Fruit Pigmentation Studies

by

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DECLARATION

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ABSTRACT

For many apple (*Malus domestica* Borkh.) and pear (*Pyrus communis* L.) cultivars, attractive colour is essential to their profitability on export markets. This study focuses on problems related to poor green colour of 'Granny Smith' apples and insufficient red colour of bi-coloured pear cultivars.

'Granny Smith' apples often suffer from poor green colour. Green colour of fruit from various orchards was already found to differ midway through fruit development, with these differences being carried through to harvest. In a trial where nitrogen (N) fertilisers were applied using different forms at different times, there was no improvement in green colour. In another trial, artificial shading was applied to fruit only during their early development. Fruit that were shaded during this time were less green at harvest than unshaded fruit. Additional N applications may only improve colour where a deficiency exists. However, green colour may be improved by increasing light distribution early during fruit development.

Bi-coloured pears attain their maximum red colour midway through their development, and this desired red colour is mostly lost prior to harvest. Red colour can also increase transiently with the passing of cold fronts. Anthocyanins, responsible for this red colour, may have a photoprotective function which would explain this pigmentation pattern, as photosystems are particularly sensitive to light damage at low temperatures. As 'Rosemarie' fruit bent over from a vertical to hanging position during development, peel photoinhibition was reduced as anthocyanins were synthesised. 'Forelle' peel was found to be very sensitive to high light levels at low temperatures. Substantial anthocyanin development took place in 'Cripps' Pink' apples when weather conditions were cold, but clear following a cold front. A photoprotective role seems to explain daily changes in anthocyanins in response to temperature, but not the seasonal progression of colour development.

Dwarfing rootstocks are known to improve red colour of bi-coloured pears due to improved light distribution. ‘Forelle’ fruit from six rootstocks of varying vigour were harvested from exposed positions only, so as to establish the effect of rootstock on red colour development independent of the effect of rootstock on canopy light distribution. Fruit from trees on quince (*Cydonia oblonga* Mill.) rootstocks were found to have redder fruit than those from vigorous BP pear rootstocks. This may be due to higher chlorophyll and carotenoid concentrations present in the peel of fruit from BP rootstocks, whose leaf and peel N were also high. The use of quince rootstocks is recommended where red colour development of bi-coloured pears is a problem.

An early season bi-coloured cultivar with good red colour is required. Breeding trials to find such a cultivar are resource intensive. To streamline the process, a method to preselect immature seedlings for their future fruit colour is required. Fruit colour from bearing seedlings was compared with colour of their immature leaves. Trees with red leaves were likely to produce fruit that were too red for the breeders’ requirements. Trees with green or blushed leaves were capable of producing blushed fruit. It would be feasible to cull red-leaved seedlings with minimal risk of losing potential bi-coloured cultivars.

OPSOMMING

Verskeie appel (*Malus domestica* Borkh.) en peer (*Pyrus communis* L.) kultivars se winsgewendheid word bepaal deur hul aantreklike kleur. In hierdie studie word die swak groen kleur van ‘Granny Smith’ appels asook rooi kleurontwikkeling van blospere ondersoek.

Die groen kleur van ‘Granny Smith’ appels is dikwels onvoldoende. Verskille in groen kleur tussen boorde was reeds gedurende vroeë vrugontwikkeling aanwesig, en hierdie verskille het voortgeduur tot met oes. Groen kleur kon nie deur verskillende bronne en tye van stikstofbemesting verbeter word nie. Stikstofbemesting verbeter groen kleur moontlik net in boorde met ‘n stikstoftekort. Vrugte wat gedurende hul vroeë ontwikkeling oorskadu is, se groen kleur was swakker by oes in vergelyking met vrugte wat nie oorskadu is nie. Groen kleur kan moontlik verbeter word deur ligverspreiding tydens vroeë vrugontwikkeling deur middel van snoei aksies te verhoog.

Blospeerkultivars bereik hul maksimum rooi kleur halfpad deur hul ontwikkeling, maar is geneig om hul rooi kleur grootliks voor oes te verloor. Rooi kleur mag egter kortstondig toeneem in reaksie op die lae temperature gepaardgaande met koue fronte. Antosianiene, wat verantwoordelik is vir die rooi kleur, het moontlik ‘n beskermende funksie teen hoë ligvlakke, en hierdie funksie mag moontlik die bogenoemde patroon van rooikleurontwikkeling verklaar. Die natuurlike buiging van ‘Rosemarie’ pere van hul aanvanklike regop oriëntasie tot hul karakteristieke hangende posisie, is gekenmerk deur ‘n afname in fotoinhibisie van die skil en ‘n gelyklopende sintese van antosianien. ‘Forelle’ skil was uiters sensitief vir hoë ligvlakke in kombinasie met lae temperature (16 ºC). ‘Cripps’ Pink’ appels het ‘n vinnig toename in rooi kleur getoon met die koue, maar helder, weerstoestande wat gevolg het op ‘n kouefront.

Dit is welbekend dat dwergende onderstamme die rooi kleur van blospere verbeter deur ligverspreiding in die boom te verhoog. Ten einde die effek van onderstam op rooi kleurontwikkeling onafhanklik van die effek van onderstam op ligverspreiding te ondersoek, is ‘Forelle’ pere wat blootgestel was aan vol son geoes van bome geënt op ses onderstamme met verskillende groeikrag. Kweperonderstamme (*Cydonia oblonga* Mill.) het rooi kleur verbeter in vergelyking met die groeikragtige BP peeronderstamme. ‘n Moontlike rede vir die verbetering is die laer chlorofiel- en karotenoïedkonsentrasies in die skil van vrugte op kweperonderstamme. Bome op peeronderstamme het ook hoër blaar- en skil stikstofvlakke gehad. Kweperonderstamme word aanbeveel in gevalle waar rooi kleurontwikkeling van blospere ‘n probleem mag wees.

Die RSA vrugtebedryf benodig ‘n vroeë blospeerkultivar met goeie rooi kleurontwikkeling. Die teling van so ‘n kultivar is hulpbronintensief en baie duur. Ten einde die teelproses meer effektief te maak, word ‘n metode benodig om saailinge al voor uitplanting in die boord te selekteer na gelang van hul toekomstige vrugkleur. Die vrugkleur van oesryp pere van draende saailinge is vergelyk met die kleur van hul onvolwasse blare. Bome met rooi blare is geneig om vrugte te dra wat té rooi is om te kwalifiseer as blospere. Die meerderheid blospere is afkomstig van bome met blos of groen onvolwasse blare. Dit is prakties haalbaar om rooiblaarsaailinge uit te dun, met net ‘n klein, aanvaarbare risiko om ‘n moontlike blospeerkultivar in die proses te verloor.

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OVERALL OBJECTIVE

The South African pome fruit industry is plagued by a number of problems with fruit colour that reduces profitability of certain cultivars.

'Granny Smith' apples need to be a dark green colour to be desirable for consumers, but whitening of the peel before harvest is common (Warrington, 1994). Previous trials conducted in our lab to improve colour with the application of nutrient and hormone sprays have been relatively ineffective (Griessel, 1991). Light and nitrogen play an important role in green colour because they are essential for chlorophyll synthesis (Purohit and Ranjan, 2002). The loss of colour with maturity has been studied, but little is known of green colour development earlier in the season (Mussini et al., 1985). We measured the colour of fruit from various orchards to try to pinpoint a causal factor of poor colour. Differences in colour were already determined midway through fruit development. Nitrogen and shading trials were conducted to measure the effects of these factors on green colour during early fruit development and at harvest. I also chose to do my literature review on the effect of light and nitrogen on 'Granny Smith' green colour, as a number of recent literature reviews in our department have covered general fruit colour and anthocyanins in depth.

Bi-coloured pears are often downgraded due to insufficient red colour development (Huysamer, 1998). Bi-coloured pears are reddest midway through fruit development, but red colour can also show a transient increase with passing cold fronts (Steyn et al., 2004) The red pigment, anthocyanin, has the ability to afford photoprotection to underlying tissues (Smillie and Hetherington, 1999), and this may explain the seasonal and daily pigmentation patterns. To confirm whether maximum red colour occurs midseason, because that is when fruit seem to be most at risk to photodamage, change of colour and photoinhibtion with fruit bending were measured, along with the response of previously shaded peel to sudden exposure to sunlight. The reaction of 'Forelle' pear peel and leaves to

light stress during a simulated cold front, and ‘Cripps’ Pink’ apple colour development in the field during an actual cold front were also measured to see if the photoprotective function of anthocyanins may explain the daily pigmentation pattern.

Light is essential for anthocyanin synthesis, so bi-coloured pears from trees on dwarfing rootstocks are known to have better red colour due to improved light distribution within the tree (Du Plooy and Van Huyssteen, 2000). It has been suggested that dwarfing rootstocks may impart other characteristics, not related to light interception, that affect colour of the scion (Jackson, 1967). We measured colour of mature fruit sampled from fully exposed positions from ‘Forelle’ trees grafted to six different rootstocks of varying vigour. By selecting fruit from exposed positions we hoped to negate the light effects of the rootstocks in order to bring any underlying differences to light.

The pear industry requires a bi-coloured pear that matures early in the season and has reliable red colour development (Human, 2005). Breeding trials are underway, but they are highly resource intensive because they require planting and maintaining thousands of seedlings for at least six years until they fruit. Breeders are lucky if they can find one suitable cultivar from such a trial. In order to reduce costs, and improve the odds of success, a method is required to cull undesirable seedlings when only one year old. Breeders observed that immature seedlings with red immature leaves would produce red fruit, and that new leaves on fruiting seedlings would be the same colour that their leaves were when they were one year old. Colour of fruit and immature leaves from fruiting seedlings were compared to see if there may be any correlations that could be used to cull unnecessary seedlings.

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LITERATURE REVIEW:

THE ROLE OF LIGHT AND NITROGEN IN GREEN COLOUR OF 'GRANNY SMITH' APPLES.

'Granny Smith' is the most widely planted apple cultivar in South Africa (24% of the total area planted), accounting for 27% of export volume (Deciduous Fruit Producers' Trust, 2008). The skin of these fruit is an intense dark, green, which becomes greenish-yellow with maturity (Warrington, 1994). In order to qualify as Class one fruit, fruit should be uniformly green, but pale skin is a common problem (Hirst et al., 1990). Chlorophyll is responsible for the green colour in plants and its role is to harvest the light used in photosynthesis (Willows, 2004). Yellowing of the fruit before harvest is a result of chlorophyll degradation revealing the carotenoids present in the peel as opposed to an increase in carotenoid synthesis (Mussini et al., 1985). Thus, in order to deepen our understanding of green colour development of 'Granny Smith' apples we will briefly touch on chlorophyll biosynthesis, the presence and behaviour of chlorophyll in fruit, and then focus on light and nitrogen as the most important factors that influence chlorophyll synthesis, and as factors that can be manipulated by growers to improve green colour.

Chlorophyll biosynthesis and degradation.

There are two types of chlorophyll pigment (chlorophyll *a* and chlorophyll *b*) and they are nearly identical in their structure. Chlorophylls are found in plastids called chloroplasts that are synthesised readily in young, developing tissue. Within the chloroplasts, chlorophylls are contained in the thylakoid membranes. The role of chlorophyll is to capture the light used to drive photosynthesis reactions. Chlorophylls appear green because they absorb violet, blue, orange and red wavelengths of light, but reflect 20% of green light (Salisbury and Ross, 1992).

The steps of chlorophyll synthesis are summarised in Figure 1. The first important process of chlorophyll synthesis is the conversion of glutamatic acid to aminolevulinic acid (ALA), a five-carbon compound. Eight ALA molecules are used to synthesise the basic tetrapyrole structure. Protoporphyrin IX is the last compound in the pathway that is shared by the chlorophyll and haem biosynthesis pathways. The steps that take place from the insertion of magnesium into the protoporphyrin IX up until the final addition of the phytol tail to chlorophyllide *a* to produce chlorophyll *a*, are unique to chlorophyll's biosynthesis pathway. Chlorophyll *a* is oxidised to form chlorophyll *b*, and interconversion is cyclical (Reinbothe and Reinbothe, 1996; Willows, 2004).

The pathway of chlorophyll degradation is not fully understood because many of the by-products are colourless and hence, difficult to study. The first step for both chlorophyll *a* and *b* is the removal of the phytol tail, chlorophyllase being the catalyst. Next, magnesium is removed with the help of magnesium dechelatase. Chlorophyll is degraded in senescing tissues so that nutrients can safely be recycled from photosynthetic proteins without running the risk of the free chlorophyll causing photo-oxidation (Willows, 2004).

Occurrence of green fruit.

Although nearly all fruit are green when unripe, their colour upon ripening tends to vary, with many fruit being shades of red, orange, yellow or blue (Gross, 1987). However, some fruit retain much of their chlorophyll at maturity and can be classified as green-ripe (Gross, 1987). This can be as a background colour as for some apple and pear cultivars, or the green colour can also occur inside the fruit like in avocados, kiwi fruit and some melons (Gross, 1987). The most important of these to consider would be fruit whose entire peel remains green despite maturity (Gross, 1987). 'Granny Smith' most likely originated as a seedling from open-pollinated 'French Crab' apple. It was selected for its excellent cooking, storage and bearing qualities, and has remained unchanged in cultivation ever since (Warrington, 1994). Because 'Granny Smith' was artificially

selected for its qualities, we cannot argue an adaptive advantage to its green peel colour. However, by studying the occurrence of green fruits in the wild, we may gain some knowledge that would assist us in improving the green colour of ‘Granny Smith’.

Fruit that are green when ripe tend to be large with large seeds, quite odorous, have protective outer layers and are generally dispersed by mammals. Cipollini and Levey (1991) hypothesised that there would be no visual dispersal benefit to fruit being green, therefore, there must be an alternative evolutionary benefit to fruit maintaining their chlorophyll through to maturity. They found in a survey of wild fruits in a tropical forest that green-ripe fruits were significantly larger than fruits from species that are bright-ripe. They suggested that green fruit have the advantage of being able to photosynthesise and thus contribute to their own carbon demands. This in turn would lead to larger fruit, constituting a greater food reward for frugivores.

Fruit photosynthesis, and particularly that of apples, has been widely researched, and well reviewed by Aschan and Pfanz (2003) and Blanke and Lenz (1989). Apple peel contains a functioning photosynthetic system (Aschan and Pfanz, 2003), although, chloroplasts and stomata are sparsely distributed in the peel compared to leaves. But, per unit chlorophyll, apple fruit photosynthetic rates are proportionate to those of leaves (Blanke and Lenz, 1989). Although there are no such figures for apples, Pavel and DeJong (1993) showed that developing peach fruit contributed 9% of their total carbon requirement through photosynthesis, and it is certain that fruit photosynthesis reduces the strain on fruit trees for energy requirements during phases of rapid growth (Aschan and Pfanz, 2003). Photosynthesis very early during fruit development may be critical to fruit development, as Vemmos and Goldwin (1994) showed that even the removal of photosynthetically active flower sepals reduced apple fruit set. Although Vemmos and Goldwin (1994) have no data on photosynthesis of recently set fruit, the

effect of photosynthesis of floral accessories suggests that photosynthesis of newly developing fruit would most likely also be important.

Chloroplast and chlorophyll changes during apple fruit ripening.

Chloroplasts in the apple peel are found in the hypodermis, in five to six layers below the epidermis (Clijsters, 1969). These chloroplasts can be elliptical or disc shaped, and are smaller than those present in leaves. The grana also have far fewer thylakoids than those from leaves (Blanke and Lenz, 1989). Clijsters (1969) found that apple peel chloroplasts showed good lamellar structure up until 60 days after full bloom. As the fruit continued to mature, the presence of globules in the chloroplasts became increasingly dominant. During this time, the lamellae became vacuole-like and grana structure was lost. Clijsters (1969) suggested a relationship between chlorophyll breakdown and the appearance of the globules, which coincided with the disintegration of the lamellar structure.

Vemmos and Goldwin (1993) measured the chlorophyll concentration of the receptacles, the precursor to the apple fruit, of 'Cox's Orange Pippin' apple flowers during flowering. Measurements were taken from green cluster stage until 12 dafb. The chlorophyll concentration increased from green bud to pink bud stage over five days. From the balloon stage to 12 dafb, which took 21 days, the receptacle chlorophyll concentration gradually decreased again. Because the aim of the trial was to study chlorophyll in flowers, no further measurements of receptacle and developing fruitlet chlorophyll concentrations were taken.

During cell division, chlorophyll is rapidly synthesised (Gross, 1987). As fruit growth slows, chlorophyll synthesis decreases. Total fruit chlorophyll may increase as the fruit expands, but the concentration is reduced due to dilution, and fruit will appear less green despite a higher total chlorophyll content (Gross, 1987). Loss of peel chlorophyll in maturing apples has been well-documented, and as chlorophyll is lost, the yellow colour of the carotenoids present in peel becomes evident (Gorski and Creasy, 1977; Knee, 1972). Knee (1972) found no

conclusive differences between the degradation of chlorophyll *a* and chlorophyll *b*. Mussini et al. (1985) also reported that peel chlorophyll levels decrease during ripening of 'Granny Smith' apples, while carotenoid concentrations remain fairly constant. This results in a loss of green colour, causing fruit to be either yellow or white, depending on the concentration of the unmasked carotenoids. Paradoxically, Gorski and Creasy (1977) found that an equal mixture of green and yellow pigments appeared greener to human subjects than pure green pigment. Thus, the presence of carotenoids in the peel should not be considered disadvantageous to 'Granny Smith' green colour.

Light.

Feedback inhibition, phytochrome, temperature, cytokinins, abscisic acid, photo-oxidative stress, the circadian clock and tissue age have all been implicated in the regulation of chlorophyll synthesis (Willows, 2004). However, light plays the most important role in regulating synthesis of chlorophyll in angiosperms (Fig. 1). The most critical step is the reduction of monovinyl protochlorophyllide to chlorophyllide. This penultimate step in the synthesis of chlorophyll is catalysed by the enzyme NADPH:protochlorophyllide oxidoreductase, which requires light in order to be acitivated (Lebedev and Timko, 1998). Light is also known to be a transcription regulator for glutamyl-tRNA reductase, as well as an upregulator of gene expression of magnesium chelatase (Willows, 2004). The synthesis of chloroplast ultrastructure is also regulated by light (Kasemir, 1979).

Apples covered with bags that prevented the transmission of light during their development had less peel chlorophyll and were much paler in colour than uncovered control fruit (Gorski and Creasy, 1977; Hirst et al., 1990). However, 'Granny Smith' apples from the lowest, innermost areas of the tree were greener than those from brighter areas of the canopy (Tustin et al., 1988; Warrington et al., 1989). Fruit from the outer canopy were far paler than those from inside the canopy. This could be because the inside fruit were less mature than their compatriots that received more sunlight and chlorophyll breakdown is known to

increase with increasing fruit maturity (Knee, 1972; Mussini et al., 1985). However, Tustin et al. (1988) hypothesised that fruit subjected to higher light levels undergo faster chlorophyll cycling or suffer from more photodegradation. 'Granny Smith' fruit receiving more than 40% light transmission also suffered from red blush development (Warrington et al., 1989). Iszo and Larsen (1990) found the lowest chlorophyll concentrations and lightest colour in 'Granny Smith' fruit from full sun and heavy shade treatments. They suggested that 37 to 70% of full sun would be the optimal irradiance for good green colour development.

Nitrogen.

