower values. F_v/F_m ratios in exposed peel of the two reddest cultivars, 'Bon Rouge' and 'Forelle', were comparable to those of shaded peel (Table 2, 3). However, a slight depression of F_v/F_m was evident in exposed peel of cultivars with less red pigmentation (Table 2, 3). The extent of naturally occurring photoinhibition correlated with hue, increasing with decreasing redness of peel (Fig. 2, 3 and 4).

Photoinhibition studies: Experiments 1 and 2. Both photoinhibitory treatments markedly reduced Fv/Fm ratios in pear peel (Table 2, 3; Fig. 2, 3). Exposed peel was generally more resistant to photoinhibition and also recovered faster than shaded peel. 'Bon Rouge' peel was consistently more resistant to photoinhibition compared to other cultivars. Generally, initial differences between cultivars in the F_v/F_m ratios of

their exposed peel were continued after photoinhibitory treatment (Table 2, 3; Fig. 2, 3). However, the percentage decrease in F_v/F_m in response to high light treatment did not differ between cultivars, with the exception of 'Bon Rouge', which showed less photoinhibition than the other cultivars (Table 3). Although fruit of different pear cultivars were at various stages of development when collected (Fig. 1), whatever differences there was in fruit maturity did not seem to influence results (Table 2, 3).

Induction of photoinhibition at moderate light resulted in a similar depression of F_v/F_m (14-23%) in shaded peel of different cultivars (Table 2). However, the degree of photoinhibition sustained by shaded peel during three hours at high light differed between cultivars and these differences corresponded with differences in the redness of exposed peel (Table 3). Differences between cultivars in the percentage reduction of F_v/F_m were lost following the recovery period in both shaded and exposed peel (Table 3).

Photoinhibition and pigment bleaching at high and low temperature. The chlorophyll, carotenoid and anthocyanin concentrations of exposed and shaded 'Forelle', 'Kieffer' and 'Packham's Triumph' pear peel before and after severe photoinhibitory treatment (24 hours at 10°C or 40°C under 1200 μmol m^{-2} s^{-1} PPFD) are presented in Table 4. Concentrations of all three pigment groups were higher in exposed compared to shaded peel. Cultivars differed with regard to carotenoid and anthocyanin levels, but had similar chlorophyll concentrations. Photoinhibitory treatment resulted in a 10-16% loss of chlorophyll, but carotenoid levels were unaffected. The extent of chlorophyll degradation did not differ between cultivars and was similar in exposed and shaded peel. Considerable degradation of anthocyanin occurred at 40°C.

Photoinhibition was more severe at 10°C than at 40°C and in shaded compared to exposed peel (Fig 5). The extent of photoinhibition incurred at both temperatures correlated with the redness of exposed peel (Fig 4). However, differences in photoinhibition between exposed 'Forelle' and 'Kieffer' peel were not significant (Fig. 5). The strength of the correlation between anthocyanin concentration and F_v/F_m was lower after photoinhibitory treatment than before, and the extent of photoinhibition incurred at 40°C did not correlate with the anthocyanin concentration of exposed peel (Table 5). The extent of photoinhibition in shaded peel did not correlate with pigment

concentration or hue and did not differ significantly between cultivars (Fig 4B). A comparable small recovery of F_v/F_m occurred in shaded peel of all three cultivars at 20ºC in darkness (Fig. 5). Exposed peel of all three cultivars displayed considerable recovery from photoinhibition at both temperatures. However, 'Packham's Triumph' seemed to recover more slowly from photoinhibition at 10ºC compared to the other two cultivars (Fig. 5).

Fluorescence light response curves and quenching. Values of ϕ_{PSII}, q_p, and especially q_{np}, fluctuated considerably between dates and were, therefore, averaged over fruit development to give a general indication of differences between cultivars. ϕ_{PSII} and q_p decreased while q_{np} increased with increasing actinic light level (Table 6-8). ϕ_{PSII} and q_p were lower in shaded than in exposed peel (Table 6 and 7). 'Bon Rouge' and 'Forelle' peel, generally, displayed the highest and 'Packham's Triumph' the lowest ϕ_{PSII} (Table 6). Values of q_p did not differ between cultivars in exposed peel except at the highest light level where 'Rosemarie' displayed higher photochemical quenching than 'Bon Rouge' and 'Packham's Triumph' (Table 7). Shaded 'Bon Rouge' peel displayed greater q_p than the other three cultivars. Shaded 'Forelle' and 'Packham's Triumph' peel displayed lower values of q_{np} at all light levels (Table 8). q_{np} in exposed 'Forelle' peel was also lower at all except the highest light level where 'Rosemarie' displayed the highest q_{np} values.

Discussion

Measurement of F_v/F_m indicated that exposed pear peel incurred a slight degree of photoinhibition under natural conditions in the orchard (Table 2, 3; Fig. 2, 3). Since fruit were picked before 08:00 in the morning, photoinhibition was not due to midday depression of photosynthesis, but was a longer-term effect. The extent of photoinhibition increased with decreasing redness of peel (Fig. 2, 3), suggesting that anthocyanins in pear fruit are able to afford protection against light stress. This result corresponds with the consistently higher F_v/F_m ratios found on exposed faces of purple *Bauhinia variegata* pods compared to exposed faces of green pods (Smillie and Hetherington, 1999). The accentuation of initial differences in F_v/F_m on the exposed sides of fruit by exposure to moderate to severe photoinhibitory conditions (Table 2, 3; Fig. 2-4) provided further support for anthocyanin photoprotection in pear

peel. Exposed 'Packham's Triumph' peel also underwent greater photoinhibition than the red 'Forelle' and 'Kieffer' peel (Fig. 5). Similarly, Smillie and Hetherington (1999) found that short exposure to severe photoinhibitory conditions as well as longer exposure to milder conditions resulted in a 25 to 55% greater decrease in F_v/F_m in green compared to red *Bauhinia* pods. The positive correlation between the redness of peel and the extent of photoprotection (Fig. 2-4) suggests that higher anthocyanin concentrations afforded more protection. Increasing redness of exposed pear peel was accompanied by a corresponding increase in darkness (Table 2, 3), indicating that the presence of anthocyanin increased light absorbance. Previously, Neill and Gould (1999) found that the extent of light attenuation in leaves of seven plant species was proportional to anthocyanin concentration.

A greater extent of photoinhibition on mango fruit exposed to sunlight during the afternoon compared to fruit that received direct light during the morning suggested to Hetherington (1997) that photoinhibitory stress in the orchard was predominantly incurred under high temperatures. Since ϕ_{PSII}, measured at the equator of fruit, was consistently higher in purple compared to green mango fruit, Hetherington (1997) inferred that anthocyanins were able to afford protection against photoinhibition at high temperatures. Some of our results support this reasoning. Firstly, red 'Forelle' and 'Kieffer' peel showed greater resistance to photoinhibition at 40ºC compared to green 'Packham's Triumph' peel (Fig. 5). Furthermore, since fruit were collected from the western sides of trees, the slight degree of photoinhibition evident in exposed faces of pears (Table 2, 3) was most probably also incurred during the afternoon, which during summer in the pear producing regions of the Western Cape is typically very hot. Though high temperatures can inactivate photosynthetic processes in the absence of light, a combination of high light levels and high temperatures are even more damaging (Al-Khatib and Paulsen, 1989). Hence, it is probable that the attenuation of light by anthocyanin should also afford some protection against photoinhibition at high temperatures.

