INFLUENCE OF PARTIAL ROOTZONE DRYING ON ASPECTS OF GRAPE AND WINE QUALITY

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SUB CRUCE LUMEN

A thesis submitted to The University of Adelaide in fulfilment of the requirement for the degree Doctor of Philosophy

April 2004

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ABSTRACT

The effect of partial rootzone drying (PRD) on the composition of grape and wine secondary metabolites was investigated. At harvest, total anthocyanin and phenolic concentration in fruit of *Vitis vinifera* L. cvs Shiraz and Cabernet Sauvignon was either unaltered or increased in response to PRD over two seasons. Where an increase in anthocyanin concentration was found with PRD, this was found to be independent of berry size. Rather, increases in anthocyanin concentration were most likely due to an increase in anthocyanin production in the berry skins. In Cabernet Sauvignon wine made from one vintage, total colour was enhanced by 10% in response to the PRD treatment, although in that season total anthocyanin concentration was unaltered in the fruit or wine.

This colour enhancement was due to an increase in coloured pigments in copigmented or polymeric form, that is in association with other anthocyanins or phenolic compounds. This is a significant finding, as it shows that the colour of wine is not dependent upon anthocyanin concentration alone, but rather on reactions taking place during winemaking and wine-ageing. The colour enhancement was found to be maintained after a year's ageing in the bottle. A key question which arises from this work is which component(s) of total phenolics in PRD-treated wines is responsible for this effect. Preliminary investigation of seed proanthocyanidins and flavan-3-ols showed that their content per berry was unaltered by the PRD treatment. However, where berry weight was decreased due to PRD, the concentration of these seed tannins was increased on a per gram basis. This could potentially lead to an increased contribution of seed tannins to total wine phenolics in PRD wines. The effect of this is two-fold. Firstly, seed tannins are known to be weak copigments and could in part account for the increased copigmentation and polymerisation of anthocyanins observed with PRD. Secondly, an increased concentration of seed tannins in PRD wines could alter wine astringency and mouthfeel properties.

In both fruit and wine samples, PRD was found to cause a decrease in the contribution malvidin-glucosides to total anthocyanins. Thus, levels of non-malvidin glucosides, namely delphinidin and cyanidin, were increased by PRD. This effect was investigated as fruit matured post-veraison, and was evident from early in berry development. Preliminary results indicate that this response requires the presence of high incident light levels to the fruit during development, but is not mediated by increased bunch exposure alone. Shading of fruit led to a significant decrease in all anthocyanin types, and caused a shift in the ratio of acetyl- and 3p-coumaryl-glucosides to mono-glucosides. The PRD treatment, however, did not cause changes in the proportions of acetyl-, 3p-coumaryl- and mono-glucoside anthocyanins. These results show that the response of the anthocyanin pathway to the PRD treatment is most likely mediated by physiological signals within the fruit and vine, rather than due to a change in bunch zone microclimate.
An important class of potential flavour and aroma compounds in grapes are the C_{13}-norisoprenoids. As the precursors to these compounds are fruit-derived, rather than by-products of fermentation, the potential exists for their manipulation using viticultural practices. Previously, bunch exposure has been thought to be the primary factor mediating the final levels of C_{13}-norisoprenoids in fruit. The current study has shown that total shading of developing bunches using artificial means led to a significant decrease in the levels of these compounds in both fruit and wine samples of Shiraz. However, when fruit of this variety was subjected to natural changes in bunch exposure, resulting from alterations in canopy structure, the response of the C_{13}-norisoprenoids to these conditions was either small or insignificant. Of the three C_{13}-norisoprenoids studied, namely ß-damascenone, ß-ionone and TDN, only TDN showed a significant response to bunch exposure under these conditions.