Nitrogen is the fourth most abundant element in plant tissues after hydrogen, carbon and oxygen, and usually occurs at a concentration of approximately 1.5% in dry tissue (Salisbury and Ross, 1992). Each chlorophyll molecule contains four nitrogen atoms. Without nitrogen, plants exhibit a yellowing, known as chlorosis, because chlorophyll cannot be synthesised when nitrogen is deficient (Salisbury and Ross, 1992). Nitrogen is very mobile within the plant, with preferential allocation to new growth causing chlorosis to occur in older tissues (Salisbury and Ross, 1992). Magnesium, which occurs at the centre of the chlorophyll molecule, is also essential for chlorophyll synthesis and deficiencies of iron, manganese, zinc and copper can also lead to chlorosis as they are required during photosynthesis reactions (Salisbury and Ross, 1992). Only the effect of nitrogen on green colour will be covered in this review, as its role is the most significant. Evans (1989) stated that a close positive linear relationship exists between nitrogen and chlorophyll leaf concentrations in various species. Around the same time, Minolta developed the SPAD meter that enables non-destructive measurements of leaf chlorophyll (Uddling et al., 2007). Over 200 studies about the use of this device have been published since then. It is a popular tool with agronomists, who use it to make nitrogen fertiliser recommendations based on the relationship between leaf chlorophyll and nitrogen contents (Uddling et al., 2007).

A number of researchers have studied the role of nitrogen in the colour of ‘Golden Delicious’ apples. Golden Delicious is a pale yellow-green cultivar, and dark green skin colour is considered undesirable (Drake et al., 2002; Neilsen et al., 1984; Williams and Billingsley, 1974). Williams and Billingsley (1974) found a consistent positive correlation between both leaf colour and fruit green colour against leaf nitrogen. In a related trial, Raese and Williams (1974) showed a very strong correlation ($r = 0.95$) between green fruit colour and percentage leaf nitrogen. In both studies, a leaf nitrogen concentration of more than 2 % resulted in undesirably green fruit. Strong negative and positive correlations were found at harvest with leaf N and ‘Golden Delicious’ peel lightness and hue angle, respectively (Drake et al., 2002). Also, Neilsen et al. (1984) found that ‘Golden Delicious’ fruit from trees receiving substantial nitrogen fertiliser showed a slower loss of skin chlorophyll. Daugaard and Grauslund (1999) examined the various orchard factors that affect colour of Mutsu, a green apple cultivar that consumers consider desirable when more yellow in colour. They found a significant positive correlation between leaf nitrogen content and green fruit colour and a significant negative correlation between leaf nitrogen and yellow fruit colour. The aim of these studies was to find ways to reduce the green appearance of the cultivars in question.

Ruiz (1986) reported that on low nitrogen soils, the problematic yellowing of ‘Granny Smith’ could be reduced through nitrogen fertiliser applications. Meheriuk (1990) faced the same problem of ‘Newton’ apples not being green enough. His results showed that both calcium nitrate and urea foliar sprays, applied five times during the season, significantly improved green skin colour compared to the control. After 90 and 180 days of storage, the fruit from trees that received the urea treatment had substantially less loss of green colour than fruit from trees that were treated with calcium nitrate, which in turn lost less green colour than the control fruit. However, the calcium nitrate treated fruit displayed bitter pit-like lesions, rendering the calcium nitrate redundant as an option for improving green colour. While studying the factors affecting colour development

of ‘Fuji’ apples, Marsh et al. (1996) found a positive relationship between fruit nitrogen and skin chlorophyll concentrations. Around the same time, while working on methods to improve red colouration of ‘Gala’ apples, Reay et al. (1998) applied eight weekly 1% urea foliar sprays to measure the effect on skin colour and chlorophyll concentrations. The urea treated fruit had higher nitrogen concentrations than the control. At harvest, the ground colour of the urea treated fruit was significantly greener than the untreated control. These studies showed that urea sprays could improve green colour, but applying upwards of five foliar sprays in order to see an effect is neither practical nor economical. When applying only one 1.5% preharvest urea foliar spray, Griessel (1991) was able to increase ‘Granny Smith’ peel chlorophyll concentrations in an orchard where vigour was poor, but not in an orchard displaying normal growth. In a separate trial, the same author was again able to increase chlorophyll concentrations and make a very slight improvement in green colour with a single 1.5% preharvest urea foliar spray. Although urea sprays appear to show more of an effect on green colour after prolonged storage, and this would facilitate marketing of the fruit, this improvement still fails to solve the problem of poor green colour at harvest.

Meheriuk et al. (1996) were unable to find an improvement in ‘Granny Smith’ green colour with a soil application of ammonium nitrate in spring. They were, however, able to significantly improve ‘Granny Smith’ green colour with four preharvest 1% urea foliar applications, although the improvement was only very slight. Fruit nitrogen content increased with the foliar application, but not with the soil application. Oland (1963) was unable to increase summer leaf nitrogen with a spring calcium nitrate soil application, but there was an improvement in summer leaf nitrogen with a 4% postharvest urea foliar spray.

In other fruits, ‘Valencia’ orange trees supplied with high amounts of nitrogen fertiliser showed more on-tree regreening of fruit than those that received less or no nitrogen (Jones and Embleton, 1969). In mangoes, where green skin is

unwelcome, all orchards that received soil applications of ammonium nitrate had significantly greener fruit, with higher skin concentrations of chlorophyll, than orchards that received no nitrogen fertiliser (Nguyen et al., 2004). However, in orchards prone to green fruit, foliar applications of ammonium nitrate resulted in even more green fruit than the highest of the soil applications. The strong correlation of leaf nitrogen and green colour found in apples was not found in mangoes. Increased nitrogen fertilisation was also found to improve green colour of cucumbers (Jasso-Chaverria et al., 2005).

The nitrogen required for late spring and early summer growth in apples is largely supplied by reserve nitrogen (Little et al., 1966) and according to Guak et al. (2003), by full bloom, 80% of new growth is funded by reserve nitrogen. Nitrogen uptake in the spring only begins around three weeks after budbreak, depending on soil temperature (Dong et al., 2001). Autumn nitrogen applications increase nitrogen reserves in the tree, and it is not known whether the reserve nitrogen status of the tree affects spring uptake. Also, early spring application of nitrogen may not result in immediate uptake because of low soil temperatures (Dong et al., 2001). Toselli et al. (2000) found that nitrogen remobilised from the previous season and nitrogen taken up during spring of the current season contributed equally to total fruit nitrogen. These researchers all refer to colder temperate conditions, and these reported timings of nitrogen uptake may not be applicable to South Africa, due to our warmer soil temperatures. However, Kangueehi (2008) drew similar conclusions from research performed in South Africa, finding that efficiency of N uptake by young apple trees during spring and early summer was limited.

Oland (1963) suggested that urea would make be an effective method of increasing the nitrogen reserves in apple trees before the onset of winter. High concentrations of nitrogen could be applied, without fearing material damage to the leaves, which would soon drop. Uptake would be quick and the nitrogen could be relocated to permanent tissues prior to leaf drop. Shim et al. (1972)

found that after 48 hrs, 80% of foliar applied urea was absorbed by leaves. Dong et al. (2002) showed that when urea was applied to apple leaves in autumn, the nitrogen concentration in the leaves peaked after two days, and then declined, while root and bark nitrogen increased all the time. Of the total nitrogen applied to the leaves, 35% was absorbed, of which 64% was translocated from the leaves within 20 days. Dong et al. (2002) suggest that their amount of urea absorption was lower than what Shim et al. (1972) had recorded, because the leaves they used had a higher nitrogen content to start with. In his trial, Oland (1963) found that a post-harvest urea spray resulted in higher yields for the treated trees in the following season, compared to trees that received a spring calcium nitrate soil application and the no-nitrogen control. This was caused by a greater fruit set, as opposed to increased fruit size, suggesting that the additional nitrogen reserves played a pivotal role during the early stages of fruit growth. Shim et al. (1972) also found an increased fruit set on trees treated with post-harvest urea sprays, and attributed this to the ability of stored nitrogen to supply the trees during the spring. Little et al. (1966) and Delap (1967) both found that a post-harvest urea spray had no effect on yield. They attributed this to the higher N levels of the trees used in their trials compared to those used by Oland (1963).

Both light and nitrogen have an important role to play in chlorophyll synthesis and hence green colour development of ‘Granny Smith’ apples. However, should these resources not be available to the tree at the time of maximum chlorophyll synthesis, in the correct quantities, green colour development will not be improved.

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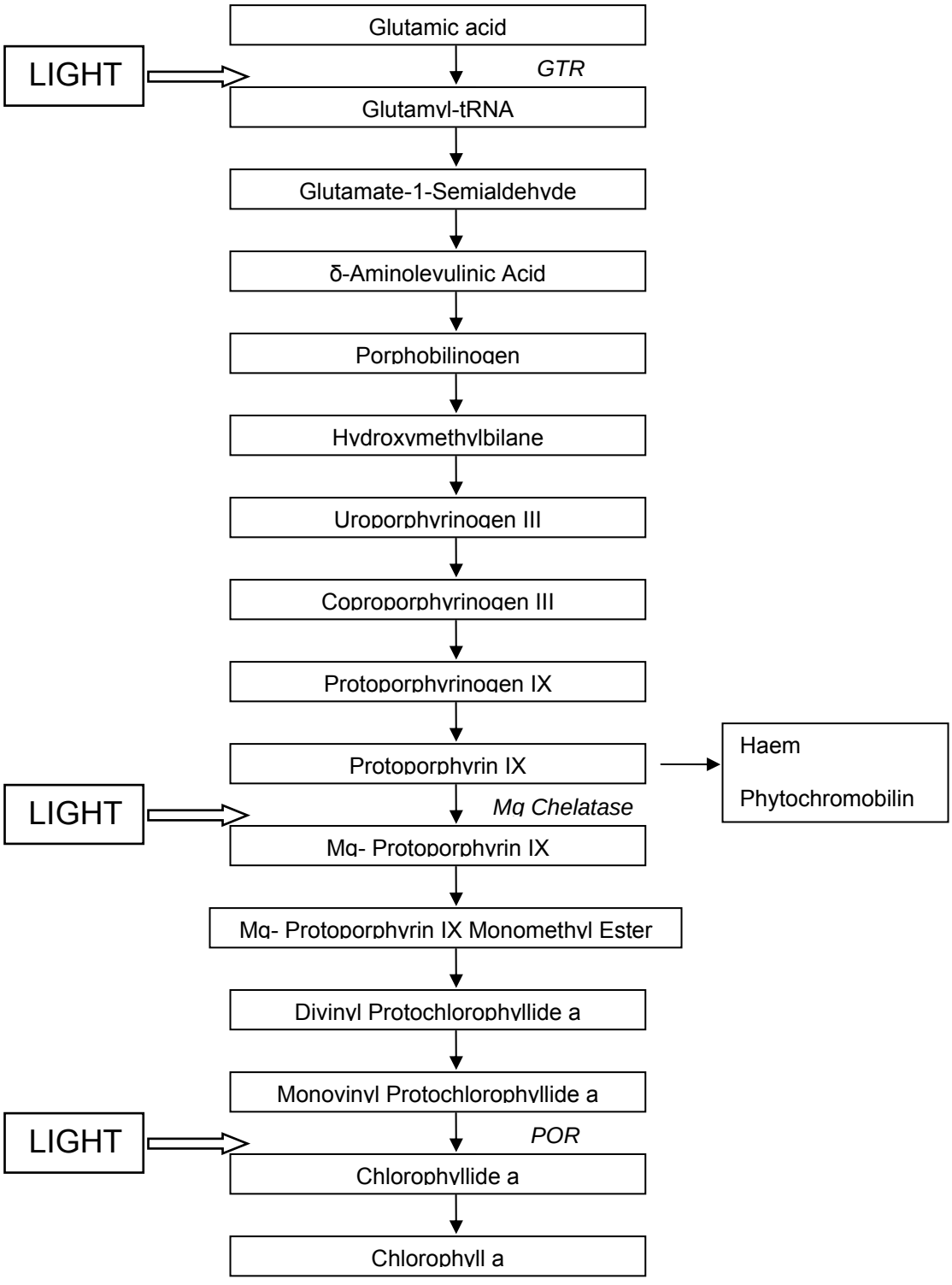

Fig 1. The pathway of chlorophyll biosynthesis, including the enzymes that are regulated by light, based on the review by Willows (2004). Abbreviations: GTR, glutamyl-tRNA reductase; POR, protochlorophyllide oxidoreductase.

PAPER 1:

IMPROVEMENT OF GREEN COLOUR OF 'GRANNY SMITH' APPLES AT HARVEST

***Abstract*. 'Granny Smith' apples (*Malus domestica* Borkh.) with a uniform dark green colour are desired by the market, but many producers struggle with fruit becoming pale before harvest. Chlorophylls present in the peel give the fruit their green colour. Fruit were sampled from 20 orchards that were selected based on their green colour performance in the previous season. Fruit from orchards where colour had been good the previous season, had significantly greener fruit, more peel chlorophyll and more leaf nitrogen (N) than the poor orchards at both 80 days after full bloom (dafb) and harvest (170 dafb). We concluded that green colour is determined during the early stages of fruit development. In the following season, we conducted a trial where different forms of N were applied at different rates and times, to improve green colour. Some of the treatments showed significant differences in green colour compared to the control at 40 dafb, 80 dafb and harvest (160 dafb), but the results were inconsistent, and so slight as to be of no commercial value. None of the treatments increased chlorophyll, peel N or leaf N. Another trial was conducted to establish the effect of early-season shading on fruit colour. Fruit were covered with 40% shadecloth from 14 until 56 dafb. There was a significant loss of green colour and chlorophyll for unshaded fruit from 14 to 56 dafb. At 56 dafb and harvest (160 dafb), unshaded fruit were significantly greener than shaded fruit. This suggests that a strategy to replace summer pruning with spring pruning may improve green colour.**

'Granny Smith' is the most widely planted apple cultivar in South Africa, accounting for 24% of apple plantings in 2007 (Deciduous Fruit Producers' Trust,

2008). The peel of these fruit is an intense dark green, which becomes lighter and greenish-yellow with maturity (Warrington, 1994). In order to be suitable for class 1 grading, the fruit should be uniformly green, but whitening of the skin is a common problem (Hirst et al., 1990). Unpublished data from our lab shows that green colour varies not only from region to region, but even between orchards on the same farm. Marsh et al. (1996) found similar regional differences for red colour of 'Fuji' apples in New Zealand. The green colour of 'Granny Smith' observed is a result of the combination of chlorophyll and carotenoid pigments present in the fruit epidermis. Chlorophyll gives the peel its green colour, and yellowing of the fruit is a result of chlorophyll degradation revealing the carotenoids as opposed to an increase in carotenoid synthesis (Mussini et al., 1985).

Light and N play an essential role in chlorophyll synthesis and subsequent photosynthesis. Although there are other limiting factors, light and N are the most critical (Purohit and Ranjan, 2002). Evans (1989a) found a strong correlation between chlorophyll concentrations and N content in the leaves of numerous crops. High N levels have also been associated with greener colour and higher chlorophyll concentrations in other apple cultivars (Marsh et al., 1996; Raese and Williams, 1974; Reay et al., 1998) and other fruit, including cucumber (Jasso-Chaverria et al., 2005) and mango (Nguyen et al., 2004). However, N is preferentially allocated to leaves where there is more light available for photosynthesis (Evans, 1989b). Hirst et al. (1990) found that subjecting 'Granny Smith' apples to deep shade resulted in white fruit. In contrast, excess light can be to the detriment of 'Granny Smith' green colour, as it causes chlorophyll degradation. Thus, fruit on the outside of the canopy tend to be paler than those from slightly shaded positions (Tustin et al., 1988).

The aim of this study was to establish whether orchard with good or poor green colour at harvest are consistent over subsequent seasons and whether fruit of these orchards differ in their colour during early fruit development. This was done

in order to narrow the range of possible factors affecting green colour and to determine when the application of any ameliorant treatments would be most beneficial. Based on these findings, we conducted trials to investigate the effects of nitrogen fertiliser and light levels on green colour.

Materials and methods

Comparison of good and bad orchards. Data was collected in 2005 from a commercial packhouse (Two-A-Day Ltd.) to rank the performance of their growers' orchards concerning 'Granny Smith' green colour. Ten of the best and ten of the worst of these orchards for green colour were selected in the Grabouw (lat: 34°10′S, long: 19°03′E) and Villiersdorp (lat: 33°59′S, long: 19°18′E) regions, in the Western Cape Province of South Africa. The orchards selected had various row directions, planting dates, planting densities, rootstocks and soils.

Fruit were sampled randomly on the same side of the trees, from two rows in the middle of each orchard at approximately 80 dafb (19 and 20 Dec 2005) and at the onset of commercial harvest (20 and 21 Mar 2006). Fruit were sampled from either the the eastern or southern sides of rows, depending on row direction, in order to avoid blushed and sunburnt fruit. Fruit were sampled from the outside and inside of the canopy by selecting 20 fruit from each of these positions. Good and bad orchards represent two treatments. On 5 and 6 Apr. 2006, leaves were sampled at shoulder height from the middle of the current season's shoots, which had a length of ~ 0.75 m.

At 80 dafb, fruit were stored overnight at -0.5 °C before laboratory work commenced. Fruit colour was measured at the darkest green point on the fruit equator using a chromameter (Model CR-400; Minolta Co. Ltd., Tokyo). The lightness value describes how light or dark green the fruit is, with a lower number representing a darker colour. Hue angle ranges between 0 ° = red-purple, 90 ° = yellow, 180 ° = bluish-green and 270 ° = blue, and is the most appropriate

method of reporting fruit peel colour (McGuire, 1992). Average fruit mass per replicate was determined with a one decimal scale, and diameters of 10 fruit were measured using an electronic calliper. Fruit were peeled by removing a strip of peel from the fruit equator on both the light and dark side of the fruit, using a vegetable peeler. Remaining flesh was then scraped off the peel strips, and individual fruit peels were pooled together within each replicate.

At harvest, fruit were stored at -0.5 °C for 7 days prior to laboratory work. Fruit colour was measured with a chromameter as for 80 dafb. The Colour Chart for Apples and Pears (Unifruco Research Service [Pty] Ltd.) was also used as a subjective measurement of green colour, where values range from 0.5 to five as colour changes from green to yellow. Average fruit mass per replicate was determined with a one decimal scale. Flesh firmness was determined on pared, opposite cheeks with a fruit texture analyser (GÜSS; Strand, South Africa), using an 11 mm tip. Flesh segments were cut from fruit, pooled together within the replicate, juiced, and total soluble solids (TSS) measured with a digital refractometer (PR32; ATAGO, Tokyo). The starch conversion of fruit was measured by applying iodine to the calyx end of each fruit and comparing it with a Starch Conversion Chart for Pome Fruit (Unifruco Research Service [Pty] Ltd). Fruit were peeled as described above.

Effect of nitrogen fertiliser on green colour. The colour of ‘Granny Smith’ apples was measured at 40 dafb, 80 dafb and again at harvest (160 dafb) during the 2006/2007 season, after different forms, amounts and timing of N fertiliser were applied. The experiment was conducted near Villiersdorp (lat: 33°59′S, long: 19°18′E), Western Cape province, South Africa. The orchard was selected based on the producer’s leaf analyses indicating a chronic N deficiency. For ‘Granny Smith’ apples, normal leaf N in January is 2.2% to 2.8%, where this orchard had leaf N of: 1.85%, 1.89%, 2.3% and 1.9% for the previous four seasons respectively. Trees were planted in 1975, in a sandy-loam soil, on seedling rootstock, in a north-south direction and trained to a palmette trellis system.