However, the anthocyanin concentration of exposed 'Forelle', 'Kieffer' and 'Packham's Triumph' peel did not correlate with the extent of photoinhibition incurred at 40º even though the initial correlation between anthocyanin concentration and F_v/F_m before photoinhibitory treatment was very strong (Table 5). Since high

temperatures are generally negatively associated with the accumulation and stability of anthocyanin (Paper 2; Haselgrove et al., 2000; Marais et al., 2001), it is dubious whether photoprotection at high temperature is an acceptable explanation for the presence of anthocyanin in fruit. For example, 'Rosemarie' pears increased in redness with the passing of cold fronts while red colour rapidly faded during intermittent warmer periods (Paper 2) and high temperatures accelerated anthocyanin degradation in detached apples and pears (Table 4; Paper 2; Marais et al., 2001). The presence of anthocyanin may also become detrimental at high temperatures. Increased susceptibility of shoulders of purple mango fruit to sunburn and photoinhibition compared to green-fruited cultivars was ascribed to the higher heat-absorbing capacity of anthocyanin-containing peel (Hetherington, 1997). The peel temperature on sunlit sides of grape berries, which was ~11°C above air temperature, was raised by a further 5°C by application of black ink to berries (Smart and Sinclair, 1976). We took care to prevent radiant heating of peel, which probably explains why anthocyanin had no negative effect on pear peel at 40°C. Reduced carbon gain due to reduction of light levels may also exceed any potential photoprotective function that anthocyanins might have at moderate temperatures (Burger & Edwards, 1996; Gould et al., 2002). However, anthocyanin pigmentation is usually of more permanence in organs or tissues that do not have a primary function in photosynthesis (Harborne, 1965).

Though resistance to photoinhibition appeared to correlate with the redness of exposed peel, we considered that photoprotection may not be due to the presence of anthocyanin. Causality between the presence of anthocyanin and increased resistance to photoinhibition in *Bauhinia* pods was established by inducing photoinhibition with red and blue-green actinic radiation (Smillie and Hetherington, 1999). Red light, which is not absorbed by anthocyanin, resulted in similar levels of photoinhibition in green and purple pods. Blue-green light, which is absorbed by anthocyanin, induced a much greater extent of photoinhibition in green pods. We made use of the absence of anthocyanin (except in 'Bon Rouge') from shaded pear peel to determine whether the increased resistance of red peel to photoinhibition was due to the presence of anthocyanin, which would be the case if there were no correlation between the extent of photoinhibition induced in shaded and exposed

peel. There were indications from these results that photoprotection should not only be ascribed to the presence of anthocyanin.

Firstly, not only the exposed peel, but also the shaded peel of the fully red cultivar 'Bon Rouge' was more resistant to photoinhibition compared with other cultivars (Table 2, 3). Though this could be due to the presence of anthocyanin in shaded 'Bon Rouge' peel, the extent of photoprotection afforded did not differ from that afforded by much redder peel on the exposed sides of fruit (Table 2, 3). Hence, 'Bon Rouge' appeared to possess an inherently greater ability to tolerate high light. The apparently greater susceptibility of shaded 'Rosemarie' peel to photoinhibition might relate to its consistently higher L values compared to other cultivars (Table 2, 3). However, the similarity in lightness of green shaded peel between other cultivars (Table 2, 3) suggests that cultivars, generally, contained comparable levels of plastid pigments.

F_v/F_m values in shaded pear peel of all cultivars, except 'Packham's Triumph', initially corresponded to the value of 0.83 typical of healthy leaves with unimpaired photochemistry (Table 2, 3) (Björkman and Demmig, 1987). Moderate light at 10°C induced a similar degree of photoinhibition in the shaded peel of the different cultivars with the exception of 'Bon Rouge', which showed higher resistance to photoinhibition (Table 2). Subjecting shaded peel of 'Forelle', 'Kieffer' and 'Packham's Triumph' pears for 24 hours to high light at 10°C or 40°C resulted in a similar decline and subsequent recovery of F_v/F_m (Fig. 5). However, when exposed for three hours to 10°C and high light, differences in the susceptibility of shaded peel of different cultivars corresponded to differences in the susceptibility of their exposed peel (Table 3), thus suggesting that the inherent ability of different cultivars to tolerate high light increased with their ability to accumulate anthocyanin, but that resistance to photoprotection in exposed peel was not necessarily related to the presence or absence of anthocyanin.

Analysis of photosystem II efficiency (ϕ_{PSII}) and the quenching coefficients, q_p and q_{np}, with increasing actinic light levels in 'Bon Rouge', 'Forelle', 'Rosemarie' and 'Packham's Triumph' pear peel did not provided conclusive evidence of anthocyanin-associated photoprotection (Table 6-8). Increased diversion of absorbed light energy from photochemical to non-photochemical pathways and a subsequent reduction in

the photon yield of photosynthesis is generally indicative of greater excitation pressure and inhibition of photosystem II (DeEll et al., 1999). Correspondingly, Smillie and Hetherington (1999) found that anthocyanin in *Bauhinia* pods reduced diversion of absorbed photon energy to non-photochemical pathways and allowed greater allocation to photochemistry under irradiation with actinic blue-green light. Shaded pear peel of the four cultivars studied displayed less efficient utilisation of absorbed light energy for photochemistry than exposed peel (Table 7). This explains the lower ϕ_{PSII} (Table 6) and increased susceptibility of shaded peel to photoinhibition relative to exposed peel (Table 2, 3; Fig. 5). In contrast, differences between cultivars in values of ϕ_{PSII}, q_p and q_{np}, were generally small, indicating no major differences in their ability to utilise light through photosynthesis (Table 6-8). However, higher values of ϕ_{PSII} in 'Bon Rouge' and 'Forelle' (Table 6) seem to correspond with the increased resistance of these cultivars against photoinhibition (Table 2, 3; Fig. 5). Since there was no interaction between cultivar and the side of fruit (Table 6), higher values of ϕ_{PSII} in 'Bon Rouge' and 'Forelle' were not linked to the presence of anthocyanin. The greater ability of shaded 'Bon Rouge' peel to utilise light through photochemical processes as indicated by higher values of q_p, especially at higher light levels, correspond with its increased resistance to photoinhibition (Table 2, 3), which might or might not be due to anthocyanin as discussed above. Although cultivars differed in the diversion of absorbed light energy to non-photochemical processes (Table 8), these differences did not relate to differences in peel pigmentation and ability to tolerate photoinhibition.

Photoinhibition of photosynthesis can result in the production of highly reactive oxygen species (Foyer et al., 1994), which may degrade chlorophyll and carotenoids through oxidation (Wise and Naylor, 1987). Merzlyak and Chivkunova (2000) found that the presence of anthocyanin in senescent apple peel reduced photobleaching of chlorophyll under severe light stress and, possibly also under natural conditions. A slight loss of chlorophyll occurred in exposed and shaded peel of 'Forelle', 'Kieffer' and 'Packham's Triumph' pears at both 10°C and 40°C during 24 hours irradiation at high light (1200 μmol m^{-2} s^{-1} PPFD) (Table 4). Since the loss of chlorophyll occurred irrespective of the presence or absence of anthocyanin (Table 4), anthocyanins evidently did not prevent or reduce chlorophyll destruction in pear peel. In fact,

Hetherington (1997) found that thermal damage to photosynthesis and bleaching of plant pigments was more pronounced on the shoulders of purple compared to green mango fruit. Merzlyak and Chivkunova (2000) remarked on the high resistance of anthocyanin to bleaching by high light levels in apple fruit compared to the apparent susceptibility of chlorophyll. On the contrary, we have previously found that light participated in anthocyanin degradation in detached apples, especially at higher temperatures (Paper 2). The degradation of anthocyanin at 40°C (Table 4) was in concurrence with our previous results (Paper 2).

It has to be kept in mind when interpreting these results that horticultural crops with increased pigmentation are usually selected for aesthetic purposes (Harborne, 1995). Hence, the presence of anthocyanin does not necessarily confer an adaptive advantage and any function of anthocyanin in photoprotection might simply be fortuitous. The same applies to the studies on *Bauhinia* pods (Smillie and Hetherington, 1999) and apple peel (Merzlyak and Chivkunova, 2000). Though studies of anthocyanin function in horticultural crops can indicate the mechanism by which anthocyanin photoprotection might act, it cannot be used to establish causality. However, the ability to accumulate anthocyanin seems to be linked with tolerance to high light, even though increased resistance to light stress might have a different basis.

A recent molecular study suggested that anthocyanin forms part of a greater and interrelated response to light stress. Iida et al. (2000) described a gene that was rapidly induced in proportion to the intensity and duration of light stress. Apart from constitutive high light tolerance, over-expression of this gene also resulted in the accumulation of anthocyanin and other adaptive phenotypic responses. Furthermore, fully red pear cultivars, which are selected bud mutations of green cultivars, are often unproductive and difficult to grow. Martin et al. (1997) established that this debility of red pear cultivars might be attributed to the lower mean maximum net photosynthetic rate and Rubisco activity found in green, mature leaves of three red-fruited sports compared to their respective green-fruited parents. Evidently, mutations giving rise to increased fruit colour in pears may also affect photochemistry and other plant processes.

In conclusion, our results suggested that the ability of exposed surfaces of pear fruit to tolerate photoinhibitory conditions correlates with, but was not necessarily due to the presence of anthocyanin. Though fully red and blushed cultivars have been selected with aesthetic considerations, these cultivars appear to be more tolerant of photoinhibitory conditions, suggesting that the ability to accumulate anthocyanin forms part of a general plant response to light stress.

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Table 1. Layout of photoinhibition experiments conducted on fruit of various pear cultivars.

	Experiment 1	Experiment 2	Experiment 3
Light intensity [z]	500	1200	1200
Temperature (ºC)	10	10	10 and 40
Duration (h)	3	3	24
Cultivars			
Bon Rouge	x	x	
Forelle	x	x	x
Flamingo	x	x	
Rosemarie	x	x	
Kieffer	x	x	x
Early Bon Chretien	x	x	
Packham's Triumph	x	x	x

[z] μmol m^{-2} s^{-1} PPFD

Table 2. F_v/F_m ratios and the percentage photoinhibition (PI) in peel disks of pear fruit of different hue angles before and after exposure to moderate light (500 μmol m^{-2} s^{-1} PPFD) at 10°C for three hours. Hue angles reported vary from 0° (red-purple) to 120° (yellow-green). Means were separated by LSD (5%).

Cultivar	Lightness	Hue (°)	F_v/F_m Before	F_v/F_m After	PI (%)
Exposed faces					
Bon Rouge	28 a	17 a	0.827 ab	0.777 a	6.0 a
Forelle	33 b	26 b	0.823 b	0.757 a	7.9 ab
Flamingo	35 b	36 c	0.799 c	0.707 bc	11.6 abc
Rosemarie	41 c	41 c	0.789 c	0.669 cde	15.3 cd
Kieffer	43 cd	58 d	0.768 d	0.635 de	17.4 cd
EBC [z]	45 d	72 e	0.762 d	0.632 de	17.1 cd
PT [y]	54 e	98 f	0.749 d	0.623 e	16.9 cd
Shaded faces					
Bon Rouge	45 d	71 e	0.832 ab	0.727 ab	12.7 b
Forelle	60 g	111 g	0.840 a	0.681 bcd	19.0 de
Flamingo	57 fg	108 fg	0.829 ab	0.682 bcd	17.7 cde
Rosemarie	66 h	112 g	0.826 ab	0.635 de	23.0 e
Kieffer	56 ef	110 g	0.823 ab	0.708 bc	14.0 cd
EBC	57 fg	112 g	0.824 ab	0.674 bcde	18.3 de
PT	58 fg	108 g	0.756 d	0.632 e	16.3 cd
Contrasts					
Side	0.0001	0.0001	0.0001	0.0414	0.0001
Cultivar	0.0001	0.0001	0.0001	0.0001	0.0002
Side * Cultivar	0.0001	0.0001	0.0012	0.0005	0.0056

[z] Early Bon Chretien

[y] Packham's Triumph

Table 3. F_v/F_m ratios and the percentage photoinhibition (PI) in peel disks of pear fruit of different hue angles before and after exposure to high light (1200 μmol m^{-2} s^{-1} PPFD) at 10°C for three hours. Hue angles reported vary from 0° (red-purple) to 120° (yellow-green). Means were separated by LSD (5%).