When the trial was conducted, there were 799 trees/ha, at a planting density of 4.74 x 2.74 m. The trial consisted of six treatments: no N (control), postharvest limestone ammonium nitrate (LAN) soil application, full-bloom LAN soil application, a combination of postharvest and full-bloom LAN soil applications, a combination of postharvest urea foliar spray and full-bloom LAN application and a preharvest urea foliar spray (Table 1). Treatments were applied to three-tree plots, with treatments replicated once each in seven blocks. A guard tree separated each plot, with guard rows on either side of treated rows. Postharvest LAN was applied on 18 Apr. 2006 and full-bloom LAN on 19 Oct. 2006. LAN was applied at a rate of 187.5 g/tree, which was equivalent to N at 42 kg·ha^{-1} (LAN, 28% N; Omnia Fertilizer Ltd., Bryanston, South Africa). After each LAN application, 2 mm of irrigation was applied to dissolve the granules. The postharvest urea foliar spray consisted of two applications, applied on 18 Apr. and 3 May 2006. Urea was applied at a rate of 1.5 kg·100 L^{-1} (Low-biuret urea, 46% N; Omnia Fertilizer Ltd, Bryanston, South Africa) with 10 ml·100 L^{-1} Aqua-Wet a.i. nonyl phenol ethoxylate, glycol ether and fatty acids (Ag-Chem Africa [Pty] Ltd., Totiusdal, South Africa). The foliar spray was applied using a truck-mounted, motorised, high-pressure sprayer until run-off (≈ 2.5 L/tree). The preharvest urea spray was applied once, 5 weeks before harvest, on 19 Feb. 2007. For this treatment the urea was applied at a rate of 1 kg·100 L^{-1}. The wetter used was 100 ml·100 L^{-1} Volcano 90 a.i. alkylated phenol-ethylene oxide (Volcano Agroscience [Pty] Ltd., Mt. Edgecombe, South Africa). This spray was applied using a backpack mist blower until run-off (≈ 1.3 L/tree).

Fruit were sampled at 40 dafb (30 Nov. 2006), 80 dafb (9 Jan. 2007) and commercial harvest (28 Mar. 2007). Fruit were sampled from all three trees of each plot, with 20 fruit from each side of the row pooled to form a replicate. Fruit were sampled randomly at shoulder height from the outside of the canopy. However, at commercial harvest, fruit were sampled from inside the canopy in order to avoid sunburnt fruit. Leaves were sampled on 31 Jan. 2007, selecting leaves from only the eastern side of the trees, at shoulder height, from the middle

of the current season's shoots. Soil was sampled on 22 Feb. 2007. A hand-held soil auger was used to sample topsoil and subsoil, at depths of 0.3 and 0.6 m respectively. Soil was sampled from underneath the central tree of the plots where either no N or two LAN applications were applied. This was repeated in three out of the seven blocks. On the same day, all trees that formed part of the trial were visually rated for vigour on a scale of one (highly vigorous) to three (slightly vigorous). The rating was performed by two people who did not know which trees had been subjected to which treatment. On 5 Apr. 2007, the trees were visually rated according to the percentage of fruit that were discoloured because of excessive sunlight. Two people performed the rating, one of whom was unaware of which trees had received which treatments. For both visual ratings, the three-tree plots were evaluated as a whole, and the scores of each assessor were combined to give an average. Trunk circumferences were measured 10 cm above the graft union on 30 May 2007.

For each sampling date, fruit were stored overnight at room temperature before colour and maturity were assessed. At 40 and 80 dafb fruit colour, mass and diameter were measured as previously described for 80 dafb fruit. Fruit were peeled with a knife, removing only the pigmented layers of peel, and individual fruit peels were pooled together within each replicate. At 40 dafb a strip of peel around the whole equator of the fruit was removed, while at 80 dafb fruit were big enough to remove only a strip of peel from dark and light side. At commercial harvest fruit colour was measured with the chromameter at both the greenest and least green sides of the fruit. Subjective colour and maturity were assessed according to the method mentioned earlier. Fruit were peeled by removing a strip of peel from the fruit equator on both the dark and light side of the fruit, using a vegetable peeler. Remaining flesh was then scraped off the peel strips, and individual fruit peels were pooled together within each replicate.

Effect of early season shading on green colour. Fruit were shaded from 14 until 56 dafb and compared with unshaded fruit at 56 dafb and commercial harvest

during the 2006/2007 season. This trial was conducted near Somerset West (lat: 34°05′S, long: 18°51′E), Western Cape province, South Africa. The 'Granny Smith' trees were planted on seedling rootstock in 1982, at a spacing of 4.5 x 1.5 m, in a north-south direction. The two treatments were repeated in five rows, each row representing a block. At 14 dafb (24 Oct. 2007), 20 clusters of fruitlets per row were enclosed in 40% woven green shadecloth. All clusters were in the light-exposed outer canopy of the western sides of the trees. Photosynthetic photon flux density was measured with a quantum meter (LI-189; LI-COR, Lincoln, Nebraska) at 42 dafb at 1300 HR. PPFD was in the range of 2000 to 2200 $\mu mol \cdot m^{-2} \cdot s^{-1}$ in full sunlight, and 1000 to 1200 $\mu mol \cdot m^{-2} \cdot s^{-1}$ under the shadecloth. Due to shoot growth, some of the clusters that had been in full sunlight at 14 dafb fell into dappled sunlight by 42 dafb. In these instances PPFD was 700 to 800 $\mu mol \cdot m^{-2} \cdot s^{-1}$ for unshaded bunches, and 350 to 450 $\mu mol \cdot m^{-2} \cdot s^{-1}$ underneath the shadecloth. On the same day, 100 fruitlets from each block were sampled from positions similar to those of covered fruit. Colour of 20 fruitlets was measured with a chromameter on the darkest green side of the fruit. Average fruit mass (100 fruitlets) and diameter (10 fruitlets) were measured as reported earlier. Fruitlets were peeled, using a knife to remove a strip of peel around the equator of the fruit. Fruitlets were sampled, measured and peeled in the same way at 28, 42 and 56 dafb using 40, 20 and 20 fruitlets for each replicate, respectively. In all cases, 20 fruit were used for colour measurements, all fruit for average mass and 10 fruit for diameter. At 56 dafb, the shadecloth was removed, and 20 previously shaded fruit were also sampled from each block. On 22 Mar. 2007, at commercial harvest (160 dafb), previously shaded fruit and unshaded fruit from similar positions were sampled. In many instances the shadecloth caused fruitlet abscission or had been dislodged, with the result that not enough previously shaded fruit were available at harvest. One of the previously shaded replicates had only four fruit, while the other four replicates each had at least 16 fruit. There were 20 fruit for all unshaded replicates at harvest.

After harvest, fruit were stored at -0.5 °C for 7 days. Thereafter, colour was measured with the chromameter on both sides of the fruit, while care was taken to avoid sun-blemished regions. Fruit were evaluated for the presence of either red blush, sunburn or bronzing, and this was reported as percentage of fruit with sun blemish. The background colour chart was only used to measure the back of the fruit, as many of the fruit were blemished by the sun on their exposed side. Average fruit mass and firmness were measured as mentioned previously. Fruit were peeled using a vegetable peeler to remove a strip of peel from the fruit equator on both the front and shaded sides. Remaining flesh was then scraped off the peel strips, and individual fruit peels were pooled together within each replicate. However, peel from the exposed and shaded side of each replicate were kept separate, and where sun blemish occurred the exposed side was not peeled.

Peel from all trials was immediately frozen in liquid nitrogen and stored at -80 °C. Peel was ground by hand in liquid nitrogen, using a mortar and pestle, and returned to -80 °C until pigment analysis.

Pigment analysis. Chlorophylls and carotenoids were extracted from ≈ 0.3 g peel in 3 ml acetone for 24 h at 4 °C in the dark. The extract was centrifuged at 10 000 g_n for 15 min and decanted, whereafter 2 ml of solvent was added to the sample, which was again centrifuged and decanted in the same manner. The decanted extracts were combined, filtered through a 0.45 µm filter (Millex-HV; Millipore Coroporation, Milford, Mass.) and absorption measured with a spectrophotometer (Cary 50 Series, Varian; Mulgrave, Australia) at 470, 645 and 662 nm. The extinction coefficients of Lichtenthaler (1987) were used to calculate chlorophyll and carotenoid concentrations, which were then expressed as $\mu g \cdot g^{-1}$ fresh weight of peel.

Leaf chlorophyll analysis: Leaves sampled for all experiments were measured with a leaf chlorophyll meter (CCM-200, Opti-Sciences; Tyngsboro, Mass.). Leaf

chlorophyll concentrations were determined using a standard curve. This standard curve was established by measuring five leaf samples with the chlorophyll meter and extracting the chlorophylls to determine their concentration. Chlorophyll was determined using the same method as for the peels, using 0.05 g of sample.

Mineral analysis. Mineral analysis of all peel, leaves and soil was carried out using inductively-coupled plasma-emission spectroscopy at an analytical laboratory (Bemlab [Pty] Ltd. Strand, South Africa).

Statistical analysis. Analysis of results was carried out using the General Linear Models (GLM) procedure of SAS 9.1 (SAS Institute Inc., 2004; Cary, N.C.).

Results and discussion

Comparison of orchards. There was a significant difference in green colour between the two groups of orchards at both 80 dafb and at harvest, at 170 dafb (Table 2). However, from our personal observations when measuring colour, only a difference of more than 2 L values would be clearly visible to the consumer. Hence, throughout the trials, where a difference of less than 2 L values is statistically significantly different, we did not consider it to be of commercial value as it would not sufficiently alter the customer's impression of the fruit. Thus, although there were significant differences in L value between the two groups of orchards at both dates, only the difference at harvest would have been obviously visible to the consumer. The orchards that previously had good colour reflected an increase of 2.3 L values over the season, compared to an increase of 3.3 L values for the poor orchards. Hue angle also decreased by 0.4 and 0.6 ° over the season for the respective orchards. Thus, orchards that started the season with good colour had darker and greener fruit at harvest, and they tended to increase in lightness and lose their green colour less than poor orchards. This suggested to us that green colour is determined early during fruit development and may tie

in with Clijster's (1969) finding that the dismantling of apple peel chloroplasts began at 60 dafb. According to the background colour chart measurements (Table 2), fruit colour did not differ significantly between the two groups of orchards. This is because the fruit were harvested very early during the picking window and many of the fruit were still far greener than the chart allowed for. Starch conversion (Table 2) of the good orchards was slightly higher than for the poor orchards, which is unusual as more advanced maturity would normally result in less green colour (Griessel, 1991; Mussini et al., 1985). There were no differences in average fruit mass, firmness or TSS (data not shown). The colour data are confirmed by the difference in chlorophyll concentrations (Table 3), which are significantly lower for poor orchards at both 80 dafb and harvest. There were no significant differences in carotenoid concentrations at either date (Table 3). There were strong, significant correlations for L value and peel chlorophyll concentration at 80 dafb; but less so at commercial harvest (Table 4).

There was no difference between treatments for peel N levels at either sampling date, while leaf N concentrations of good orchards at harvest were significantly higher (Table 5). Differences in leaf chlorophyll concentrations were non-significant (data not shown). There were strong, significant correlations for leaf N with peel L value and peel chlorophyll at commercial harvest, while there were no correlations with leaf N at 80 dafb or with peel N at either date (Table 4). This correlation of leaf N, but not peel N, with green colour, may have been caused by variances in the peeling process. However, we did observe that darkest green fruit came from vigorous orchards, and perhaps the correlation with leaf N points to tree vigour playing a role in green colour that is unrelated to N levels. Marsh et al. (1996) found that chroma of red colour of 'Fuji' apples, which is influenced by chlorophyll present in the peel, correlated with tree vigour and leaf N, but that vigour and N did not correlate with one another. The orchards that had poor colour can be classified as N deficient (Table 5), although it should be borne in mind that these norms refer to leaf mineral content in late January, while these leaves were sampled in early April. Our results agree with those of Raese and

Williams (1974) working on ‘Golden Delicious’, where they also saw that trees with higher leaf N had greener fruit. Treatment differences for all other peel and leaf minerals were non-significant (data not shown).

Effect of nitrogen fertiliser on green colour. The results of the orchard comparison experiment prompted us to investigate different fertiliser strategies to increase available N during early fruit development. The role of in N in apple green colour is well-documented (Marsh et al., 1996; Raese and Williams, 1974; Reay et al., 1998). Autumn and spring fertiliser applications were compared because leaf growth and flower development of apple trees in spring is largely supplied by remobilised N (Guak et al., 2003; Little et al., 1966), whereas apple fruit were found to contain equal amounts of autumn and spring applied N (Toselli et al., 2000). Conventional soil applied N was compared with autumn foliar urea applications, as they have been shown to be an excellent method of increasing apple tree N reserves (Dong et al., 2002; Oland, 1963).

At 40 dafb (Table 6), the two treatments where N was applied both postharvest and at full bloom had significantly greener colour than the control, according to the L value and hue angle measurements. The other treatments showed no difference from the control. For the colour measurements, trunk circumference was a significant covariate. For L value, the contrasts for all N treatments against the control, N amount, and N time, were all significant. Notably, the contrast for preharvest urea foliar spray against other N applications was non-significant. At this point, the preharvest urea foliar spray had not yet been applied, and could still be counted as a control. This seems to indicate that there was a great amount of natural variation. For hue angle, only the contrast for N amount was significant. No differences between the treatments could be found for peel chlorophyll and carotenoid concentrations. This is unsurprising considering the only very slight difference in colour measured and the variance that occurs with pigment analysis.

At 80 dafb (Table 7), the L value for the double LAN application was significantly, albeit marginally, better than the control. Both the treatments where N was applied both postharvest and at full bloom had significantly better hue angles than the control. At 80 dafb, trunk circumference was no longer a significant covariate. For hue angle, the contrast of all N applications against the control was significant, while the contrast of March urea foliar sprays against other N applications was significant for both L value and hue angle. The lack of significant difference for the contrast of all N against the control for L value throws doubt over whether these results are meaningful. At this stage preharvest urea and the no N control have both received no N, yet the contrasts appear to contradict this. These contrasts show that the N applications have had a tendency to improve fruit colour slightly, when compared to the two control treatments at this point. Differences between pigment concentrations were again insignificant as a result of substantial variance. Leaf chlorophyll concentrations in January were also non-significant (data not shown). There was also no difference in soil N in February between the control and the double LAN treatment (data not shown).

At commercial harvest (Table 8), the treatment comprising of postharvest LAN only and the combination treatment of postharvest urea plus full-bloom LAN treatment, both had significantly lower L values than the control. However, this difference is less than 2 L values, and thus would not be easily visible to the customer. Only the contrast of L value for LAN timing was significant. The preharvest urea spray did not improve green colour compared to the control. However, the contrast for other N applications compared to preharvest urea, which was significant at 80 dafb, was no longer significant at harvest, indicating that the urea may have caused a slight improvement in colour in relation to the other treatments. According to the hue angle and background chart, the treatments had no effect on colour compared to the control. Differences in pigment concentrations were again insignificant. There were no significant differences for average fruit mass, flesh firmness, TSS or starch conversion (data not shown). The visual ratings of tree vigour and sunburn and measured trunk

circumferences were also non-significant (data not shown). The N fertiliser applications were neither able to increase peel N at any of the sampling dates, nor January leaf N (Table 9). The contrasts for N amount and time were significant for 40 dafb peel, and N amount for January leaf. This is caused by the inexplicably low N levels for the postharvest LAN treatment compared to the other treatments. The orchard for this experiment had been selected on the grounds of being deficient for leaf N; however, it is evident from the leaf N levels of the control (Table 9) that the trees fall well within the norm for 'Granny Smith' leaf N.

In a 3-year trial using 30, 60 and 180 kg $N \cdot ha^{-1}$ in spring, Neilsen et al. (1984) were only able to increase leaf N and 'Golden Delicious' green colour in one season with 180 kg $N \cdot ha^{-1}$. Subsequently, Meheriuk et al. (1996) were unable to find an improvement in 'Granny Smith' green colour when applying N in spring at rates of 80 and 160 $kg \cdot ha^{-1}$. Oland (1963) was also unable to increase leaf N when applying 62 kg $N \cdot ha^{-1}$ in spring. However, Oland (1963) was able to increase leaf N with a 4% postharvest urea foliar spray in the preceding season. Little et al., (1966) and Delap (1967) both found that their applications of a post-harvest urea spray had no effect on yield. They attributed this to the higher nitrogen levels of the trees used in their trials than those used in Oland's (1963) work. Meheriuk (1990) and Meheriuk et al. (1996) were able to improve 'Granny Smith' green colour significantly with five and four preharvest 1% urea foliar applications respectively, although the improvement was only very slight. Eight preharvest urea foliar applications were enough to increase 'Gala' apple fruit N, with a slight improvement in peel chlorophyll concentration and green background colour (Reay et al., 1998). When applying only one 1.5% preharvest urea foliar spray, Griessel (1991) was able to increase 'Granny Smith' peel chlorophyll concentrations in an orchard where vigour was poor, but not in an orchard displaying normal growth. In a separate trial, the same author was again able to improve chlorophyll concentrations and green colour with a single 1.5% preharvest urea foliar spray. However, it should be noted that, although

significantly different from the control, this improvement in green colour was too little to be of commercial value.

Effect of early season shading on green colour. This experiment was also founded on our results from the orchard comparison experiment. The aim was to investigate the effect of different light levels on green colour during early fruit development. Apples that were covered with opaque bags for the duration of fruit growth as well as those covered later on in their development had pale skin and low peel chlorophyll concentrations at harvest (Gorski and Creasy, 1977; Hirst et al., 1990), but no trials have been done with shading only during the first few weeks of fruit growth.

From 14 until 56 dafb, there was a significant decrease in L value, along with a loss of chlorophylls and carotenoids in peel of unshaded fruit (Table 10). The green colour loss was evident at each two-weekly interval; however, the only significant change in pigments was between 14 and 28 dafb (Table 10). Mussini et al. (1985) found that 'Granny Smith' peel chlorophyll concentration decreases from December (southern hemisphere) until harvest, but there is no previous research regarding chlorophyll or green colour loss prior to 60 dafb. Anthocyanin synthesis in the fruitlet peels was responsible for the lower hue and significant quadratic contrast at 28 dafb, as the fruitlets were so small that the blushed areas could not be avoided when measuring colour. The quadratic contrast for pigments was significant due to the loss of chlorophyll being much lower from 28 to 42 days being much smaller than the loss from 14 to 28 days.

At 56 dafb (Table 11), unshaded fruit were significantly greener than shaded fruit, while differences between pigment concentrations were significant at $p<0.1$. At harvest (Table 12), there was a significant difference for L value and hue of the sun-exposed side of fruit between the two treatments, indicating that unshaded fruit were darker green in colour. For the shaded side, there was no significant difference for L value, but according to the colour chart, unshaded fruit were

greener (Table 13). There were no significant differences at harvest between the two treatments for pigment concentration (Tables 12 and 13). Blemishes caused by excess sunlight were significantly higher at harvest for fruit that had been shaded (Table 12). These findings concur with those of Hirst et al. (1990), where they had shaded 'Granny Smith' apples from 60 dafb. Izso and Larsen (1990) conducted a shading trial and found that apples with the lowest peel chlorophyll and lightest colour were from the full sun and 95% shade treatments. They suggested that optimal green colour would occur at a light level between 37 and 70% full sun. This experiment simulated the exposure of previously shaded fruit to light, that summer pruning would produce, and our results echo those of Morgan et al. (1984) where summer pruning of 'Gala' apples resulted in more red blush, but no increase in green background colour. No mention of sunburn was made in this study.