Cultivar	Lightness	Hue (°)	F_v/F_m Before	F_v/F_m After	PI (%)	PI after 24 hours (%)
Exposed						
Bon Rouge	27 a	15 a	0.816 ab	0.752 a	7.9 a	1.2 a
Forelle	31 b	24 a	0.819 ab	0.726 ab	11.4 abc	1.8 a
Flamingo	36 c	40 b	0.777 e	0.608 cde	21.9 cd	2.3 a
Rosemarie	42 d	45 b	0.782 de	0.614 cde	21.6 bcd	2.4 a
Kieffer	40 d	56 c	0.785 cde	0.654 bcd	16.7 abc	2.3 a
EBC [z]	51 f	84 d	0.761 e	0.593 de	22.1 cd	3.3 ab
PT [y]	54 fg	102 e	0.774 e	0.582 de	24.7 cde	1.9 a
Shaded						
Bon Rouge	47 e	75 d	0.827 ab	0.689 abc	16.7 abc	7.4 abc
Forelle	59 h	113 f	0.840 a	0.599 de	28.6 de	12.8 cd
Flamingo	58 gh	108 ef	0.841 a	0.578 de	31.3 def	10.0 bcd
Rosemarie	65 i	110 ef	0.833 ab	0.472 fg	43.4 g	14.5 cd
Kieffer	55 g	111 ef	0.813 abc	0.548 ef	32.6 ef	10.5 bcd
EBC	61 h	109 ef	0.807 bcd	0.444 g	44.8 g	15.5 d
PT	60 h	109 ef	0.783 de	0.462 fg	40.8 fg	10.1 bcd
Contrasts						
Side	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001
Cultivar	0.0001	0.0001	0.0001	0.0001	0.0001	0.6627
Side * Cultivar	0.0001	0.0001	0.0603	0.4264	0.3570	0.8195

[z] Early Bon Chretien

[y] Packham's Triumph

Table 4. Changes in pigment concentration (μg g^{-1} fr wt) in exposed and shaded faces of 'Forelle', 'Kieffer' and 'Packham's Triumph' pears subjected for 24 hours to high light (1200 μmol m^{-2} s^{-1} PPFD) at 10° or 40°C. Means were separated by LSD (5%).

	Chlorophyll	Carotenoids	Anthocyanin
Cultivar			
Forelle	31.2	10.5 b	18.0 a
Kieffer	31.3	13.3 a	3.6 b
Packham's Triumph	30.8	9.1 c	0.1 c
Side			
Sun	33.1 a	11.9 a	13.4 a
Shade	29.1 b	9.9 b	1.1 b
Treatment			
Control	34.0 a	10.5	7.7 a
10°C	30.5 b	11.2	8.1 a
40°C	28.8 b	11.1	5.9 b
Contrasts			
Cultivar	0.9543	0.0001	0.0001
Side	0.0036	0.0002	0.0001
Treatment	0.0069	0.4263	0.0110
Cult. * Side	0.2940	0.3910	0.0001
Cult. * Treatment	0.9885	0.8496	0.0519
Side * Treatment	0.8447	0.4610	0.0441
3-Factor interaction	0.9147	0.7964	0.2166

Table 5. Correlation between pigment concentrations of exposed faces of different pear cultivars, and hue, F_v/F_m before and after photoinhibitory treatment and the percentage photoinhibition.

	Hue (°)	Initial F_v/F_m	F_v/F_m after treatment	Photoinhibition (%)
10°C				
Anthocyanin	-0.85 **	0.86 **	0.75 *	-0.69 *
Chlorophyll	-0.25	0.30	0.13	-0.08
Carotenoids	-0.58	0.61	0.60	-0.58
40°C				
Anthocyanin	-0.85 **	0.96 ***	0.79 *	-0.51
Chlorophyll	-0.18	0.25	0.30	-0.27
Carotenoids	-0.24	0.37	0.42	-0.36

[z] Pearson Correlation Coefficients / Prob > |R| under Ho: Rho=0 / N where N = 9.

*, ** and *** denote correlation coefficients significant at P = 0.05, 0.01 and 0.001, respectively.

Table 6. PSII efficiency of exposed and shaded 'Bon Rouge', 'Forelle', 'Rosemarie' and 'Packham's Triumph' pear peel at increasing light levels.

	PPFD (μmol m^{-2} s^{-1})			
	28	96	300	885
Cultivar				
Bon Rouge	0.615 a	0.491 a	0.271 a	0.099 a
Forelle	0.622 a	0.485 a	0.253 ab	0.088 b
Rosemarie	0.572 b	0.451 b	0.252 ab	0.091 ab
Packham's Triumph	0.574 b	0.447 b	0.242 b	0.085 b
Side				
Exposed	0.620 a	0.536 a	0.320 a	0.122 a
Shaded	0.572 b	0.401 b	0.188 b	0.060 b
Contrasts				
Cultivar	0.0003	0.0057	0.0478	0.0353
Side	0.0001	0.0001	0.0001	0.0001
Cultivar * Side	0.2372	0.7935	0.1351	0.0844

Table 7. Photochemical quenching at increasing light levels in exposed and shaded 'Bon Rouge', 'Forelle', 'Rosemarie' and 'Packham's Triumph' pear peel.

	PPFD (μmol m^{-2} s^{-1})			
	28	96	300	885
Exposed peel				
Bon Rouge	0.852 a	0.738 a	0.456 a	0.188 b
Forelle	0.847 a	0.752 a	0.469 a	0.196 ab
Rosemarie	0.858 a	0.763 a	0.496 a	0.209 a
Packham's Triumph	0.858 a	0.739 a	0.459 a	0.187 b
Shaded peel				
Bon Rouge	0.780 b	0.594 b	0.318 b	0.116 c
Forelle	0.768 b	0.526 c	0.234 c	0.075 d
Rosemarie	0.723 c	0.523 c	0.262 c	0.093 d
Packham's Triumph	0.762 bc	0.542 bc	0.266 c	0.091 d
Contrasts				
Cultivar	0.3670	0.4442	0.0850	0.0694
Side	0.0001	0.0001	0.0001	0.0001
Cultivar * Side	0.1851	0.0901	0.0090	0.0093

Table 8. Non-photochemical quenching at increasing light levels in exposed and shaded 'Bon Rouge', 'Forelle', 'Rosemarie' and 'Packham's Triumph' pear peel.

	PPFD ($\mu mol\ m^{-2}\ s^{-1}$)			
	28	96	300	885
Exposed peel				
Bon Rouge	0.395 a	0.481 ab	0.601 ab	0.698 bc
Forelle	0.319 c	0.401 d	0.535 c	0.677 bc
Rosemarie	0.385 a	0.489 ab	0.608 a	0.713 a
Packham's Triumph	0.381 ab	0.466 bc	0.564 bc	0.671 c
Shaded peel				
Bon Rouge	0.392 a	0.515 a	0.635 a	0.733 a
Forelle	0.272 d	0.354 e	0.455 d	0.613 d
Rosemarie	0.346 bc	0.482 ab	0.595 ab	0.707 ab
Packham's Triumph	0.337 c	0.430 cd	0.540 c	0.668 c
Contrasts				
Cultivar	0.0001	0.0001	0.0001	0.0001
Side	0.0017	0.1673	0.0573	0.2709
Cultivar * Side	0.3056	0.0363	0.0108	0.0068

Fig. 1. Horizontal lines give the growth periods of 'Forelle' (FR), 'Kieffer' (KF), 'Packham's Triumph' (PT), 'Flamingo' (FL), 'Bon Rouge' (BR), 'Rosemarie' (RM) and 'Early Bon Chretien' (EBC) pears, from anthesis until harvest. Arrows denote the collection dates of fruit used in three photoinhibition experiments relative to anthesis and harvest dates. All seven cultivars were used in the first two experiments while only FR, KF and PT were used in the final experiment.

Fig. 2. Correlation between hue and F_v/F_m ratios of exposed (A) and shaded (B) faces of 'Bon Rouge' (BR), 'Forelle' (FR), 'Flamingo' (FL), 'Rosemarie' (RM), 'Kieffer' (KF), 'Early Bon Chretien' (EBC) and 'Packham's Triumph' (PT) pears before and after exposure to 500 μmol m^{-2} s^{-1} PPFD at 10ºC for three hours. Hue angles reported vary from 0º (red-purple) to 120º (yellow-green). When larger than the symbol, ± SE are shown, n = 3 replicates of two fruit each.

Fig. 3. Correlation between hue and F_v/F_m ratios of exposed (A) and shaded (B) faces of 'Bon Rouge' (BR), 'Forelle' (FR), 'Flamingo' (FL), 'Rosemarie' (RM), 'Kieffer' (KF), 'Early Bon Chretien' (EBC) and 'Packham's Triumph' (PT) pears before and after exposure to 1200 μmol m^{-2} s^{-1} PPFD at 10ºC for three hours. Hue angles reported vary from 0º (red-purple) to 120º (yellow-green). When larger than the symbol, ± SE are shown, n = 3 replicates of two fruit each.

Fig. 4. Correlation between hue and F_v/F_m ratios of exposed (A, C) and shaded (B, D) faces of 'Forelle' (FR), 'Kieffer' (KF), and 'Packham's Triumph' (PT) pears before and after exposure for 24 hours to 1200 μmol m^{-2} s^{-1} PPFD at 10ºC (A, B) or 40ºC (C, D). Hue angles reported vary from 0º (red-purple) to 120º (yellow-green). When larger than the symbol, ± SE are shown, n = 3 replicates of four fruit each.

Fig. 5. Fv/Fm ratios in exposed (A and C) and shaded (B and D) peel of 'Forelle' (FR), 'Kieffer' (KF) and 'Packham's Triumph' (PT) pears before and after exposure for 24 hours to high light (1200 μmol m^{-2} s^{-1} PPFD) at 10ºC (A, B) or 40ºC (C, D) as well as after 24 hour recovery in darkness at 20ºC. When larger than the symbol, ± SE are shown, n = 3 replicates of four fruit each.