Conclusions

It would appear that 'Granny Smith' green colour at harvest is primarily determined during the early stages of fruit development. Thus, any practices aimed at improving green colour should aim to maximise chlorophyll synthesis early during development, rather than to try to rectify poor colour just before harvest. Our N applications were most likely unsuccessful due to the high N status and vigour of the trees. We would thus only suggest N fertiliser as a method of improving green colour where trees are N deficient. Likewise, although previous studies have shown that pre-harvest urea foliar sprays may cause a slight improvement in green colour at harvest, and more so after storage, the improvement in colour is so small that such applications cannot be economically justified. The results from the shading of the young fruit show that if canopy light penetration is insufficient early in the season, green colour will suffer. Rectifying light penetration through summer pruning will only result in more sunburnt and blushed fruit, without improving green colour. Future studies should focus on establishing the role of vigour in green colour development and pruning trials

should be conducted to optimise canopy light distribution during spring while the fruit are still very young. Also, work needs to be done on finding a more accurate method of peeling fruit and extracting peel chlorophyll, as our current method resulted in poor correlations of chlorophyll concentrations with colour measurements.

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Table 1. Summary of treatments applied for nitrogen fertiliser trial during 2006/2007.

	Treatment Stage and Method	Nitrogen Source and Total Nitrogen Applied	Dates
1	Control	No N	-
2	Postharvest Soil	LAN (N = 42 kg·ha^{-1})	18 Apr. 2006
3	Full-bloom Soil	LAN (N = 42 kg·ha^{-1})	19 Oct. 2006
4	Postharvest Soil + Full-bloom Soil	LAN (N = 42 kg·ha^{-1}) + LAN (N = 42 kg·ha^{-1})	18 Apr. 2006 + 19 Oct. 2006
5	Postharvest Foliar + Full-bloom Soil	Urea 1.5% (14 kg·ha^{-1}) x 2 + LAN (N = 42 kg·ha^{-1})	18 Apr., 3 May 2006 + 19 Oct. 2006
6	Preharvest Foliar	Urea 1% (5 kg·ha^{-1})	19 Feb. 2007

Table 2. Lightness (L) values and hue angles of 'Granny Smith' apples at 80 dafb and commercial harvest, and background chart colour and fruit starch conversion at commercial harvest, for 2005/2006 season, for orchards that were previously (2004/2005 season) good or poor for green colour. Means in columns were separated by LSD (5%).

Treatment	80 dafb		Commercial harvest			
	L value	Hue (°)	L value	Hue (°)	Background colour chart [z]	Starch conversion (%)
Good orchards	53.1 b	118.7 a	55.4 b	118.3 a	0.54 [NS]	19 a
Poor orchards	54.4 a	118.3 b	57.7 a	117.7 b	0.77	12 b
			Pr>F			
Treatment	0.0062	0.0135	<0.0001	0.0088	0.0861	0.0325

[NS] Non-significant
[z] Values ranging from 0.5 for a green background to 5 for a yellow background.

Table 3. Peel chlorophyll and carotenoid concentrations of 'Granny Smith' apples at 80 dafb and commercial harvest (170 dafb) for 2005/2006 season, for orchards that were previously (2004/2005 season) good or poor for green colour. Values are an average of fruit from the inner and outer canopy. Means in columns were separated by LSD (5%).

Treatment	80 dafb		Commercial harvest	
	Chlorophyll ($\mu g \cdot g^{-1}$)	Carotenoids ($\mu g \cdot g^{-1}$)	Chlorophyll ($\mu g \cdot g^{-1}$)	Carotenoids ($\mu g \cdot g^{-1}$)
Good orchards	293 a	63 [NS]	199 a	48 [NS]
Poor orchards	273 b	59	165 b	41
		Pr>F		
Treatment	0.0245	0.1220	0.0344	0.0674

[NS] Non-significant

Table 4. Correlations of fruit lightness (L) value and peel chlorophyll with leaf and peel N for 'Granny Smith' apple for 20 orchards used in the 2005/2006 season.

	80 dafb		Commercial harvest	
Correlation	*r* [z]	*Pr>F*	*r*	*Pr>F*
L value with chlorophyll	-0.70	0.0009	-0.71	0.0006
L value with peel N	0.05	0.8466	-0.30	0.2165
L value with leaf N	-0.05	0.8281	-0.79	<0.0001
Chlorophyll with peel N	-0.02	0.9400	0.23	0.3418
Chlorophyll with leaf N	0.19	0.4241	0.73	0.0004

[z] Pearson Correlation Coefficients / Prob > |R| under Ho: Rho=0 / N where n = 20.

Table 5. Nitrogen concentrations for 'Granny Smith' fruit peel at 80 dafb and commercial harvest and leaves at commercial harvest (170 dafb) in the 2005/2006 season, for orchards that were previously (2004/2005 season) good or poor for green colour. Means in columns were separated by LSD (5%).

Treatment	80 dafb peel (%)	Commercial harvest peel (%)	Commercial harvest leaves (%)
Good orchards	0.88 [NS]	0.51 [NS]	2.33 a
Poor orchards	1.07	0.49	2.04 b
		Pr>F	
Treatment	0.0897	0.4716	0.0009

[NS] Non-significant

Table 6. Peel lightness (L) values, hue angles and chlorophyll and carotenoid concentrations of 'Granny Smith' apples at 40 dafb for trees treated with different rates, forms and timing of N fertiliser, in the 2006/2007 season. Means were adjusted using trunk circumference as a covariate where necessary. Means in columns were separated by LSD (5%). See Table 1. for treatment dates.

	Treatment	L value	Hue (°)	Chlorophyll ($\mu g \cdot g^{-1}$)	Carotenoids ($\mu g \cdot g^{-1}$)
1	Control (no N)	57.3 a	118.6 b	227[NS]	52[NS]
2	LAN[x] (Postharvest)	57.0 a	118.6 b	202	47
3	LAN (Full-bloom)	57.7 a	118.5 b	204	49
4	LAN (Postharvest + Full-bloom)	56.2 b	118.9 a	228	55
5	Urea foliar 1.5%[y] (Postharvest) + LAN (Full-bloom)	56.4 b	118.9 a	244	55
6	Urea foliar 1%[z] (Preharvest)	56.9 a	118.6 b	261	61
		Pr > F			
Trunk circumference		0.0001	0.0092	-	-
Treatment		<0.0001	0.0013	0.3156	0.3184
Contrasts					
N vs no N		0.0150	0.0761	0.9753	0.7712
6 vs other N		0.8345	0.1457	0.0805	0.0626
LAN vs Urea (4 vs 5)		0.2410	0.8599	0.5913	0.5913
N amount (2,3 vs 4,5)		<0.0001	0.0001	0.1213	0.1213
LAN time (2 vs 3)		0.0063	0.2653	0.9292	0.9292

[NS]Non-significant

[x]187.5 g/tree of LAN (28% N) = N at 42 $kg \cdot ha^{-1}$

[y]2.5 L/tree of low-biuret urea (46% N) 1.5% x 2 applications = N at 28 $kg \cdot ha^{-1}$

[z]1.3 L/tree of low-biuret urea (46% N) 1% = N at 5 $kg \cdot ha^{-1}$

Table 7. Fruit peel lightness (L) values, hue angles and chlorophyll and carotenoid concentrations of 'Granny Smith' apples at 80 dafb for trees treated with different rates, forms and timing of N fertiliser, in the 2006/2007 season. Means were adjusted using trunk circumference as a covariate where necessary. Means in columns were separated by LSD (5%). See Table 1. for treament dates.

	Treatment	L value	Hue (°)	Chlorophyll ($\mu g \cdot g^{-1}$)	Carotenoids ($\mu g \cdot g^{-1}$)
1	Control (no N)	57.7 ab	117.9 bc	196 [NS]	49 [NS]
2	LAN [x] (Postharvest)	56.9 bc	118.2 ab	243	58
3	LAN (Full-bloom)	57.0 bc	118.1 abc	209	52
4	LAN (Postharvest + Full-bloom)	56.4 c	118.4 a	230	52
5	Urea foliar 1.5%[y] (Postharvest) + LAN (Full-bloom)	56.7 bc	118.3 a	253	60
6	Urea foliar 1%[z] (Preharvest)	58.4 a	117.87 c	208	53
		Pr > F			
Trunk circumference		-	-	-	-
Treatment		0.0034	0.0108	0.1161	0.1862
Contrasts					
N vs no N		0.1194	0.0476	0.0704	0.1359
6 vs other N		0.0002	0.0026	0.1625	0.4655
LAN vs Urea (4 vs 5)		0.5289	0.5037	0.3253	0.0775
N amount (2,3 vs 4,5)		0.2786	0.1124	0.3319	0.6747
LAN time (2 vs 3)		0.7557	0.9405	0.1499	0.2073

[NS]Non-significant

[x]187.5 g/tree of LAN (28% N) = N at 42 $kg \cdot ha^{-1}$

[y]2.5 L/tree of low-biuret urea (46% N) 1.5% x 2 applications = N at 28 $kg \cdot ha^{-1}$

[z]1.3 L/tree of low-biuret urea (46% N) 1% = N at 5 $kg \cdot ha^{-1}$

Table 8. Fruit peel lightness (L) values, hue angles and chlorophyll and carotenoid concentrations of 'Granny Smith' apples at commercial harvest (160 dafb) for trees treated with different rates, forms and timing of N fertiliser, in the 2006/2007 season. Means were adjusted using trunk circumference as a covariate where necessary. Means in columns were separated by LSD (5%).

	Treatment	L value	Hue (°)	Background colour chart [w]	Chlorophyll ($\mu g \cdot g^{-1}$)	Carotenoids ($\mu g \cdot g^{-1}$)
1	Control (no N)	61.8 a	116.6 [NS]	1.77 [NS]	214 [NS]	52.4 [NS]
2	LAN [x] (Postharvest)	59.9 b	117.2	1.67	242	60.9
3	LAN (Full-bloom)	61.7 a	116.7	1.73	220	53.9
4	LAN (Postharvest + Full-bloom)	60.7 ab	117.0	1.60	229	55.4
5	Urea foliar 1.5% [y] (Postharvest) + LAN (Full-bloom)	60.2 b	117.0	1.70	211	53.1
6	Urea foliar 1% [z] (Preharvest)	61.4 ab	116.8	1.72	227	55.2
		Pr > F				
	Trunk circumference	-	-	-	-	-
	Treatment	0.0459	0.0808	0.1863	0.6955	0.4346
	Contrasts					
	N vs no N	0.0713	0.0643	0.0968	0.4594	0.3341
	6 vs other N	0.2091	0.2092	0.4304	0.9477	0.8606
	LAN vs Urea (4 vs 5)	0.4106	0.9468	0.1125	0.3902	0.5970
	N amount (2,3 vs 4,5)	0.5089	0.8429	0.3160	0.4528	0.3181
	LAN time (2 vs 3)	0.0157	0.0262	0.4078	0.2969	0.1124

[NS] Non-significant

[w] Values ranging from 0.5 for a green background to 5 for a yellow background

[x] 187.5 g/tree of LAN (28% N) = N at 42 $kg \cdot ha^{-1}$

[y] 2.5 L/tree of low-biuret urea (46% N) 1.5% x 2 applications = N at 28 $kg \cdot ha^{-1}$

[z] 1.3 L/tree of low-biuret urea (46% N) 1% = N at 5 $kg \cdot ha^{-1}$

Table 9. Nitrogen concentrations of fruit peels and leaves of 'Granny Smith' trees treated with different rates, forms and timing of N fertiliser, in the 2006/2007 season. Means were adjusted using trunk circumference as a covariate where necessary. Means in columns were separated by LSD (5%).

	Treatment	40 dafb peel	80 dafb peel	Harvest peel	January leaf
1	Control (no N)	1.08 [NS]	0.61 [NS]	0.70 [NS]	2.62 [NS]
2	LAN [x] (Postharvest)	1.11	0.73	0.82	2.55
3	LAN (Full-bloom)	0.83	0.69	0.75	2.46
4	LAN (Postharvest + Full-bloom)	1.16	0.73	0.79	2.66
5	Urea foliar 1.5%[y] (Postharvest) + LAN (Full-bloom)	1.13	0.75	0.66	2.58
6	Urea foliar 1%[z] (Preharvest)	1.11	0.67	0.78	2.55
		Pr > F			
Trunk circumference		-	-	-	-
Treatment		0.0841	0.5480	0.3752	0.1607
Contrasts					
N vs no N		0.8760	0.0963	0.5012	0.3343
6 vs other N		0.5455	0.4562	0.5394	0.8418
LAN vs Urea (4 vs 5)		0.8001	0.7482	0.0986	0.2486
N amount (2,3 vs 4,5)		0.0441	0.6501	0.1148	0.0405
LAN time (2 vs 3)		0.0204	0.6245	0.0806	0.2127

[NS] Non-significant

[x] 187.5 g/tree of LAN (28% N) = N at 42 kg·ha^{-1}

[y] 2.5 L/tree of low-biuret urea (46% N) 1.5% x 2 applications = N at 28 kg·ha^{-1}

[z] 1.3 L/tree of low-biuret urea (46% N) 1% = N at 5 kg·ha^{-1}

Table 10. Peel lightness (L) values, hue angles and chlorophyll and carotenoid concentrations of 'Granny Smith' apples at two-weekly intervals during the early phases of fruit development in the 2006/2007 season. Means in columns were separated by LSD (5%).

Days after full bloom	L value	Hue (°)	Chlorophyll ($\mu g \cdot g^{-1}$)	Carotenoids ($\mu g \cdot g^{-1}$)
14	48 d	117.7 b	280 a	79 a
28	50 c	114.7 c	230 b	55 b
42	54 b	118.4 a	226 b	53 b
56	56 a	118.5 b	214 b	52 b
		Pr>F		
Treatment	< 0.0001	< 0.0001	0.0007	< 0.0001
Contrasts				
Linear	<0.0001	< 0.0001	0.0002	< 0.0001
Quadratic	0.8267	< 0.0001	0.0454	0.0005

Table 11. Peel lightness (L) values, hue angles and chlorophyll and carotenoid concentrations of 'Granny Smith' apples at 56 dafb, that were either covered with 40% shadecloth from 14 to 56 dafb, or left unshaded, in the 2006/2007. Means in columns were separated by LSD (5%).

Treatment	L value	Hue (°)	Chlorophyll ($\mu g \cdot g^{-1}$)	Carotenoids ($\mu g \cdot g^{-1}$)
No shading	55.8 b	118.4 a	214 ^{NS}	52 ^{NS}
Shadecloth	58.3 a	117.9 b	172	41
		Pr>F		
Treatment	0.0005	0.0081	0.0828	0.0702

^{NS} Non-significant

Table 12. Peel lightness (L) values, hue angles and chlorophyll and carotenoid concentrations of the exposed side of 'Granny Smith' apples at harvest (160 dafb) that were either covered with 40% shadecloth from 14 to 56 dafb, or left unshaded, in the 2006/2007. Means in columns were separated by LSD (5%).

Treatment	L value	Hue (°)	Sun blemish [z]	Chlorophyll ($\mu g \cdot g^{-1}$)	Carotenoids ($\mu g \cdot g^{-1}$)
No shading	57.5 b	113.3 [NS]	64 b	228 [NS]	56 [NS]
Shadecloth	60.7 a	110.6	87 a	185	47
			Pr>F		
Treatment	0.0273	0.4233	0.0334	0.2787	0.0989

[NS] Non-significant
[z] Sunburn, blush or bronzing

Table 13. Peel lightness (L) values, hue angles and chlorophyll and carotenoid concentrations of the shaded side of 'Granny Smith' apples at harvest (160 dafb) that were either covered with 40% shadecloth from 14 to 56 dafb, or left unshaded, in the 2006/2007. Means in columns were separated by LSD (5%).

Treatment	L value	Hue (°)	Background chart [z]	Chlorophyll ($\mu g \cdot g^{-1}$)	Carotenoids ($\mu g \cdot g^{-1}$)
No shading	60.4 [ns]	116.5 [ns]	1.49 b	221 [NS]	55 [NS]
Shadecloth	61.7	116.1	1.73 a	187	47
			Pr>F		
Treatment	0.3506	0.1224	0.0206	0.2064	0.3151

[NS] Non-significant
[z] Values ranging from 0.5 for a green background to 5 for a yellow background.

PAPER 2:

THE PHOTOPROTECTIVE FUNCTION OF ANTHOCYANINS IN PEARS

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***Abstract*. The profitability of bi-coloured pear (*Pyrus communis* L.) cultivars is limited by poor red colour development. Red colour, imparted by anthocyanins in the peel, reaches a maximum midway through fruit development and declines until harvest. Transient improvements in colour can also occur with the passing of cold fronts. Here we report on trials established to determine whether the photoprotective function of anthocyanins can explain these pigmentation patterns. Colour and photoinhibition of 'Rosemarie' pears was measured as fruit gradually bent over from upright to hanging during development. As shaded peel was exposed to light, red colour increase was concomitant with a reduction in photoinhibition. Chlorophyll quenching was also performed on 'Forelle' pear peel and leaves at a range of temperatures. Peel was very sensitive to excess light at 16 and 24 °C. Peel was also more sensitive to excess light than leaves. 'Cripps' Pink' apples (*Malus domestica* Borkh.) were also used to test our hypothesis, and were measured for red colour response to a passing cold front. Red colour improved very quickly under the high light, low temperature conditions experienced after the passing of the front. Photoprotection may explain both the presence of anthocyanins when fruit are most exposed to sunlight during development, as well as temporary accumulation with passing cold fronts.**

Anthocyanins are responsible for the red colour of bi-coloured pear cultivars, considered desirable by consumers. However, producers struggle to produce blushed pears with enough red colour to satisfy class 1 grading requirements, with the cultivar Rosemarie being particularly problematic (Huysamer, 1998). Peel anthocyanins in 'Rosemarie' reach a maximum in November and then

decline until harvest in January. However, anthocyanins do increase transiently with the passing of cold fronts, but are lost again as temperatures recover afterwards (Steyn et al., 2004a). To develop relevant technologies to solve the problem of poor red colour, we need a deeper understanding of why anthocyanins in the peel respond to the environment in this way. The function of anthocyanins in mature fruit is usually explained by their ability to attract seed dispersers (Harborne, 1965). However, this can only explain anthocyanin accumulation as fruit reach maturity, as is the case for many apple cultivars (Saure, 1990), but not why maximum anthocyanin concentrations in pear peels would be reached early during fruit development, and lost again before harvest. However, we may be able to use apples when we search for an explanation. Apples also display anthocyanin synthesis early in the season like bi-coloured pears, and this is separate from the anthocyanin synthesis that occurs with maturity (Saure, 1990). We cannot propose an evolutionary cause and effect explanation for the presence of anthocyanin in bi-coloured pears, because they are cultivated varieties selected specifically for their appearance, and not their ability to survive. Unlike pears, the early reddening of apples was not selected for in breeding, so it may have an evolutionary purpose. Anthocyanins in apples and pears require light for their synthesis, and when combined with low temperatures, this synthesis is increased (Reay, 1999; Steyn et al. 2004b; Walter, 1967). Any theories proposing a purpose for the anthocyanins' presence would need to incorporate these requirements.

Anthocyanins have numerous protective functions in leaves, including protection against high levels of visible light, UV and herbivory (Gould, 2004). Merzlyak and Chivkunova (2000) suggested that anthocyanins had a photoprotective function in apples, and Smillie and Hetherington (1999) showed that in pods of *Bauhinia variegata,* anthocyanins were an effective light screen, able to prevent damage to chlorophyll caused by high levels of light. When other stressors such as low temperature are also present, photoinhibition can even occur at moderate light levels (Powles, 1984). Our hypothesis is that anthocyanins in pear peel offer a

photoprotective function to the underlying chlorophyll in the pear peel, as this would best explain the reason for anthocyanin synthesis responding to both light and temperature. We suspect that both the seasonal and daily pigmentation pattern of the pears can be explained by a photoprotective function. We propose that anthocyanins accumulate mid-season because that is the point at which the fruit is horizontally orientated, with the largest surface perpendicular to sunlight, and thus most at risk for photodamage. We further propose that the reason for anthocyanin accumulation in response to a cold front is that conditions just after a cold front, when it is still cold, yet sunny, render photosystems very susceptible to damage, i.e., conditions of low temperature and high light intensity. Apples were used to test this hypothesis, due to a lack of suitable weather during pear development.