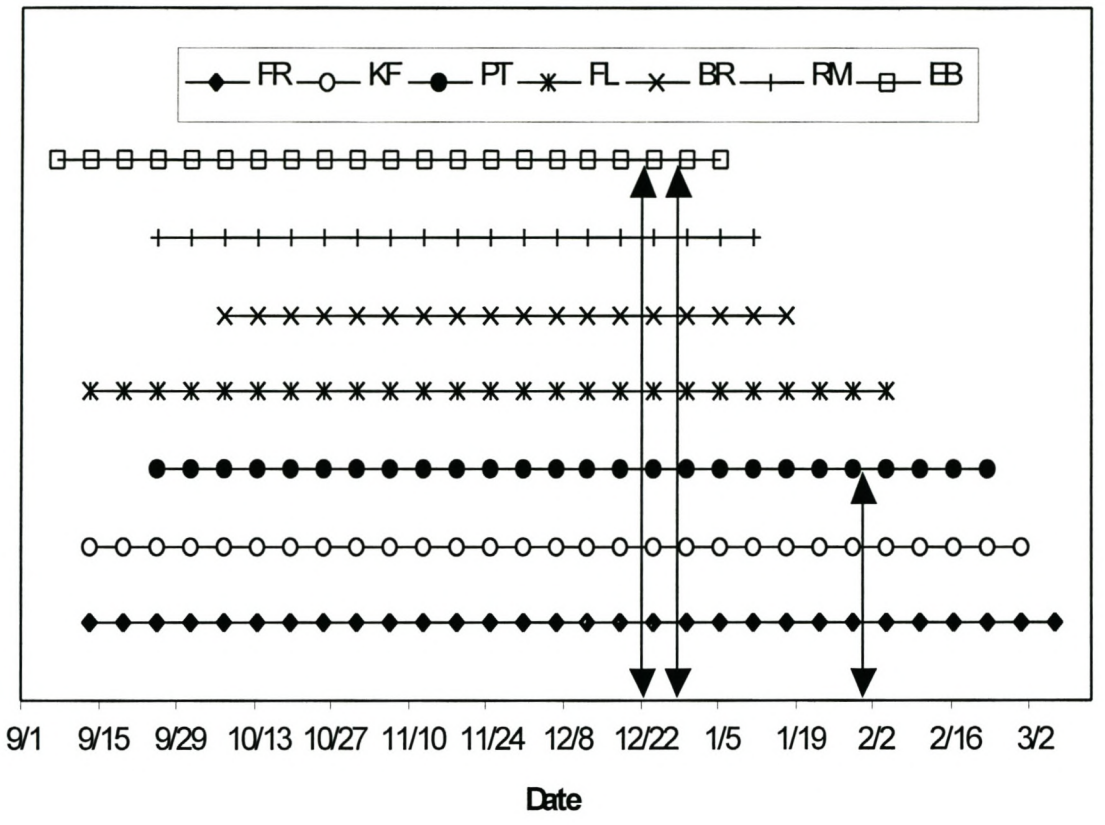


Fig. 1 Paper 4

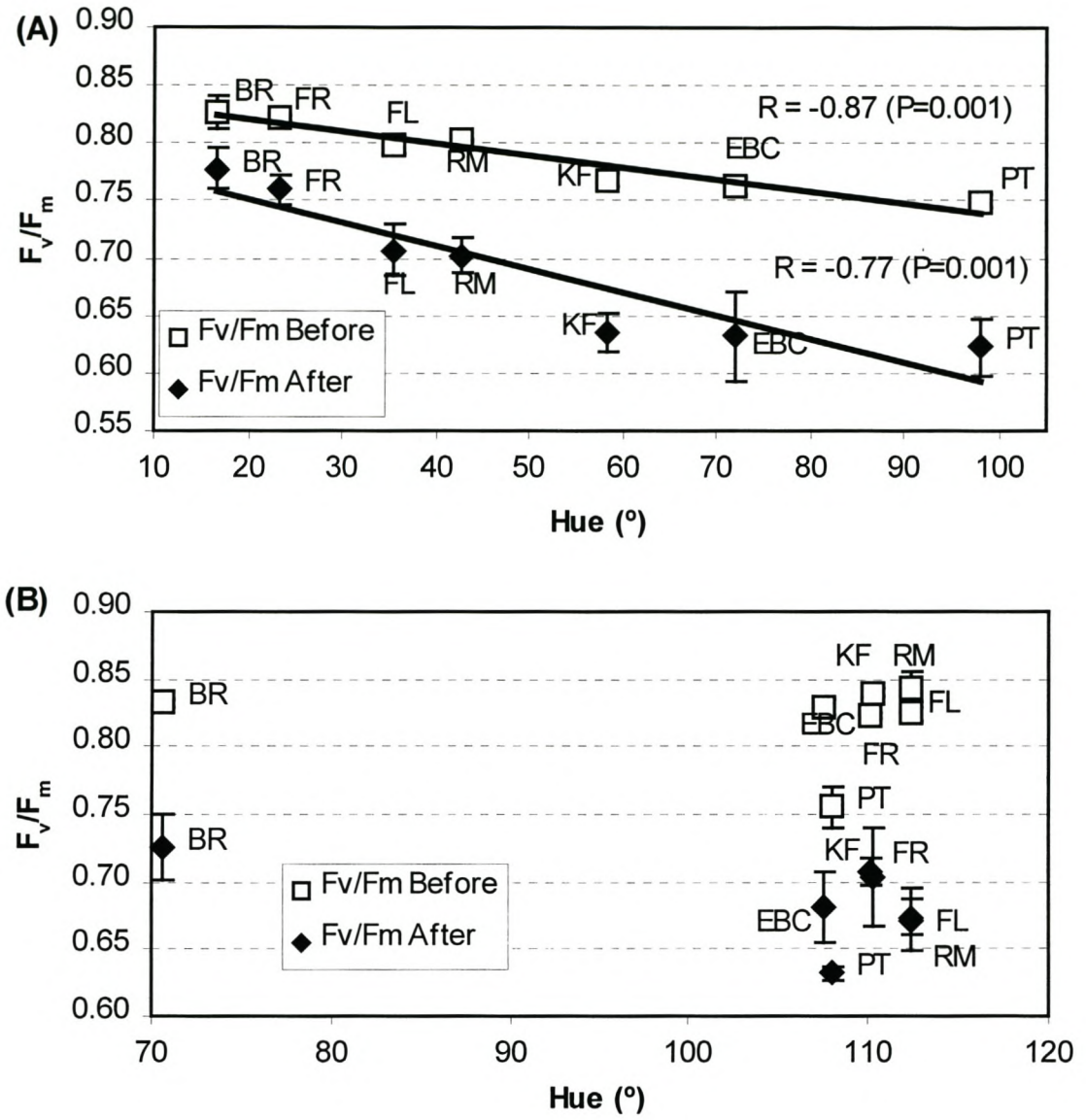
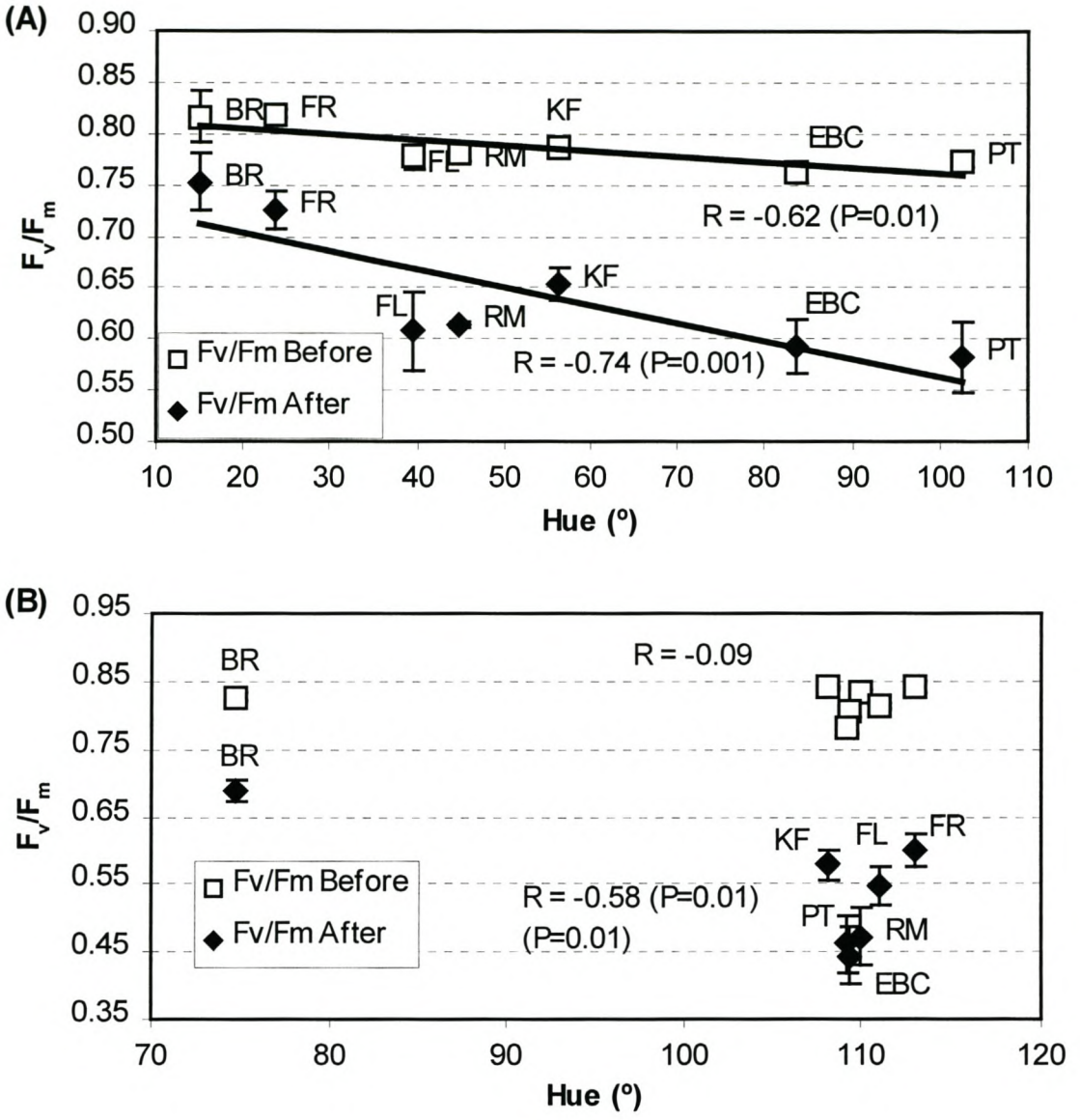


Fig. 2 Paper 4



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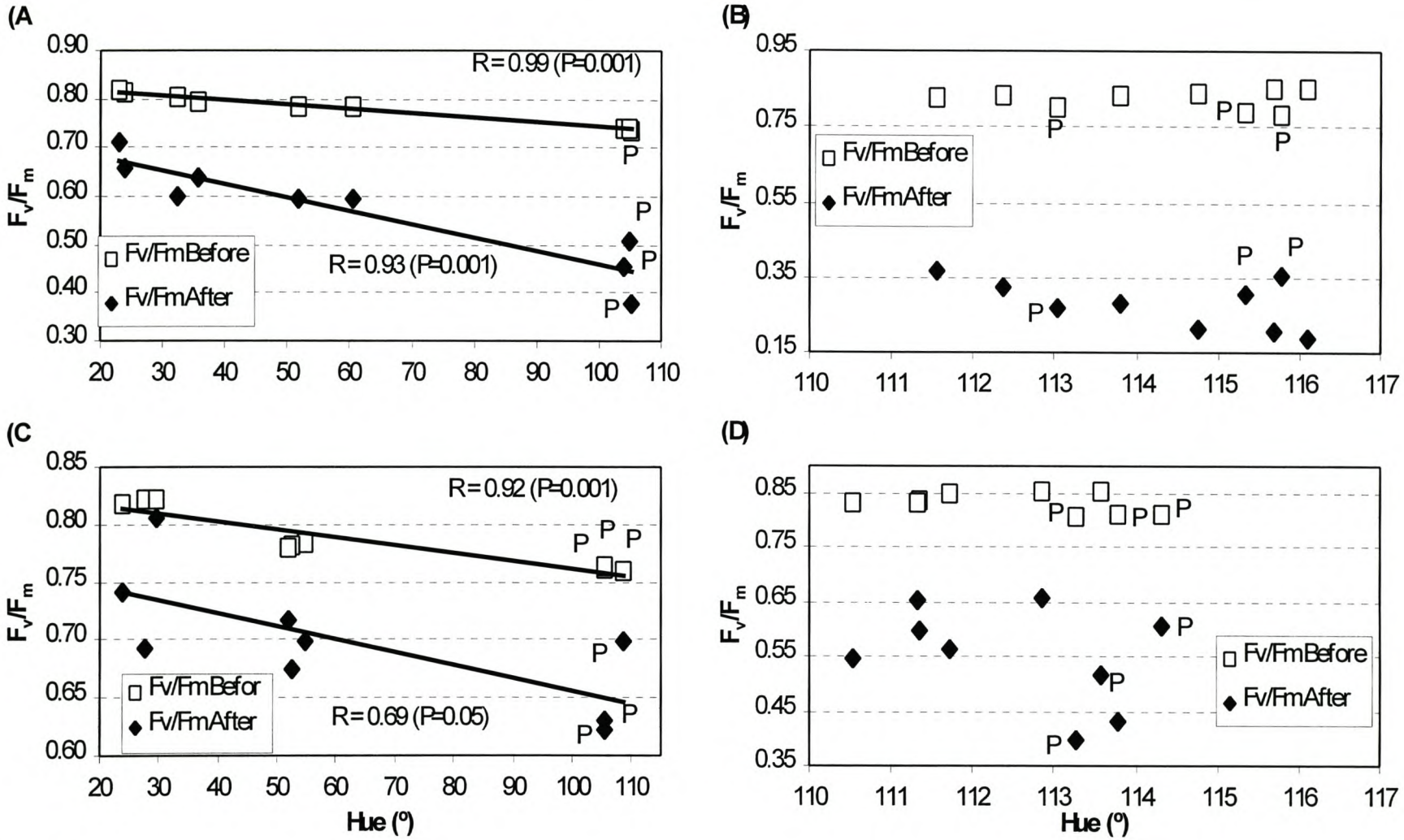

Fig. 4 Paper 4

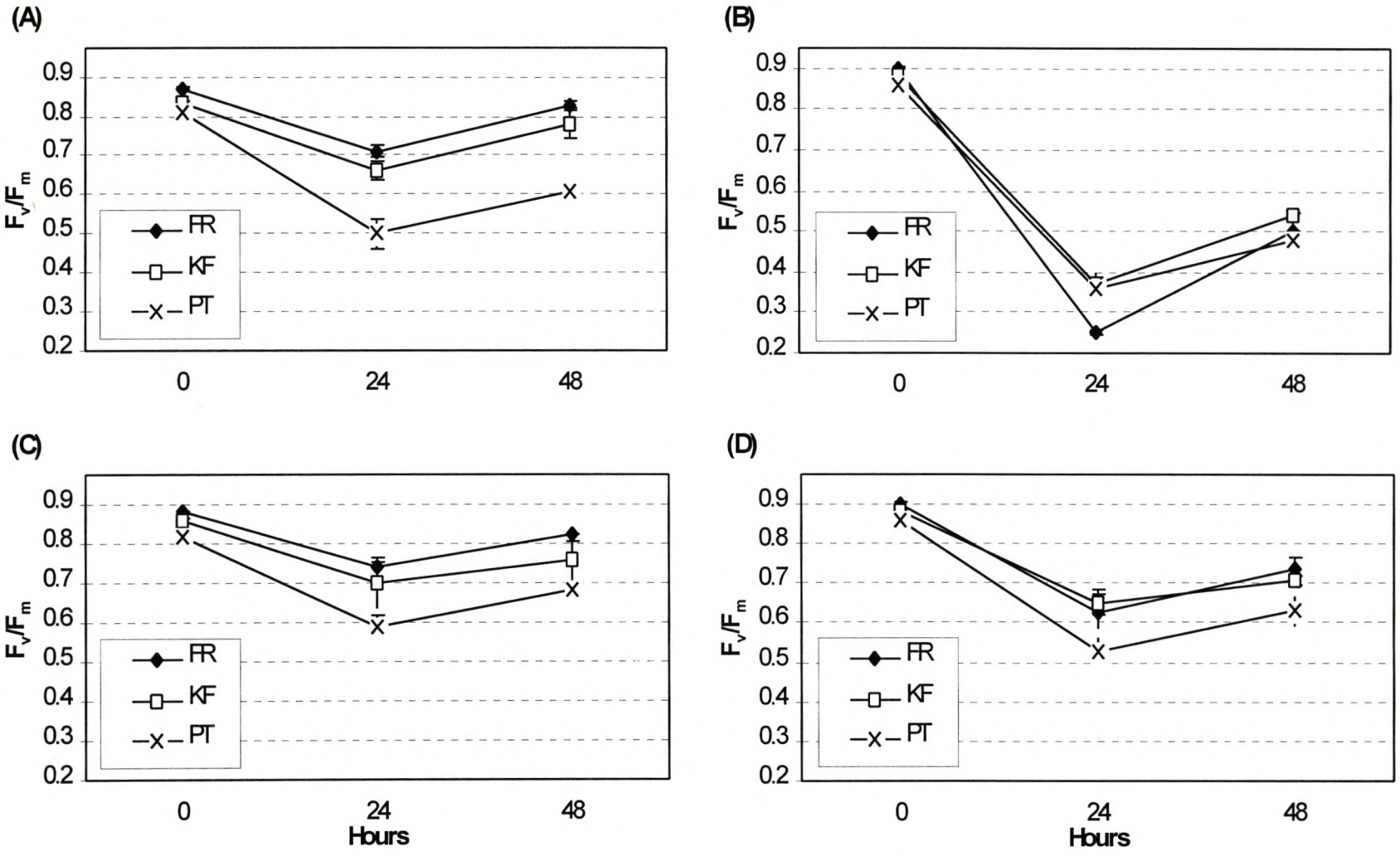

Fig. 5 Paper 4

GENERAL SUMMARY AND CONCLUSIONS:

Red colour loss.