Materials and methods

Plant material. Field experiments were conducted in apple and pear orchards located at Welgevallen experimental farm, Stellenbosch, Western Cape province, South Africa (lat: 33°58′S, long: 18°50′E). Fruit used for laboratory studies were sampled from the same orchards. All experiments conducted and all fruit sampled were from fully exposed positions from the western periphery of trees planted to a palmette system with a north-south row orientation. Temperature and radiation data were obtained from the Nietvoorbij automatic weather station situated ≈4 km from the trial site.

Experiment 1. On 7 Nov. 2005, 10 fruit, representative of fruit in the orchard, were selected from fully exposed positions on the western side of the trees. These fruit were then marked, and used as a reference point throughout the experiment. When sampling fruit over the following weeks, fruit were selected from similar positions to, and were also orientated at the same angle as the marked fruit. Sampling of 10 fruit took place in the same manner on 7, 14, 21 and 28 Nov., and 5 Dec. 2005. All fruit were sampled before sunrise, and were then dark-adapted for 30 min at 20 °C. The angle of fruit orientation was recorded.

Fruit were randomly divided into two groups of five, where one group was used to measure the previously shaded position of the peel, and the other group used to measure the reddest position of the peel. Fruit diameters were measured with an electronic caliper. Lightness and hue values were measured using a chromameter (Model CR-400, Minolta Co. Ltd., Tokyo, Japan). The lightness value describes how light or dark green the fruit is, with a lower number representing a darker colour. Hue angle ranges between 0 ° = red-purple, 90 ° = yellow, 180 ° = bluish-green and 270 ° = blue, and is the most appropriate method of reporting fruit peel colour (McGuire, 1992). Chlorophyll fluorescence was measured using a pulse-modulated fluorimeter (FMS2; Hansatech Instruments Ltd., Norfolk, England). Maximum photon yield of photosystem II photochemistry was measured as F_v/F_m. In order to determine F_v/F_m, the minimal yield of fluorescence (F_0), was measured in the absence of photosynthetic active radiation. A saturating light pulse (10,800 $\mu mol \cdot m^{-2} \cdot s^{-1}$ photosynthetic photon flux density (PPFD) of 0.7 s) was then applied to induce maximum fluorescence (F_m). Variable fluorescence (F_v) was calculated as F_m - F_0. Fruit were placed in a plastic dish with water (5 mm deep) to prevent water loss. The dish was then placed in a growth cabinet for 30 min at irradiance of 855 $\mu mol \cdot m^{-2} \cdot s^{-1}$ PPFD, with the relevant sides of fruit exposed to the light. After fruit were removed from the growth cabinet, L value, hue and fluorescence (F_v/F_m) were measured again. Photoinhibition (PI) was determined by subtracting the post-irradiance F_v/F_m value from the dark-adapted F_v/F_m value.

Experiment 2: Chlorophyll quenching of fruit peel and leaves was measured at a range of temperatures to determine the susceptibility of fruit peel to photoinhibition at low temperatures compared to leaves. ‘Forelle’ pears were used in this trial. The orchard was established in 1998, on Quince A rootstock. Fruit peel was measured on 3 days from 16 to 18 Jan. 2007. Fruit were sampled before sunrise from the western side of the trees, and dark adapted fro 30 min at 20 °C. A slice of the reddest section of peel was removed and placed in a petri dish with a little water to prevent dehydration. Growth chambers were set to 16,

24, 32 and 40 °C. Peel sections, still in the petri dish, were moved to the growth chamber 30 min before measurement, so as to reach the temperature of the growth chamber. The order of temperature treatments was randomized each day. At each temperature, chlorophyll quenching was carried out on the peel sections at increasing actinic light levels (40, 140, 470 and 1400 $\mu mol \cdot m^{-2} \cdot s^{-1}$ PPFD) using a pulse-modulated fluorimeter (FMS2; Hansatech Instruments Ltd., Norfolk, England). Maximum fluorescence at these actinic light levels was measured as F_m', and minimum fluorescence as F_0'. The effective photon yield of photosystem II was measured as F_v'/F_m', where $F_v' = F_m' - F_0'$. Once a steady state level of fluorescence (F) was reached at each of these light levels, F was recorded, followed by measurements of F_m' and $F_0' - F_m'$. The photon yield (actual efficiency) of PS II photochemistry (Φ_{PSII}) was measured as (Fm'-F)/Fm' (Genty et al., 1989) at stepwise increasing actinic radiation to a maximum of 1400 $\mu mol\ m^{-2}\ s^{-1}$ PPFD. The photochemical chlorophyll fluorescence quenching coefficient, q_p, was determined as $(F_m' - F)/(F_m'-F_0')$ and the non-photochemical chlorophyll fluorescence quenching coefficient, q_{np}, as $(F_m' - F_m')/(F_m - F_0')$. The same procedure was carried out for mature, exposed leaves taken from spurs from 7 to 9 Feb. 2007.

Experiment3. Fruit colour was measured daily at 07h00, 13h00 and 19h00 with the passing of a cold front, to assess whether anthocyanins accumulate rapidly enough to confer photoprotection. 'Cripps' Pink' apples were used in this trial. The trees were planted in 1998 on M793 rootstock. Measurements were made on 40 exposed fruit on the western side of the trees, from 8 to 13 Feb. 2007. A cold front had been forecast to arrive on 9 Feb. 2007. An area on the exposed side of each fruit was marked, and the same point measured throughout the trial. Colour measurements were performed using a chromameter (Model CR-400, Minolta Co. Ltd., Tokyo, Japan). Colour data are presented as hue angle.

Statistical analysis. Analysis of results was carried out using the General Linear Models (GLM) procedure of SAS 9.1 (SAS Institute Inc., 2004; Cary, N.C.).

Results and discussion

Experiment 1: All fruit were upright (0°) at the start of the trial (28 days after full bloom). By 56 dafb, the fruit had bent below the horizontal, with average fruit orientation of ≈105° (Fig. 1a). As fruit bent over, areas of peel that had been shaded were exposed to the sun, so that by 56 dafb these positions were nearly identical. As the previously shaded areas of peel became exposed to light, their colour changed from yellow-green to red as anthocyanins were synthesised (Fig. 1a). At 28 dafb, with the start of the trial, there was an appreciable difference in colour between the exposed and shaded peel areas. By 56 dafb these differences between the exposed and shaded peel areas diminished as fruit bent into the light. During this period, the difference in hue between the two treatments decreased from 53° to 11° (Fig. 1a). From 28 until 42 dafb, after experiencing simulated light stress, the previously shaded positions had more PI than exposed (Fig. 1b). This shows that these peel areas did not have the mechanisms to cope with excess light. By 49 dafb there were no significant differences in PI between exposed and previously shaded peel, indicating that the previously shaded areas of peel were able to cope with the excess light just as well as the reddest peel areas. This improvement coincided with the previously shaded peel accumulating anthocyanins and turning red in colour. Anthocyanins are effective at reducing photoinhibition, but our results do not prove whether they were solely responsible for this improvement.

Plants have numerous mechanisms for coping with excess light, most of which are not visible. These can include the accumulation of xanthophylls and other antioxidants, or the heightened activity of antioxidant enzymes (Adams III and Demmig-Adams, 1992). Xanthophylls can be synthesised very quickly in apple peel to help alleviate photoinhibition under stressful conditions (Ma and Cheng, 2004). Nevertheless, it has been shown that anthocyanins can play a significant role in photoprotection by absorbing excess white and blue-green light (Smillie and Hetherington, 1999). Li and Cheng (2009) showed that anthocyanins present

in shaded peel of red ‘Anjou’ pear help to alleviate photoinhibition under conditions of high light and temperature. Because the peel had always been shaded, there would have been few other mechanisms, apart from the anthocyanins, in place to mitigate this stress. Li et al. (2008) determined that for sun-exposed peel of red ‘Anjou’ pear, despite increased presence of xanthophylls and anti-oxidant enzymes, anthocyanins in the peel were predominantly responsible for reducing photoinhibition.

Experiment 2. The aim of this experiment was to simulate the cold, but bright conditions experienced by pears in the field after the passing of a cold front, and compare this with their response to normal and high summer temperatures they would ordinarily experience during the growing season. The red peel of ‘Forelle’ fruit and mature ‘Forelle’ leaves were compared for their responses to chlorophyll quenching carried out at a range of light levels and temperatures. For all but the lowest light level, Φ_{PSII} for peel was most impaired at 16 °C. At 24°C, Φ_{PSII} was also reduced at the two highest light levels, compared to 32 and 40 °C (Table 1). For 40 and 140 $\mu mol \cdot m^{-2} \cdot s^{-1}$, q_p was reduced at 16 °C. At the maximum light level, q_p was significantly lower for all other temperature treatments compared to the 40 °C treatment (Table 2). This means that the high light conditions combined with lower temperatures caused the closure of photosynthetic reaction centres, but this did not reduce the efficiency of PS II to the same extent. For 40 and 140 $\mu mol \cdot m^{-2} \cdot s^{-1}$, q_{np} of peel chlorophyll was reduced at 16 °C, and also 24 °C for 140 $\mu mol \cdot m^{-2} \cdot s^{-1}$. There were no differences for q_{np} at the higher light levels (Table 3). This evidence that peel photo-apparatus is more sensitive to high light at low temperatures may explain why anthocyanin synthesis is increased under cold conditions (Reay, 1999; Steyn et al. 2004b) and why anthocyanin synthesis takes place with the passing of cold fronts (Steyn et al., 2004a). For leaves, the only differences in Φ_{PSII} were observed for 470 $\mu mol \cdot m^{-2} \cdot s^{-1}$ and 1400 $\mu mol \cdot m^{-2} \cdot s^{-1}$, where the 16 °C and 16 and 24 °C treatments, respectively, showed a reduction in Φ_{PSII} (Table 4). For q_p of leaves, the only difference occurred at 16 °C for the two highest light levels (Table 5). There were no significant differences for q_{np}

between treatments any light level (Table 6). Leaves followed a very similar response to peel for Φ_{PSII} and q_p, although they were better able to cope with the stressful conditions. However, at the highest light and temperature treatments, they appear to have lost their advantage over the peel (Tables 1, 2, 4, and 5). Peel also generally showed a more linear response, compared to the general quadratic response of the leaves (Tables 1, 2, 4, and 5). However, there were exceptions, including a linear response with temperature of leaf q_{np} at 470 $\mu mol \cdot m^{-2} \cdot s^{-1}$. Differences in q_p show that more reaction centers were closed under low temperature, high light conditions, whereas the general lack of difference in q_{np} for the different treatments shows that the ability of the tissues to dissipate heat is similar under all treatment conditions. These differences found between peel and leaves may be due to the processes in fruit photosynthesis differing substantially from those that take place in leaves (Blanke and Lenz, 1989; Pavel and DeJong, 1993).

Experiment 3. The hue change of 'Cripps' Pink' apples with the passing of a cold front during the 2006/2007 season is presented in Figure 2. Fruit colour was measured daily at 07h00, 13h00 and 19h00. The cold front arrived on 9 Feb., bringing cloudy, cool conditions, i.e., reduced radiation and lower temperatures (Fig. 2a). Hue already began to gradually decrease, and it is interesting to note that some red colour development could take place during these cloudy conditions (Fig. 2b). The following day, the weather cleared, but temperatures remained low (Fig. 2a.) This resulted in the fruit becoming substantially redder than they did the previous day, indicated by the decrease in hue (Fig. 2b). On 11 and 12 Feb., temperatures remained mild with partly cloudy skies (Fig. 2a) which resulted in continued red colour development (Fig. 2b). By 13 Feb., the clouds had completely cleared and temperatures returned to normal (Fig. 2a), resulting in a loss of red colour that day (Fig.2b). From 19h00 on 9 Feb. until 19h00 on 10 Feb. there was a total hue decrease of 10 ° (Fig. 2b), with a total hue decrease from 87 ° to 60 ° taking place from 07h00 on 9 Feb. to 07h00 on 13 Feb (data not shown). This confirms the findings of Steyn et al. (2004a) where daily hue

measurements on ‘Rosemarie’ pears also showed a decrease with passing cold fronts. Additionally, with the more frequent measurement of colour, our experiment was able to show that substantial anthocyanin synthesis can occur within six hours, under conditions of high light levels combined with low temperatures.

Conclusions

A photoprotective function of the anthocyanins may be explain both the seasonal and daily pigmentation patterns of red colour in peel. Although we showed that red colour increases and photoinhibition decreases when fruit are orientated to be most susceptible to light damage, this is insufficient to prove that the sole purpose of the timing of this pigmentation is to reduce photoinhibiton, and is not merely coincidental. However, the behaviour of ‘Forelle’ and ‘Cripps’ Pink’ peel under high light, low temperature conditions, strongly suggests that the purpose of daily changes in anthocyanin content is to protect peel chlorophyll from damage under stressful conditions. Our findings in these trials have placed some crucial pieces in the puzzle of pear red colour development, particularly by showing how rapidly red colour can develop under suitable conditions. Such a photoprotective role may now also support why anthocyanins are more likely to be formed where N levels are low, and knowing why anthocyanins occur in the peel may help to explain why certain methods to improve red colour may be ineffective.

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Table 1. Photon yield of PSII (Φ_{PSII}) at increasing light levels, for 'Forelle' pear peel exposed to different temperatures during the 2006/2007 season. Means in columns were separated by LSD (5%).

Temperature (°C)	PPFD ($\mu mol \cdot m^{-2} \cdot s^{-1}$)			
	40	140	470	1400
16	0.673 ^{NS}	0.475 b	0.193 c	0.066 b
24	0.725	0.592 a	0.306 b	0.089 b
32	0.758	0.684 a	0.450 a	0.154 a
40	0.733	0.692 a	0.490 a	0.191 a
	Pr>F			
Temperature	0.0678	0.0104	0.0021	0.0041
Temperature linear	0.0342	0.0024	0.0003	0.0006
Temperature quadratic	0.0723	0.1059	0.2980	0.6581

^{NS} Non-significant

Table 2. Photochemical quenching (q_p) at increasing light levels, for 'Forelle' pear peel exposed to different temperatures during the 2006/2007 season. Means in columns were separated by LSD (5%).

Temperature (°C)	PPFD ($\mu mol \cdot m^{-2} \cdot s^{-1}$)			
	40	140	470	1400
16	0.892 b	0.661 b	0.299 c	0.112 c
24	0.934 a	0.797 a	0.469 b	0.163 c
32	0.958 a	0.884 a	0.648 a	0.265 b
40	0.960 a	0.906 a	0.726 a	0.365 a
	Pr>F			
Temperature	0.0174	0.0067	0.0006	0.0023
Temperature linear	0.0043	0.0014	0.0001	0.0004
Temperature quadratic	0.1267	0.1315	0.2414	0.3905

Table 3. Non-photochemical quenching (q_{np}) at increasing light levels, for 'Forelle' pear peel exposed to different temperatures during the 2006/2007 season. Means in columns were separated by LSD (5%).

Temperature (°C)	PPFD (μmol·m^{-2}·s^{-1})			
	40	140	470	1400
16	0.361 a	0.528 a	0.688 ^{NS}	0.785 ^{NS}
24	0.218 b	0.414 a	0.689	0.812
32	0.163 b	0.236 b	0.592	0.831
40	0.207 b	0.267 b	0.583	0.852
	Pr>F			
Temperature	0.0098	0.0045	0.0970	0.1610
Temperature linear	0.0058	0.0010	0.0227	0.0916
Temperature quadratic	0.0140	0.0983	0.8890	0.8875

^{NS} Non-significant

Table 4. Photon yield of PSII (Φ_{PSII}) at increasing light levels, for 'Forelle' pear leaves exposed to different temperatures during the 2006/2007 season. Means in columns were separated by LSD (5%).

Temperature (°C)	PPFD (μmol·m^{-2}·s^{-1})			
	40	140	470	1400
16	0.694 ^{NS}	0.558 ^{NS}	0.251 b	0.077 b
24	0.785	0.729	0.458 a	0.118 ab
32	0.772	0.720	0.463 a	0.157 a
40	0.703	0.675	0.413 a	0.144 a
		Pr>F		
Temperature	0.4096	0.1283	0.0178	0.0179
Temperature linear	0.9686	0.1767	0.0275	0.1397
Temperature quadratic	0.1181	0.0603	0.0118	0.0059

^{NS} Non-significant

Table 5. Photochemical quenching (q_p) at increasing light levels, for 'Forelle' pear leaves exposed to different temperatures during the 2006/2007 season. Means in columns were separated by LSD (5%).

Temperature (°C)	PPFD ($\mu mol \cdot m^{-2} \cdot s^{-1}$)			
	40	140	470	1400
16	0.945 ^{NS}	0.808 ^{NS}	0.479 b	0.182 b
24	0.986	0.928	0.738 a	0.359 a
32	0.975	0.926	0.770 a	0.361 a
40	0.942	0.912	0.748 a	0.324 a
		Pr>F		
Temperature	0.4172	0.1064	0.0206	0.0182
Temperature linear	0.8117	0.0905	0.0124	0.0258
Temperature quadratic	0.1247	0.0824	0.0335	0.0131

^{NS} Non-significant

Table 6. Non-photochemical quenching (q_{np}) at increasing light levels, for 'Forelle' pear leaves exposed to different temperatures during the 2006/2007 season. Means in columns were separated by LSD (5%).

Temperature (°C)	PPFD (μmol·m^{-2}·s^{-1})			
	40	140	470	1400
16	0.472 ^{NS}	0.643 ^{NS}	0.855 ^{NS}	0.905 ^{NS}
24	0.232	0.356	0.766	0.900
32	0.277	0.373	0.763	0.910
40	0.337	0.371	0.765	0.904
	Pr>F			
Temperature	0.4127	0.1537	0.0736	0.6863
Temperature linear	0.4978	0.1050	0.0447	0.7287
Temperature quadratic	0.1786	0.1524	0.0934	0.8905

^{NS} Non-significant

Fig. 1. Change in hue angle (a) and percentage photoinhibition (b) of ‘Rosemarie’ pears, measured on the exposed and previously shaded areas of fruit peel subjected to simulated light stress, measured weekly during the 2005/2006 season, while fruit bent over from an upright (0 °) to a hanging (> 90 °) orientation. Hue angle decreases with increasing redness of peel. Photoinhibition decreases with increasing ability to cope with light stress. Mean change of hue and photoinhibition ±SE bars is the average for 10 fruit.

Fig. 2. Change in hue angle (a) of ‘Cripps’ Pink’ apples in relation to radiation levels (b) and air temperature (b) measured with the passing of a cold front from 8 to 13 Feb. 2007. Hue angle decreases with increasing redness of peel. Therefore, a negative change in hue on the graph indicates an increase in red colour. The mean change in hue ±SE bars for each measurement time is the average for 36 fruit. Adapted from Steyn et al. (2009).

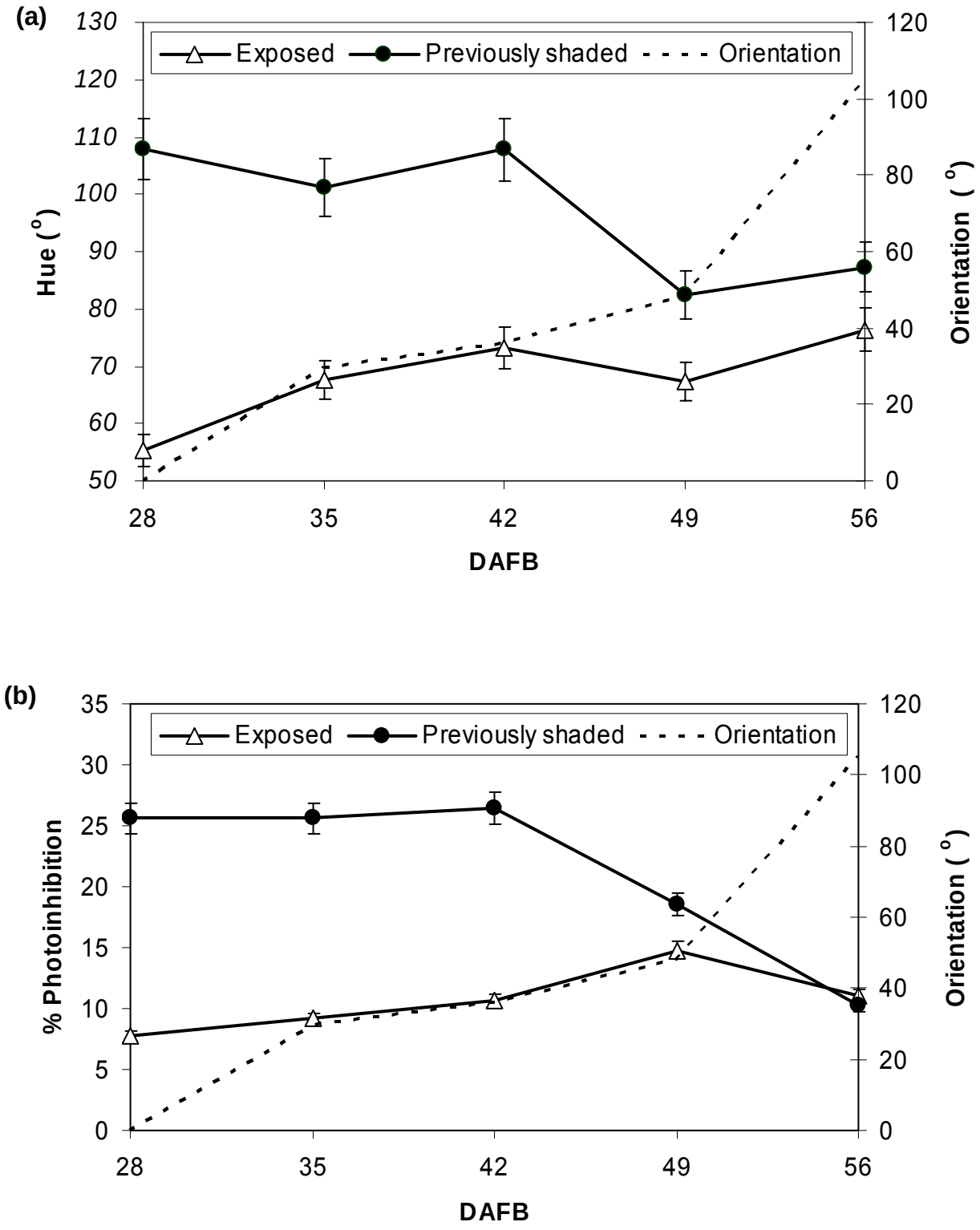
(a)
Exposed
Previously shaded
Orientation
Hue (°)
Orientation (°)
DAFB
(b)
Exposed
Previously shaded
Orientation
% Photoinhibition
Orientation (°)
DAFB

Fig. 1. Paper 2

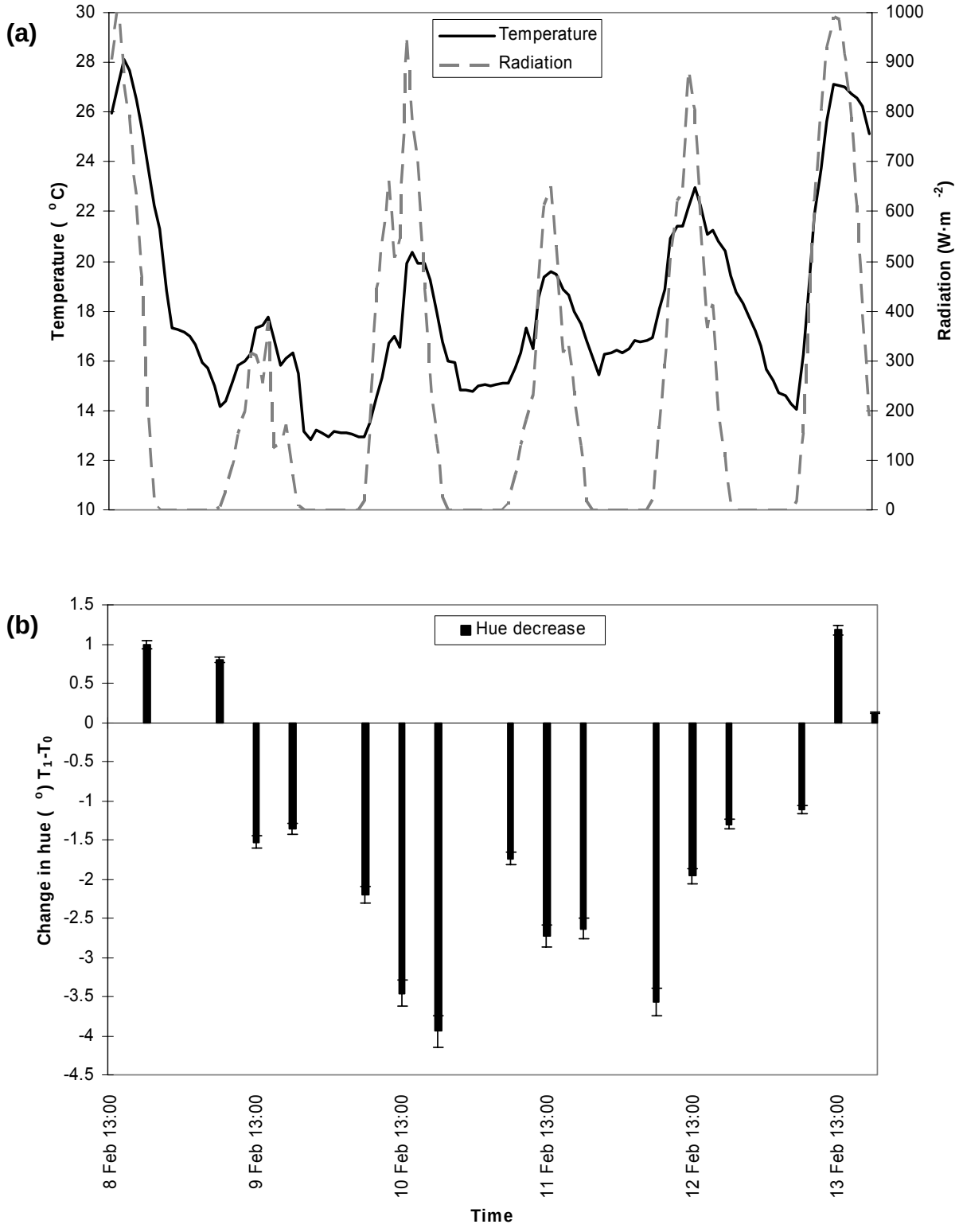
(a)
Temperature
Radiation
Temperature (°C)
Radiation (W·m -2)
30
28
26
24
22
20
18
16
14
12
10
1000
900
800
700
600
500
400
300
200
100
0
(b)
Hue decrease
Change in hue (°) T1-T0
1.5
1
0.5
0
-0.5
-1
-1.5
-2
-2.5
-3
-3.5
-4
-4.5
8 Feb 13:00
9 Feb 13:00
10 Feb 13:00
11 Feb 13:00
12 Feb 13:00
13 Feb 13:00
Time

Fig. 2. Paper 2

PAPER 3:

EFFECT OF ROOTSTOCK ON RED COLOUR OF BI-COLOURED 'FORELLE' PEARS

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***Abstract*. Insufficient red colour limits the profitability of bi-coloured pear cultivars in South Africa. Here we report on a trial conducted to establish whether red colour can be improved by choice of rootstock. We assessed the effect of the clonal pear (*Pyrus communis* L.) rootstocks BP1, BP3, Old Home x Farmingdale (OHxF) 97; and clonal quince (*Cydonia oblonga* Mill.) rootstocks Quince A, Quince C 51 and BA 29 on the colour of 'Forelle' pears over two seasons. Light interception effects caused by differences in vigour were negated by only sampling exposed fruit. Fruit from trees on BP rootstocks showed the poorest red colour. This does not appear to be related to differences in fruit maturity between the rootstocks, because firmness correlated poorly with background colour. Chlorophyll and carotenoid concentrations of the peel were significantly lower in fruit from quince rootstocks. Differences in anthocyanin concentrations between the treatments were non-significant, although they did follow colour measurement trends. Peel and leaves from trees on BP rootstocks contained significantly more nitrogen, which may explain their higher peel chlorophyll concentrations. Fruit from trees on QC 51 rootstock most likely appeared redder due to lower chlorophyll concentrations in the peel. Our conclusion is that the different rootstocks may have a direct effect on red colour that is not related to differences in light interception or maturity, but may in part result from differences in nitrogen concentrations.**

Bi-coloured pear cultivars have an attractive red blush on a green or yellow background. These bi-coloured pears are a valuable export product for South Africa, but are often downgraded due to poor red colour, leading to reduced

revenues (Huysamer, 1998). ‘Forelle’ is South Africa’s most important bi-coloured pear, accounting for 18% of exports (Deciduous Fruit Producers Trust, 2005). The red colour of bi-coloured pears is due to the presence of anthocyanins in the peel (Francis, 1970). However, perception of the fruit colour is also a result of the blending of chlorophylls, carotenoids and anthocyanins in the peel (Lancaster et al., 1994). Anthocyanin synthesis in pears is light dependant, thus good light exposure is critical for the production of red fruit (Steyn et al., 2005). Walter (1967) concluded that dwarfing rootstocks tend to produce redder apples because they allow for greater exposure of the fruit to sunlight. Du Plooy and Van Huyssteen (2000) found that ‘Forelle’ pears on a dwarfing quince rootstock had redder colour, and also attributed this to better light distribution compared to vigorous BP1 and BP3 rootstocks. However, the work of Jackson (1967) suggested that apples at identical light positions in the canopy may have better colour on dwarfing rootstocks compared to invigorating rootstocks. This led us to ask whether there may be a direct effect of rootstock on red colour and pigment levels in pear that is not a result of variances in light distribution.

Our aim in this study was to determine if there was a difference in ‘Forelle’ pear red colour when grafted to different rootstocks. The effect of differences in light distribution on red colour was to be negated by only sampling exposed fruit.

Materials and methods

Trial layout and sampling. The trial site, in Villiersdorp, Western Cape province, South Africa (lat: 33°59′S, long: 19°18′E), consisted of ‘Forelle’ pears grafted on three clonal pear rootstocks (BP1, BP3 and OHxF 97) and three clonal quince rootstocks (QA, QC 51 and BA 29). Trees were planted in 1999, in a north-south direction, and trained to a V-system. Trees were staggered in double rows on mounds. Distance between the sets of double rows was 4.2 m, while trees within the double rows were staggered at 0.5 x 0.5 m. Rootstocks were randomized in four blocks with 10 trees for each rootstock per replicate. Trunk circumferences

(Table 1) were measured 15 cm above the graft union, in June 2006. At harvest, 100 fruit were sampled per replicate. All fruit were sampled from full-sun positions in order to negate the effect of the improved light distribution within trees on dwarfing rootstocks. In 2005/2006, fruit were harvested one week before optimal maturity (21 Feb. 2006). In 2006/2007 fruit were harvested two weeks beyond optimal maturity (5 Mar. 2007). During January of both seasons, five leaves were also sampled at shoulder height from the current season's shoots, from each of the 10 trees per replicate.

Colour and maturity measurements. In 2005/2006, the colour of all 100 fruit per replicate was measured at the reddest point, using a chromameter (Model CR-400, Minolta Co. Ltd., Tokyo). The chroma value describes how vivid or dull a colour is, with a higher number representing a more vivid colour. Hue angle ranges between 0 ° = red-purple, 90 ° = yellow, 180 ° = bluish-green and 270 ° = blue, and is the most appropriate method of reporting fruit peel colour (McGuire, 1992). In 2006/07, the background green colour was also measured with the chromameter. A subsample of 25 fruit per replicate was used for further subjective colour and maturity assessments. In 2005/06 this subsample consisted of the reddest fruit, while in 2006/07 the subsample was randomly selected. Blush percentage was subjectively determined by averaging the estimated blush coverage of the peel surface for both the blushed and green sides. Background colour was measured by comparing the green side of fruit with the Colour Chart for Apples and Pears (Unifruco Research Service [Pty] Ltd.), with values ranging from 0.5 for a green background to five for a yellow background. Flesh firmness was determined on pared, opposite cheeks with a fruit texture analyser (GÜSS; Strand, South Africa), using an 11 mm tip. Fruit of each replicate were pooled, juiced and total soluble solids (TSS) measured with a digital refractometer (PR32 ATAGO, Tokyo). Blushed areas of fruit were peeled, using a knife to remove only the pigmented layers of peel, and no flesh. Peel was pooled together within a replicate, frozen in liquid nitrogen, and stored at -80 °C.

Peel was ground by hand in liquid nitrogen, using a mortar and pestle, and returned to -80 °C until pigment analysis.

Pigment analysis. Anthocyanins were extracted from ≈0.2 g sample in 5 ml 5% (v/v) 3 M hydrochloric acid in methanol at 4 °C for 1 h in the dark. The extract was centrifuged at 10 000 g_n for 10 min and decanted, whereafter 5 ml of solvent was added to the sample, which was again centrifuged and decanted in the same manner. The decanted extracts were combined, filtered through a 0.45 µm filter (Millex-HV; Millipore Corporation, Milford, Mass.) and absorption measured at 530 and 653 nm with a spectrophotometer (Cary 50 Series, Varian, Mulgrave, Australia). The 530 nm reading was corrected for the presence of chlorophyll by subtracting 24% of absorbance at 653 nm (Murray and Hackett, 1991). Anthocyanin concentrations were determined using idaein chloride (cyanidin-3-galactoside) to obtain a standard curve. Anthocyanins were expressed as $\mu g \cdot g^{-1}$ fresh weight of peel. Chlorophylls and carotenoids were extracted from ~0.3 g sample in 3 ml acetone for 24 h at 4 °C in the dark. The extract was centrifuged at 10 000 g_n for 15 min and decanted, whereafter 2 ml of solvent was added to the sample, which was again centrifuged and decanted in the same manner. The decanted extracts were combined, filtered through 0.45 µm filters and absorption measured at 470, 645 and 662 nm. The extinction coefficients of Lichtenthaler (1987) were used to calculate chlorophyll and carotenoid concentrations, which were then expressed as $\mu g \cdot g^{-1}$ fresh weight of peel.

Mineral analysis. Mineral analysis of all peel and leaves was carried out using inductively-coupled plasma-emission spectroscopy at an analytical laboratory (Bemlab [Pty] Ltd., Strand, South Africa).

Statistical analysis. Results were analysed using the General Linear Models (GLM) and Correlation (CORR) procedures of SAS 9.1 (SAS Institute Inc., 2004; Cary, N.C.).

Results and discussion

The combination of these rootstocks should have a range of approximately 40 to 100% vigour, compared to a *P. communis* seedling tree (Huysamer, 1997; Wertheim, 2002; Jacobs and Cook, 2003). The greatest circumference was for trees on BP1 and BP3, which was significantly higher than the smallest, which was for trees on QC51 and OHxF 97, with QA and BA 29 falling in-between (Table 1). Red colour was different between the rootstocks in both seasons (Tables 2 and 3). Fruit from trees on quince rootstocks generally had the reddest colour, represented by lower hue angle, higher chroma value and a greater percentage of blush. This means that not only were the fruit were redder, but the red colour was also more vivid. OHxF 97 gave colour similar to the quince rootstocks, while fruit on the BP rootstocks tended to be less red. There was a significant difference in 2006/2007 for background green colour between rootstocks, according to the background chart and background hue, with fruit from quince rootstocks being more yellow (Table 3). Since background chart and background hue gave similar results (Table 3), only correlations with background chart are presented so that comparisons can be made between the seasons (Table 5). For red colour, correlations with chroma and percent blush are not presented as their correlations were very similar to those of hue.

Pigment data were highly variable (Table 4). This is most likely because of the low number of replicates within the experiment as well as possible errors with the peeling of the fruit. It was thus necessary to combine the pigment data of both seasons for statistical analysis. Chlorophyll and carotenoid concentrations were significantly different, but anthocyanins were not significant at the 5% level (Table 4). However, pigment concentrations do follow the colour measurement trends (Tables 2, 3 and 4).

The yellow background colour of fruit from quince rootstocks is most likely due to carotenoids being more visible as a result of a lower chlorophyll concentration,

within the peel. Generally, such a yellow background colour would be attributed to chlorophyll degradation caused by advanced maturity (Knee, 1972). However, flesh firmness, the industry benchmark of pear maturity, correlates poorly with background colour (Table 5). Unlike apples, pears do not have a ripening associated peak in anthocyanins (Steyn et al., 2004). This suggests that colour differences found between the rootstocks may not result from differences in maturity.

The better red colour of fruit from quince rootstocks may, in part, be due to their more yellow background colour. Since fruit colour results from the blending of chlorophylls, carotenoids and anthocyanins in the peel (Lancaster et al., 1994), at similar levels of anthocyanin, fruit containing less chlorophyll and carotenoids should appear more intensely red. This may explain why fruit from QA appeared very red but had the lowest anthocyanin concentrations, because these fruit also had the lowest chlorophyll and carotenoid concentrations (Tables 2, 3 and 4).

TSS correlated negatively with hue and positively with background colour in 2006/2007, with no significant correlations in 2005/2006 (Table 6). Since carbohydrate accumulation and anthocyanin synthesis often respond to the same environmental stimuli, and anthocyanin synthesis is sugar inducible (Steyn et al., 2002), the poorer colour of fruit on BP rootstocks in 2006/2007 could be related to their lower TSS levels (Table 3). There also appeared to be a low crop load on the BP3 trees in 2006/2007 that may somehow have played a role in the particularly poor colour that season.

Nitrogen levels in leaves and fruit peel were significantly higher from pear rootstocks than quince rootstocks (Table 7). Peel and leaf nitrogen correlated positively with hue and negatively with background colour in 2006/2007, with no significant correlations in 2005/2006 (Tables 8 and 9). The negative correlation between nitrogen and TSS is most likely because TSS also correlates well with hue and background colour (Tables 6, 8 and 9). Williams and Billingsley (1974)

found a direct relationship between leaf nitrogen and ‘Golden Delicious’ apple colour, where higher levels of nitrogen were associated with greener fruit. In addition, Evans (1989) found a strong correlation of leaf nitrogen with leaf chlorophyll content in a number of plant species. With the addition of nitrogen, Sakomoto et al. (1994) were able to reduce anthocyanin accumulation in cell suspension cultures. Reay et al. (1998) found in apples, that urea sprays that increased peel nitrogen content resulted in reduced peel anthocyanin concentrations; and it is well documented that high nitrogen levels are associated with poor red colour development in apples (Walter, 1967). The combination of these nitrogen effects could explain the reason for colour differences observed among the rootstocks.

We would recommend that the BP rootstocks should not be used for ‘Forelle’ pears. The high N levels present in BP rootstocks most likely interfere with anthocyanin synthesis and also cause the fruit to have a ruddy, unattractive red colour because of high chlorophyll concentrations in the peel. Even with optimal light management, ‘Forelle’ trees on BP rootstocks would still have a lower innate ability to produce red fruit compared to fruit from trees on quince and OHxF 97 rootstocks.

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Table 1.Trunk circumferences of 'Forelle' trees on different rootstocks, measured in June 2006. Means in columns separated by LSD (5%).