Our investigations were primarily concerned with red colour development and loss in fully red and blushed pears. Poor fruit colour of some blushed cultivars is purportedly due to the loss of red colour during warm periods prior to harvest (Huysamer, 1998). In this study, we quantitatively confirmed the occurrence of pre-harvest red colour loss and established net anthocyanin degradation at high temperatures as the cause. Pear cultivars differed in their susceptibility to colour loss, with 'Rosemarie' being the most susceptible. We identified three main factors that contributed to red colour loss in pears namely, 1) a dependence on low temperatures for anthocyanin synthesis, 2) the inherent developmental pattern of colour development in pears and, 3) a low capacity to accumulate anthocyanin.

Differences in susceptibility to red colour loss.

Unlike apples, where red colour development in all cultivars seems to require or benefit from low temperatures (Curry, 1997), only 'Rosemarie' amongst the pear cultivars studied responded significantly to low temperatures. Assessment of the activities of enzymes related to anthocyanin synthesis confirmed that low temperatures induced anthocyanin synthesis in 'Rosemarie', but not in 'Bon Rouge'. 'Rosemarie' colour increased with the passing of cold fronts due to anthocyanin synthesis, only to fade again during intermittent warmer periods when increased degradation and reduced synthesis of anthocyanin resulted in net anthocyanin degradation.

The colour of the other blushed and red pear cultivars was much less responsive to temperature. These cultivars displayed a similar general pattern of red colour development, attaining their best red colour about a month or more before harvest, whereafter colour gradually faded towards harvest at a rate related to the maximum anthocyanin concentrations attained during fruit development. The fading of red colour appeared to be developmentally regulated. Even 'Rosemarie' displayed a reduced ability to accumulate anthocyanin in response to cold fronts closer to harvest. This corresponds with grower observations that the incidence of red colour

loss increases towards harvest (Huysamer, 1998). In fruit kinds where best red colour is attained at maturity, for example apples (Lancaster, 1992), the increasing ability to accumulate anthocyanin should limit the effect of pre-harvest red colour loss to poor fruit colour at harvest.

The visual expression of changes in anthocyanin levels depends on the initial anthocyanin concentration of fruit. We found that high initial anthocyanin concentrations buffered fruit against visible red colour loss even when considerable reductions in concentration had occurred. This is due to the exponential relationship between anthocyanin concentration and hue at high pigment levels. The relationship becomes linear at lower pigment levels. As a consequence, much larger reductions in anthocyanin concentration are required to induce a similar extent of visible colour change in well-coloured fruit with high anthocyanin concentrations compared to poorly coloured fruit containing little anthocyanin. Of all the pear cultivars studied, 'Rosemarie' displayed the lowest capacity to accumulate anthocyanin and is, therefore, most susceptible to visible red colour loss.

Improvement of red colour.

We evaluated pulsed application of overhead evaporative cooling (EC) as a measure to improve red colour of blushed pears. 'Forelle' did not benefit from EC, probably due to the stability of red colour in this cultivar. In 'Rosemarie', early initiation of EC at the end of November was ineffective in improving red colour. This was ascribed to the possible acclimation of fruit to the milder conditions and requires further research. By reducing colour loss, EC application from two weeks before harvest at air temperatures exceeding 28ºC increased the redness of 'Rosemarie' pears compared to the control. However, the beneficial effect of EC on fruit colour was relatively small compared to the extent of colour fluctuation in response to temperature. Therefore, we do not consider EC a final solution for the poor colour problem of 'Rosemarie'. New plantings should rather be confined to cool production regions. Harvesting of 'Rosemarie' could also be scheduled to coincide with the passing of a cold front, which can considerably improve final fruit colour.

We observed that cultivation of 'Rosemarie' pears on dwarfing rootstocks or on sandy soils improves red colour development. This improvement was likely due to increased

anthocyanin synthesis. Although the beneficial effect of dwarfing rootstocks on fruit colour has been reported, the underlying reason remains to be established.

Applications and future research.

Results reported here could have considerable application in the breeding and selection of new blushed pear cultivars. Breeders should aim to identify the cultivars that transfer the low-temperature requirement for anthocyanin synthesis to their progeny, since this characteristic, together with a low capacity for anthocyanin accumulation, increases susceptibility to pre-harvest red colour loss. This could easily be achieved by measuring daily changes in hue in response to cold fronts. However, since high anthocyanin concentrations mask fluctuation in anthocyanin levels, it might be necessary to also assess changes in the activity of anthocyanin synthesising enzymes. The lowest hue value attained during fruit development could also give an indication of potential susceptibility to visible red colour loss.

In most fruit kinds, anthocyanin accumulation peaks in mature fruit. Boss et al. (1996) postulated the involvement of a ripening-associated regulatory gene in grape berries. Pear cultivars have been reported in which anthocyanin accumulated towards harvest (Dussi et al., 1997). One of these cultivars is a 'Bartlett' mutation, like 'Bon Rouge' included in our study. Since an increasing ability to accumulate anthocyanin towards harvest reduces the risk for pre-harvest red colour loss, establishing the molecular basis of these contrasting pigmentation patterns may prove rewarding.

The optimum temperatures for anthocyanin synthesis in 'Rosemarie' have not been established. We also do not know if anthocyanin synthesis has the same diurnal temperature requirement as found in apples (Curry, 1997) and whether the optimum temperature for synthesis changes during fruit development. Studies of anthocyanin synthesis in pears under controlled conditions are hampered by the inability of detached pears to accumulate anthocyanin. As has previously been done in grape (Boss et al., 1996), molecular studies of gene expression could provide insight into the regulation of red colour development in pears.

Anthocyanin photoprotection in pear peel.

The attainment of maximum anthocyanin concentrations about midway between anthesis and maturity in pear peel is not congruent with the proposed function of anthocyanin in seed dispersal. Hence, the possible photoprotective function of anthocyanin in pears was assessed. We found that the resistance of fruit of different pear cultivars to light stress under natural conditions and in response to photoinhibitory treatment increased as peel increased in redness. However, there were indications that despite this association between red skin colour and increased resistance to photoinhibition, photoprotection was not necessarily due to light attenuation by anthocyanin. The basis of light stress tolerance in pears and its relation to red skin colour and productivity requires further investigation. The involvement of anthocyanin in photoprotection in pear peel could be resolved by utilising the differences in the ability of anthocyanin to absorb red and blue-green light as has been done in other systems (Smillie and Hetherington, 1999).

Conclusion

The research reported here increases our understanding of red colour development and loss in pears and could assist in future breeding and production of blushed pear cultivars. All the blushed and fully red pear cultivars grown in South Africa decrease in redness or in their ability to accumulate anthocyanin towards harvest. With the exception of 'Rosemarie', where anthocyanin synthesis requires low temperatures and, therefore, is restricted to the passing of cold fronts, fading of colour is gradual and relates to the maximum pigment levels attained during fruit development. The comparatively low anthocyanin concentrations maintained throughout fruit development in 'Rosemarie' results in the fluctuation of colour between red and green in response to climatic conditions. Anthocyanin synthesis in other blushed and fully red cultivars does not appear to require low temperatures. Hence, red colour is more stable in these cultivars.

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