Rootstock	Trunk circumference (cm)
BP3	30.7 ab
BP1	31.3 a
OHxF 97	26.9 cd
BA 29	29.8 abc
QA	27.9 bcd
QC 51	26.7 d
	Pr>F
Rootstock	0.0189

Table 2. Effect of different rootstocks on fruit colour and maturity parameters of 'Forelle' pears at harvest, for the 2005/2006 season. Means in columns separated by LSD (5%).

Rootstock	Hue (°)	Chroma	Blush (%)	Firmness ($kg \cdot m^{-2}$)	TSS (° Brix)	Background chart[z]
BP3	51 a	31 d	40 b	6.8 a	14.7[NS]	2.2[NS]
BP1	52 a	32 cd	39 b	6.7 a	14.6	2.3
OHxF 97	50 a	33 c	48 a	6.4 b	15.1	2.2
BA 29	48 ab	34 bc	46 ab	6.4 b	15.1	2.2
QA	48 ab	35 ab	44 ab	6.4 b	15.2	2.3
QC 51	42 b	36 a	48 a	6.4 b	15.2	2.4
			Pr>F			
Rootstock	0.0504	<0.0001	0.0366	0.0017	0.3136	0.2541

[NS] Non-significant

[z] Values ranging from 0.5 for a green background to 5 for a yellow background.

Table 3. Effect of different rootstocks on fruit colour and maturity parameters of 'Forelle' pears at harvest, for the 2006/2007 season. Means in columns separated by LSD (5%).

Rootstock	Hue (°)	Chroma	Blush (%)	Firmness ($kg \cdot m^{-2}$)	TSS (° Brix)	Background chart[z]	Background hue (°)
BP3	58 a	35 b	44 ab	5.84[NS]	12.6 b	2.4 c	112 a
BP1	50 ab	35 b	30 b	5.97	13.4 ab	2.7 bc	111 ab
OHxF 97	43 bc	38 ab	52 a	6.12	14.3 a	2.8 b	110 bc
BA 29	38 c	40 a	57 a	5.83	14.1 a	3.2 a	109 c
QA	42 bc	39 a	52 a	5.97	13.9 a	3.0 ab	109 c
QC 51	40 bc	40 a	55 a	6.06	14.2 a	3.1 ab	109 c
				Pr>F			
Rootstock	0.0176	0.0090	0.0200	0.1073	0.0197	0.0021	0.0034

[NS] Non-significant

[z] Values ranging from 0.5 for a green background to 5 for a yellow background.

Table 4. Effect of different rootstocks on peel pigments of 'Forelle' pears at harvest, averaged over the 2005/2006 and 2006/2007 seasons. Means in columns separated by LSD (5%).

Rootstock	Anthocyanins ($\mu g \cdot g^{-1}$)	Chlorophyll ($\mu g \cdot g^{-1}$)	Carotenoids ($\mu g \cdot g^{-1}$)
BP3	689[NS]	227 a	89 a
BP1	785	216 ab	87 ab
OHxF 97	876	185 bc	77 abc
BA 29	962	171 c	71 bc
QA	677	149 c	63 c
QC 51	1028	177 bc	76 abc
		Pr>F	
Rootstock	0.1071	0.0090	0.0448

[NS] Non-significant

Table 5. Correlations of fruit hue and maturity parameters for 'Forelle' pear on all rootstocks for the 2005/2006 season.

	2005/2006		2006/2007	
Correlation	*r* [z]	*Pr>F*	*r*	*Pr>F*
Hue with firmness	-0.0695	<0.0001	-0.09042	0.0268
Hue with background colour chart	-0.08415	0.0393	0.45449	<0.0001
Background colour chart with firmness	-0.21651	<0.0001	0.14825	0.0004

[z]Pearson Correlation Coefficients / Prob > |R| under Ho: Rho=0 / N where n = 600.

Table 6. Correlations of fruit TSS with colour for 'Forelle' pear on all rootstocks for the 2005/2006 and 2006/2007 seasons.

	2005/2006		2006/2007	
TSS correlated with:	*r* [z]	*Pr>F*	*r*	*Pr>F*
Hue °	-0.20761	0.3303	-0.86898	<0.0001
Background colour chart	-0.26812	0.2052	0.66094	0.0006

[z]Pearson Correlation Coefficients / Prob > |R| under Ho: Rho=0 / N where n = 24.

Table 7. Effect of different rootstocks on nitrogen contents of 'Forelle' fruit peel and leaves for the 2005/2006 and 2006/2007 seasons. Means in columns separated by LSD (5%).

	Peel nitrogen (%)		Leaf nitrogen (%)	
Rootstock	2005/2006	2006/2007	2005/2006	2006/2007
BP3	0.95 a	0.84 a	2.54 a	2.54 a
BP1	0.94 a	0.77 ab	2.35 b	2.48 ab
OHxF 97	0.90 a	0.70 bc	2.33 bc	2.37 b
BA 29	0.82 b	0.64 cd	2.28 bcd	2.24 c
QA	0.79 b	0.59 d	2.18 d	2.21 c
QC 51	0.82 b	0.65 bcd	2.23 cd	2.21 c
		Pr>F		
Rootstock	0.0002	0.0042	0.0001	0.0002

Table 8. Correlations of peel and leaf nitrogen with colour and maturity parameters for 'Forelle' pear on all rootstocks for the 2005/2006 season.

	Peel nitrogen		Leaf nitrogen	
Correlated with:	*r* [z]	*Pr>F*	*r*	*Pr>F*
Hue °	0.28704	0.1738	0.17261	0.4199
Background colour chart	-0.12043	0.5751	-0.40158	0.0518
TSS	-0.45396	0.0259	-0.38251	0.0651

[z]Pearson Correlation Coefficients / Prob > |R| under Ho: Rho=0 / N where n = 24.

Table 9. Correlations of peel and leaf nitrogen with colour and maturity parameters for 'Forelle' pear on all rootstocks for the 2006/2007 season.

	Peel nitrogen		Leaf nitrogen	
Correlated with:	*r* [z]	*Pr>F*	*r*	*Pr>F*
Hue °	0.77487	<0.0001	0.56850	0.0037
Background colour chart	-0.75625	<0.0001	-0.64675	0.0009
TSS	-0.59752	0.0020	-0.66034	0.0004

[z]Pearson Correlation Coefficients / Prob > |R| under Ho: Rho=0 / N where n = 24.

PAPER 4:

LEAF COLOUR OF SEEDLINGS CAN BE USED TO STREAMLINE THE BREEDING OF BI-COLOURED PEARS

***Abstract*. A breeding programme is underway to develop a new early season bi-coloured pear (*Pyrus communis* L.) with stable red colour. Fruit breeding is time and resource intensive, and breeders are looking for ways to reduce costs and increase the likelihood of success. Within these trials, immature leaf colour and fruit colour of individual seedling trees were measured, to establish whether a relationship may exist between immature leaf colour and fruit colour. Fruit from trees with red immature leaves were likely to be fully red, and thus undesirable for the breeding programme. Trees with fully green leaves or with green leaves with a red edge (bi-coloured) could produce either green or bi-coloured fruit. From our findings it would be possible to remove seedlings with red leaves at a young age, as nearly all of them will produce undesirable red fruit. However, this red leaf and fruit trait appears to be restricted to progeny of 'Bon Rouge', and more research is required to develop a universal process of culling seedlings based on immature leaf colour.**

Bi-coloured pear cultivars have an attractive red blush on a green or yellow background, and are a valuable export product for South Africa. Forelle and the locally bred Rosemarie and Flamingo are South Africa's most important blushed cultivars (Huysamer, 1998). According to Human (2002), 'Rosemarie' and 'Flamingo' were selected because they ripen earlier than 'Forelle' and widen the marketing window for bi-coloured pears. However, these cultivars suffer from poor red colour development, amongst other faults, and are often downgraded, reducing their profitability. The breeding division of the Agricultural Research Council (ARC) Infruitec-Nietvoorbij has a programme to develop new bi-coloured cultivars. However, around 5000 seedlings are produced per year in the breeding programme and these need to be maintained until they reach the end of their juvenile phase and start bearing

fruit, which takes an average of six years, but often as long as 10 years (Visser, 1964). Once the fruit from these seedlings can be evaluated, it is most likely that nearly all of them will have unsuitable colour and will not be selected for the second phase of evaluation. The maintenance of this many seedlings is very costly, and return on investment may only come after 20 years if a successful cultivar is found. In order to reduce costs and increase the likelihood of success, a method is required to select for fruit colour when seedlings are as young as possible.

Fully red pears first originated from bud mutations in the form of periclinal chimeras (Chevreau et al., 1989), and this gene is carried in the second histogenic layer of the apical meristem, with anthocyanins occurring in two to five layers below the non-pigmented epidermis (Dayton, 1966). This pigmentation is also visible in the leaves, petioles and shoots (Dayton, 1966). ‘Max-Red Bartlett’ and ‘Red Bartlett’ are the best known of these mutations, and are widely used for their red colour trait in breeding trials. In South Africa, the red-skinned, Bon Chretien mutation known as Bon Rouge, was crossed with Forelle, and gave rise to the bi-coloured cultivars Flamingo and Rosemarie (Human, 2002). The only natural red colour mutant that does not follow this pattern is the vividly red Starkrimson cultivar, where the mutation occurred in the outer histogenic layer, and anthocyanins are present almost exclusively in the epidermis. This prevents the transmission of ‘Starkrimson’s’ colour to progeny, which makes the cultivar unsuitable for traditional breeding (Dayton, 1966).

The ARC breeders noticed that one-year-old pear seedlings, from various crosses, with red leaves, tended to produce red fruit when they eventually matured. They also observed that the immature leaves of each season’s new vegetative growth of the same fruiting trees were red as they had been in seedlings. This led us to the hypothesis, that a correlation could be found between immature leaf colour and fruit peel colour, which would allow one-year-old seedlings to be culled based on leaf colour. Hence, we first had to establish the correlation between mature fruit and immature leaf colour, and then determine whether immature leaf colour could provide a reliable way to

select for bi-coloured pears. We chose to conduct colour measurements with a tri-stimulus chromameter as we needed an affordable and easy method that could be used in the field.

Materials and methods

2005/2006. We used a total of 136 seedlings from four crosses for this study: ‘Doyenne du Comice’ (‘Comice’) × ‘Rosemarie’, ‘Ceres’ × ‘Bon Rouge’, ‘Rosemarie’ × ‘Bon Rouge’ and ‘Flamingo’ × ‘Bon Rouge’. Parent fruit colour, number of seedlings and planting dates for each of the crosses are presented in Table 1. These seedlings were planted in an east-west direction at Drostersnes experimental farm, near Grabouw (lat: 34°10’S, long: 19°03’E), Western Cape province, South Africa.

On 8 and 9 Dec. 2005, three immature leaves were picked from each of the seedling trees bearing fruit, and their colour measured using a chromameter (Model CR-400, Minolta Co. Ltd., Tokyo). Hue ranges between 0 ° = red-purple, 90 ° = yellow, 180 ° = bluish-green and 270 ° = blue, and describes leaf colour most aptly (McGuire, 1992). The immature leaves were also subjectively categorised as either red, bi-coloured or green. Fruit were harvested on various dates in Jan. and Feb. 2006. Between one and 10 fruit per tree were harvested at a firmness of 6 to 8 kg (8.4 mm probe). All fruit harvested were from the northern side of the trees and were fully exposed to the sun. After harvest, fruit were stored at -0.5 °C for 8 weeks, and placed in the laboratory at room temperature for 48 h before analysis. Fruit peel colour was measured with a chromameter (NR-3000; Nippon Denshoku, Tokyo) and hue was recorded.

Fruit were placed individually on a step-up motor for measurement of colour coverage. A high-resolution digital camera (DXM1200, Nikon, Tokyo) adapted with ×0.63 relay lens was used to collect images of the two opposite sides of each fruit (most and least coloured sides). A microlite fluorescent ring light for epi-illumination was applied for optimal lighting. Total fruit area coloured with

red pigmentation was determined using Image Pro Plus 4.5 image analysis software (Media Cybernetics, 2001, Bethesda, Md.). The average hue and percentage red colouration for the fruit from each seedling were used to categorise the seedlings as having green, blushed or red fruit. Where fruit had more than 50% red colouration, they were classified as red. Fruit with less than 50% red colouration, but with a red blush of a hue less than 60 ° were considered blushed, while the remainder of the fruit were classified as green.

2006/2007. The seedlings used in 2005/2006 were unavailable for this trial in 2006/2007. In 2006/2007, 95 seedlings from five crosses were utilised: 'Flamingo' × 'Bon Rouge', 'Harrow Delight' × 'Bon Rouge' 'Harrow Delight' × 'Rosemarie', 'Comice' × 'Bon Rouge' and 'Comice' × 'Flamingo'. Parent fruit colour, number of seedlings and planting dates for each of the crosses are presented in Table 2. These seedlings were planted in a north-south direction at the Bien Donné experimental farm near Simondium (lat. 33°3′S, long. 19°9′E), Western Cape province, South Africa. Immature leaves were sampled, their colour measured and categorised on 20, 23 and 24 Nov. 2006, using the same methods as the previous season. Fruit harvesting, storage and colour measurement was the same as for 2005/2006.

Statistical analysis. STATISTICA data analysis software system (Version 8.0; StatSoft Inc., Tulsa, Okla.) was used to analyse the data. Classification tree anyalysis was used to classify seedlings according to leaf hue and determine accuracy with which fruit colour can be predicted. Receiver Operating Characteristic (ROC) curves were used to determine the ideal leaf hue cut-off point for culling purposes, and the accuracy with which fruit colour can be predicted. These analyses use 60% of the data to formulate a model, and then test that model on the remaining 40% of data.

Results and discussion

2005/2006. The number of seedlings, and their parents used in 2005/2006 is presented in Table 1. A classification tree analysis was used to categorise the leaves according to their hue (Table 3, Figure 1). The procedure classified the

leaves into three groups (Chi-square test $p < 0.0001$). There were 49 seedlings with a leaf hue less than 65 °, 64 with a hue between 65 ° and 104 °, and 23 with a hue greater than 104 °. Red-fruited seedlings accounted for 44 out of the 49 with a leaf hue less than 65 °. The other five seedlings had blushed fruit, which was equivalent to 10% of all red-leaved seedlings, or 19% of all blushed fruit. When this data is presented for seedling parents (Tables 4, 5, 6 and 7), we can see that 'Rosemarie' x 'Bon Rouge' and 'Flamingo' x 'Bon Rouge' are almost exclusively responsible for the red fruit, red leaf phenomenon, with about 50 to 60% of the seedlings displaying this pigmentation pattern. Banno et al. (2002) found that when Japanese pear (*Pyrus pyrifolia* Nakai) 'Osa Nijisseiki' was crossed with an F1 hybrid of *Pyrus pyrifolia* 'Oharabeni' and *Pyrus communis* 'Max Red Bartlett', 50% of the progeny inherited 'Max Red Bartlett's' red colour, and all progeny with red leaves developed red fruit, although the degree of expression did differ. Progeny with green leaves developed no red fruit (Banno et al., 2002). Interestingly, the likelihood for producing a blushed cultivar was around 20% for all these crosses, except for 'Ceres' x 'Bon Rouge', where it was 10%. For both seasons, subjective leaf colour classification is not presented, as the outcome was very similar to that of the classification tree analysis.

The ROC analysis for 2005/2006 is presented in (Table 8). ROC is commonly used for diagnoses in medical sciences. It is used when one wants to determine the probability of making a correct or incorrect diagnosis based on a symptom (Bewick et al., 2004). It is ideal for this experiment, as we are essentially trying to diagnose fruit colour according to the symptom of leaf colour. However, as it is not widely used in the field of horticultural research, an explanation of the terms is warranted. Firstly, this analysis can only distinguish between two categories. As we were looking at the potential of culling based on either red or green leaf colour, the data needed to be analysed twice: once for red against not red (i.e. bi-coloured or green) and again for green against not green (i.e. bi-coloured or red). The analysis uses 60% of the data to formulate a model, and then tests this model on the remaining 40% of the data. The ROC determines various probabilities for each data point (leaf hue in this case), and from this the cut-off point is

selected where these probabilities are optimised (Bewick et al., 2004). In this case, where we are using leaf hue to diagnose for fruit colour, the positive predictive value (PPV) is used to determine the probability of a red or green leaf giving rise to red or green fruit. Negative predictive value (NPV) determines the probability of a non-red or non-green leaf giving rise to a non-red or non-green fruit. The positive and negative likelihood ratios (LR^+ and LR^-) are the odds of favouring a positive or negative result, respectively (Bewick et al,. 2004).

In 2005/2006, the ROC determined hue cut-off points of 66 ° and 84 ° for red, non-red and green, and non-green, respectively (Table 8 and Fig. 1). It is important to remember that ROC data in Table 8 is based on only 40% of the data, whereas in Figures 1 and 2, data is for all seedlings used in the trial, so there are differences between what the ROC predicted and what happened in reality. The PPV for red, non-red at a hue below 66 ° shows that there is a 92.5% probability that seedlings below that hue have red fruit. The NPV shows that there is a 93.1% probability that a seedling with leaf hue above 66° will not have red fruit. The LR+ tells us that the odds for accurately predicting colour correctly at this hue cut-off are 7.45:1, while the odds against are 25:1 (0.04 divided by one). In Table 8 we can also see that to eliminate green leaf seedlings with a hue greater than 84 ° would be risky, as indicated by the lower PPV and NPV; and an LR^- that is higher than the LR^+.

2006/2007. The number of seedlings, and their parents used in 2006/2007 are presented in Table 2. The classification tree analysis was performed as for 2005/2006 (Table 9, Figure 2), the procedure was only able to classify the leaves into two distinguishable colour groups (Chi-square test $p < 0.0001$), as opposed to three in the previous season. There were only 10 seedlings with a leaf hue less than 78 °, and 78 with a hue greater than 78 °. The number of red leaf, red fruit seedlings was far lower than 2005/2006 because trees from different crosses were used. Also, because these trees were younger, we observed that many of them, especially those with red leaves, were not yet bearing, which skews these results somewhat. Bien Donné has a much warmer climate than Drostersnes, and this may also explain less red colour,

as anthocyanins are better synthesised under cool conditions (Steyn et al., 2004). Data according to different seedling families has only been included where there were more than 20 bearing seedlings per family (Tables 10 and 11). ‘Flamingo’ x ‘Bon Rouge’ had only 25% red leaf, red fruit seedlings compared to 57% in 2005/2006. This seems to indicate that climate and lower precocity of red progeny played a role in red colour expression. ‘Comice’ x ‘Flamingo’ may prove very useful for the breeders, as more than half of the progeny had bi-coloured fruit (Table 11).

The ROC data for 2006/2007 also shows how difficult it was to differentiate fruit colour from leaf colour for the greener leaves (Table 8, Figure 2). For green, non-green, fruit colour was not very predictable, and was not significant at the 5% level. For the red, non-red hue cut-off, the PPV was at a maximum of 100%, meaning that trees with red leaves are guaranteed to have red fruit. This causes the LR^- value to be equivalent to one divided by zero, hence the infinity value (Table 8).

Although the results from the two different seasons are not very consistent, and we had insufficient fruiting seedlings to work with, there is enough evidence to suggest that red-leafed seedlings can be removed without risking the loss of too many bi-coloured fruit. Cornelius et al. (1995) showed that red-leaved Eucalyptus seedlings had a slower growth rate than their green-leaved seedlings, while Williamson et al. (2006) found that yellow flesh colour of peaches could be selected for based on senescent leaf colour of juvenile seedlings. Both these authors suggested that undesirable seedlings could be successfully culled at any early age based on leaf colour. To try to remove green leaved seedlings would be too risky as many bi-coloured fruit came from trees with green leaves. Although both the classification tree and the ROC recommended culling seedlings with a leaf hue of below 65 and 66 ° degrees respectively, this is based on false-positive and false-negative diagnoses carrying an equal risk. In this case, the risk of losing a bi-coloured cultivar far outweighs the cost of rearing a few red-leafed seedlings that may only produce red fruit. Thus, we would recommend culling all seedlings with an immature leaf hue less than 55 °, in order to reduce the risk of losing

potential bi-coloured cultivars. The fact that all bi-coloured cultivars at Bien Donné had higher leaf hue angles than at Drostersnes, seems to suggest that warmer conditions may help to differentiate cultivars based on their ability to express anthocyanin synthesis. Also, seedling parents play an important role. Only progeny of 'Bon Rouge' seem to have red-leaved seedlings. With insufficient data from a variety of other seedling families, it is difficult to determine whether this pigmentation is more widespread. For now though, this method may only be useful when used for 'Bon Rouge' progeny.

Conclusions

Pear breeders at the ARC would be able to use immature leaf colour to cull undesirable red-fruited seedlings. However, this method needs to be refined. In order to do this, many more fruiting seedlings from more crosses are required for measurement. It would be interesting to investigate exposing seedlings to high temperatures. We suspect that only fully red seedlings would continue to show red colouration under these conditions, and more seedlings could then be removed without risking bi-coloured pears.

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Table 1. Details of pear seedling parentage and how planted at Drostersnes experimental farm, for which immature leaf colour and poststorage fruit colour were recorded in 2005/2006.

Parent name	Parent colour	Seedlings	Plant date
Comice × Rosemarie	Green × Bi-colour	26	1998
Ceres × Bon Rouge	Green × Red	20	1998
Rosemarie × Bon Rouge	Bi-colour × Red	48	1997 - 1999
Flamingo × Bon Rouge	Bi-colour × Red	42	1997 - 1998

Table 2. Details of pear seedlings planted at Bien Donné experimental farm, for which immature leaf colour and poststorage fruit colour were recorded in 2006/2007.

Parent name	Parent colour	Seedlings	Plant date
Flamingo × Bon Rouge	Bi-colour × Red	28	1999
Harrow Delight × Bon Rouge	Bi-colour × Red	13	2001
Harrow Delight × Rosemarie	Bi-colour × Bi-colour	15	1999
Comice × Bon Rouge	Green × Red	3	1999
Comice × Flamingo	Green × Red	36	1999

Table 3. Hue angle classification of immature leaves, determined by classification tree analysis ($p < 0.0001$), and compared with poststorage fruit colour classification for all pear seedlings in 2005/2006.

	Fruit Colour			
Leaf colour	Red	Bi-colour	Green	Leaf total
Hue ≤ 65	44	5	0	49
65 < Hue ≤ 104	5	19	40	64
Hue > 104	0	3	20	23
Fruit total	49	27	60	136

Table 4. Hue angle classification of immature leaves, determined by classification tree analysis, compared with poststorage fruit colour classification for ‘Comice’ × ‘Rosemarie’ in 2005/2006.

	Fruit Category			
Leaf category	Red	Bi-colour	Green	Leaf total
Hue ≤ 65 °	0	0	0	0
65 ° < Hue ≤ 104	0	4	11	15
Hue > 104	0	1	10	11
Fruit total	0	5	21	26

Table 5. Hue angle classification of immature leaves, determined by classification tree analysis, compared with poststorage fruit colour classification for ‘Ceres × Bon Rouge’ in 2005/2006.

	Fruit Category			
Leaf category	Red	Bi-colour	Green	Leaf total
Hue ≤ 65	2	2	0	4
65 < Hue ≤ 104	0	0	11	11
Hue > 104	0	0	5	5
Fruit total	2	2	16	20

Table 6. Hue angle classification of immature leaves, determined by classification tree analysis, compared with poststorage fruit colour classification for ‘Rosemarie × Bon Rouge’ in 2005/2006.

	Fruit Category			
Leaf category	Red	Bi-colour	Green	Leaf total
Hue ≤ 65	18	1	0	19
65 < Hue ≤ 104	5	7	12	24
Hue > 104	0	2	3	5
Fruit total	23	10	15	48

Table 7. Hue angle classification of immature leaves, determined by classification tree analysis, compared with poststorage fruit colour classification for ‘Flamingo × Bon Rouge’ in 2005/2006.

	Fruit Category			
Leaf category	Red	Bi-colour	Green	Leaf total
Hue ≤ 65	24	2	0	26
65 < Hue ≤ 104	0	7	7	14
Hue > 104	0	0	2	2
Fruit total	24	9	9	42

Table 8. Probabilities and likelihood ratios for false-positive and false-negative outcomes when selecting for fruit colour based on leaf hue, as determined by Receiver Operating Characteristic (ROC) analyses for all pear seedlings in 2005/2006 and 2006/2007.

Leaf hue	Selecting for	PPV[w]	NPV[x]	LR^{+} [y]	LR^{-} [z]	Pr>F
2005/2006						
< 66 °	Red fruit	0.925	0.931	7.45	0.04	<0.0001
> 84 °	Green fruit	0.816	0.886	5.66	6.10	0.0003
2006/2007						
< 90 °	Red fruit	0.958	1	3.50	∞	<0.0001
> 108 °	Green fruit	0.560	0.679	1.66	0.62	0.8542

[w] Positive predictive value

[x] Negative predictive value

[y] Positive likelihood ratio

[z] Negative likelihood ratio

Table 9. Hue angle classification of immature leaves, determined by classification tree analysis ($p < 0.0001$), compared with poststorage fruit colour classification for all pear seedlings in 2006/2007.

	Fruit Colour			
Leaf colour	Red	Bi-colour	Green	Leaf total
Hue ≤ 78	10	0	0	10
Hue > 78	2	38	38	78
Fruit total	12	38	38	88

Table 10. Hue angle classification of immature leaves, determined by classification tree analysis, compared with poststorage fruit colour classification for ‘Flamingo × Bon Rouge’ in 2006/2007.

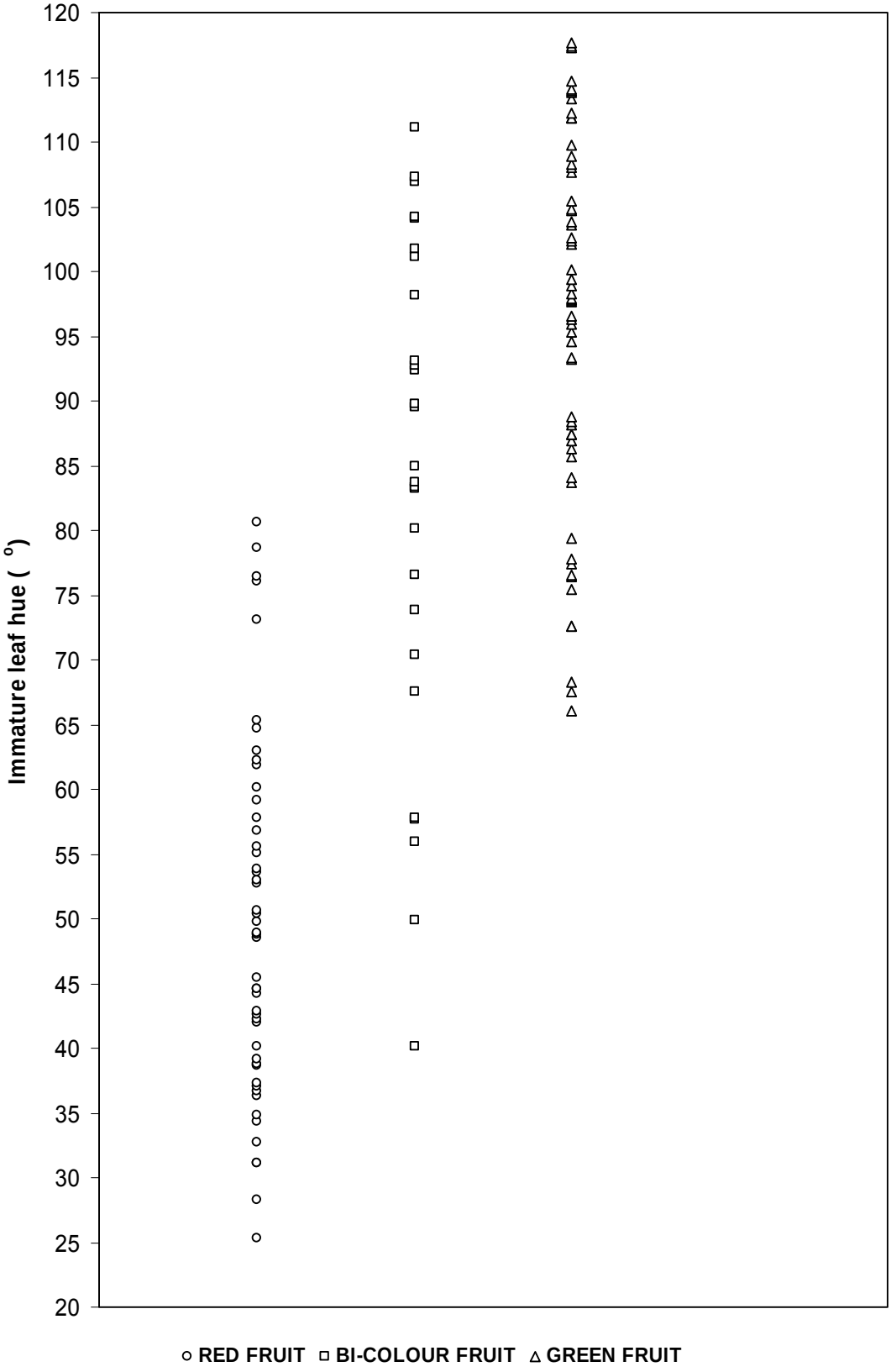
	Fruit Category			
Leaf hue	Red	Bi-colour	Green	Leaf total
Hue ≤ 74	8	0	0	8
Hue > 74	1	5	15	20
Fruit total	9	5	15	28

Table 11. Hue angle classification of immature leaves, determined by classification tree analysis, compared with poststorage fruit colour classification for ‘Comice’ × ‘Flamingo’ in 2006/2007.

	Fruit Category			
Leaf hue	Red	Bi-colour	Green	Leaf total
Hue ≤ 74	0	0	0	0
Hue > 74	0	21	15	36
Fruit total	0	21	15	36

Fig. 1. Poststorage fruit colour categories plotted against hue angle of immature leaves for all seedlings from Drostersnes in 2005/2006. The classifications determined by classification tree and Receiver Operated Characteristic (ROC) analyses are indicated .

Fig. 2. Poststorage fruit colour categories plotted against hue angle of immature leaves for all seedlings from Bien Donné in 2006/2007. The classifications calculated by Classification Tree and Receiver Operated Characteristic (ROC) analyses are indicated .


Immature leaf hue (°)
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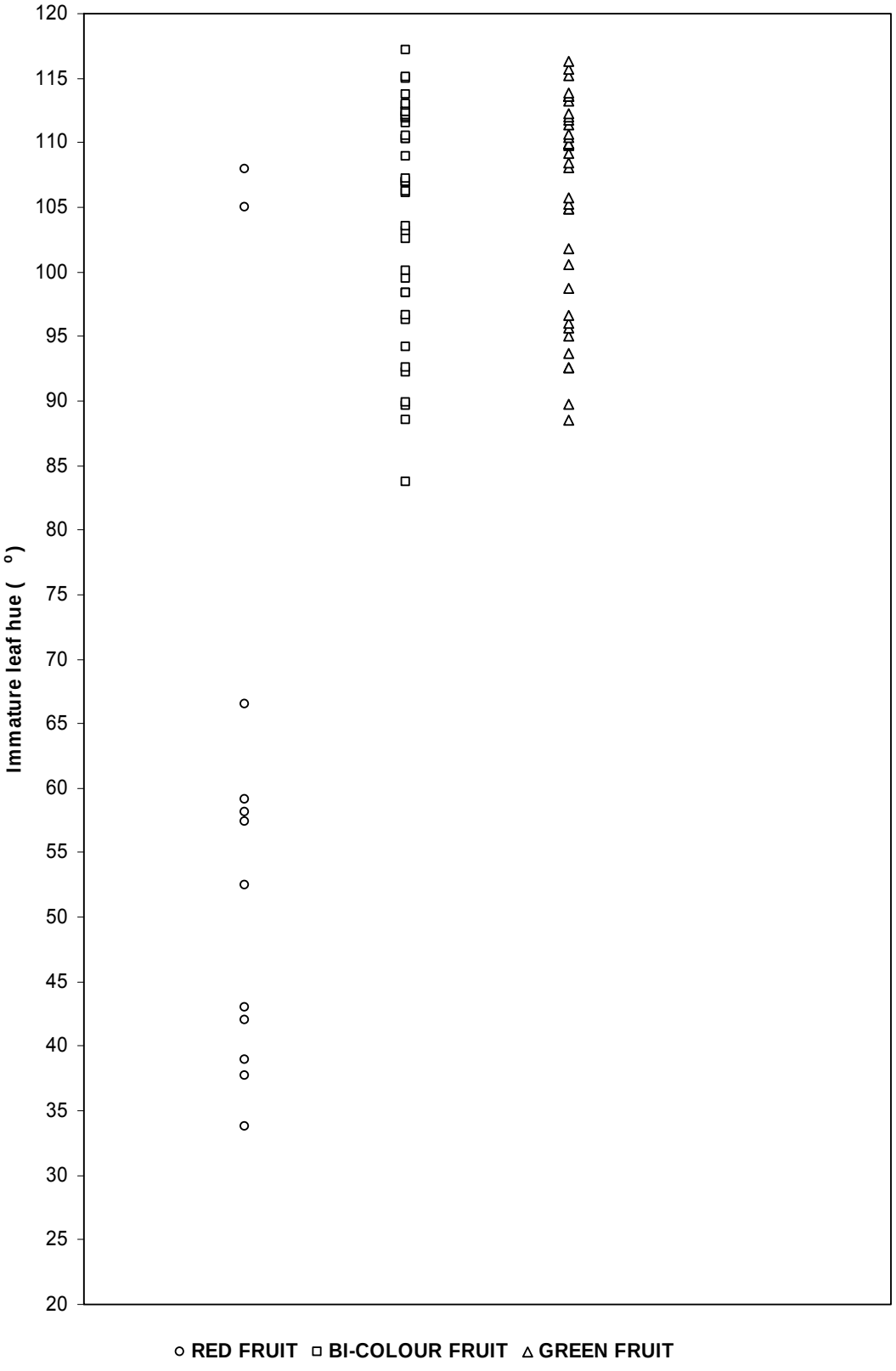
RED FRUIT
BI-COLOUR FRUIT
GREEN FRUIT

Fig 1. Paper 4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Immature leaf hue (°)
RED FRUIT
BI-COLOUR FRUIT
GREEN FRUIT

Fig 2. Paper 4

GENERAL DISCUSSION AND CONCLUSION

'Granny Smith' green colour.

It would appear that 'Granny Smith' green colour at harvest is primarily determined during the early stages of fruit development. Thus, any practices aimed at improving green colour should rather aim to maximise chlorophyll synthesis early during development, than to try to rectify poor colour just before harvest. Our nitrogen (N) applications were most likely unsuccessful due to the high N status and vigour of the trees. We would thus only suggest N fertiliser as a method of improving green colour where trees are N deficient. Likewise, although previous studies have shown that pre-harvest urea foliar sprays may cause a slight improvement in green colour, which is accentuated after storage, the improvement in colour is too small to economically justify numerous urea applications, and the problem of poor green colour at harvest is still not solved. The results from the shading of young fruit show that if canopy light penetration is insufficient early in the season, green colour will suffer. Rectifying light penetration through summer pruning will only result in more sunburnt and blushed fruit, without improving green colour. Future studies should focus on pruning post-anthesis to optimise canopy light distribution during early fruit development. Also, the role of vigour in green colour development needs to be established.

Photoprotective function of anthocyanins.

A photoprotective function of the anthocyanins responsible for red colour in pear peel appears to explain both the developmental and daily pigmentation patterns of bi-coloured pears. As shaded areas of peel bend into the light with the progress of fruit development, red colour increases with a concomitant decrease in photoinhibition. Photo-apparatus of 'Forelle' pear peel experienced the most stress under conditions of high light and low temperature, such as would be experienced with the passing of a cold front. 'Cripps' Pink' apples, with a similar early season red colouration to that of pears, showed substantial red colour development within six hours under high light and low temperature conditions following a cold front. Although the

developmental pigmentation pattern may merely coincide with fruit's increased exposure to light, and more research is required on this point, it is highly unlikely that colouration of fruit with the passing of cold fronts is not related to the photoprotective function of anthocyanins. Although we cannot propose any immediate applications of this work to improve red colour development, this additional knowledge will be valuable. The protective role may support why anthocyanins are more likely to be formed where N levels are low, and knowing why anthocyanins occur in the peel may help in explaining why certain methods to improve red colour may be ineffective.

Effect of rootstocks on red colour of 'Forelle' pears.

Choice of rootstock can affect pear colour through more than just improving light distribution within the canopy. Fruit from 'Forelle' grafted onto dwarfing quince rootstocks were far redder than those from trees on BP rootstocks. This does not appear to be as a result of more anthocyanin synthesis. Rather, it appears that lower chlorophyll and carotenoid concentrations in the peel make the anthocyanins appear brighter red in colour. We also found that fruit peel and leaves from trees on pear rootstocks had higher N levels, which may also explain why fruit from those trees had a higher peel chlorophyll concentration. Vigour appears to play a role outside of light distribution effects, in both red colour and background green colour. We would recommend that the BP rootstocks should not be used for 'Forelle' pears, because even with optimal light management, 'Forelle' trees on BP rootstocks would still have a lower innate ability to produce red fruit compared to more dwarfing rootstocks. The role of N and tree vigour corroborates our theories about 'Granny Smith' green colour.

Pear seedling leaf colour.

Pear breeders at the ARC would be able to use immature leaf colour to cull undesirable red-fruited seedlings, in order to facilitate the search for new bi-coloured cultivars. However, this strong relationship between red colour of immature leaves and red fruit for pear seedlings seems to be restricted to progeny where 'Bon Rouge' is a parent. Substantially more fruiting seedlings from various families are required for measurement in order to see if this

relationship might also occur elsewhere. Growing region appears to affect this relationship, and it would be interesting to investigate exposing seedlings to high temperatures. We suspect that only fully red seedlings would continue to show red colouration under these conditions, and more seedlings could then be removed without risking bi-coloured pears. From our observations, it would not be possible to remove seedlings with very green immature leaves, as there is a good possibility that such a tree may still produced bi-coloured fruit.

Conclusion.

The research reported here has increased our understanding with regards to various facets of apple and pear colour development. Our finding that ‘Granny Smith’ green colour is determined early during fruit growth throws new light on the subject. Previous studies on this subject have only measured colour changes closer to harvest. We believe there is great potential for manipulation of green colour early in the season, particularly with the use of pruning, nitrogen and plant growth regulators. With regards to red colour development of bi-coloured pear cultivars, we appear to be running out of options to improve the colour of our current cultivars. Although the photoprotective function of anthocyanins broadens our understanding of red colour development, and will influence future research on the topic, we stil require a solution to the problem. Even if growers make sure to grow a problematic cultivar like ‘Rosemarie’ using the best practices, e.g. rootstock selection, the industry as a whole will still struggle with red colour, particularly with the advent of global warming. We believe the answer to this problem lies in the breeding of cultivars with superior red colour retention. Our work on trying to streamline the breeding process using immature leaf colour will hopefully aid in this quest. We certainly do not have the final answer on the best way to pre-select seedlings for potential bi-coloured fruit, and far more research is still required, but we have taken the all-important first step.