Preliminary results of this study on Shiraz indicated that the concentration of the C_{13}-norisoprenoids ß-damascenone and TDN can potentially be influenced by changes in vine shoot vigour and canopy architecture, induced by a water-deficit. This finding was validated by a study on Cabernet Sauvignon, where the effect of PRD on fruit C_{13}-norisoprenoids was investigated. The concentrations of the C_{13}-norisoprenoids ß-damascenone, ß-ionone and TDN were shown to be increased by up to 30% in response

to PRD in Cabernet Sauvignon fruit over two vintages. The changes in these compounds in the fruit were associated with increased levels of the carotenoids lutein and ß-carotene, which are potential precursors to the C_{13}-norisoprenoids. The results of the current study have shown that the regulation of C_{13}-norisoprenoid concentration in grapes and wine may not be primarily due to the level of incident light on developing bunches, although the presence of light is necessary for their production. Rather, the effect of deficit irrigation practices on whole-vine physiology and vine shoot vigour holds stronger potential as a tool for the viticultural manipulation of these compounds in fruit and wine.

DECLARATION

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief contains no material previously published or written by another person, except where due reference has been made

I give consent to this copy of my thesis being available for loan and photocopying, once deposited in the University of Adelaide Library.

Signed:..... Date: 14/07/2004

Keren A. Bindon

ACKNOWLEDGEMENTS

- My supervisors Assoc. Prof. Peter Dry and Affil. Prof. Brian Loveys
- The Cooperative Research Centre for Viticulture (CRCV) and Winetech (South Africa) for their respective financial contributions to my PhD project
- Adelaide University's School of Agriculture and Wine and CSIRO Plant Industry (Horticulture Unit, Adelaide) for their wonderful staff and the use of their laboratory facilities
- The Winetac (CRCV) PhD student professional development programme, with special thanks to Peter Mansfield and Libby Boschen
- Fiona Brooks and Maria Luisa de la Hera Orts for field and laboratory assistance
- Gerhard du Toit for access to his Cabernet Sauvignon pot experiments
- Sue Maffei and Mark Downey for assistance with the HPLC
- Mike McCarthy and Shannon Pudney for management of the Nuriootpa field trial
- John Kennedy and Matt Partridge for management of the Orlando, Langhorne Creek field experiment
- Staff of the Australian Wine Research Institute for supply of deuterated standards of C_{13}- norisoprenoids, with particular thanks to Alan Pollnitz for advice and proof-reading of some of my work.
- Stephen Clarke and Chris Day for assistance in winemaking at the University of Adelaide winery
- Renata Ristic for supply of experimental wines and grape samples from the canopy-management trial and assistance with the analysis of seed tannins
- Biometrics SA for assistance with experimental design and statistical analysis
- Patrick Iland and Erika Winter for contributing their thoughts to my project
- To the other CRCV PhD students for their friendship, with particular acknowledgement of Eve Cottral who is no longer with us, and will always be remembered
- Thanks to my parents who have encouraged me from day one
- Above all, to my Lord and Saviour Jesus Christ

LIST OF ABBREVIATIONS

ABA	abscisic acid
°C	degrees Celsius
GCMS	gas-chromatography mass-spectrometry
HPLC	high-performance liquid chromatography
n	number of samples
ns	not significant
nd	not determined
P	probability for data
PAR	photosynthetically active radiation ($\mu mol.m^{-2}.s^{-1}$)
PDMS	polydimethylsiloxane
PRD	partial root-zone drying
PW	pruning weight (kg/vine)
RDI	regulated deficit irrigation
RH	relative humidity
SE	standard error of the mean
SPME	solid-phase micro-extraction
TA	titratable acidity (g/L as tartaric acid)
TDN	1,1,6-trimethyl-1,2-dihydronaphthalene
TSS	total soluble solids (°Brix)
VPD	vapour pressure deficit (kPa)
WUE	water use efficiency (tonnes/ML)
Ψ_L	leaf water potential (MPa)
Ψ_S	stem water potential (MPa)

CHAPTER 1: GENERAL INTRODUCTION

1.1 The definition of grape and wine quality

Internationally, within the wine industry no clear definition exists for optimum 'quality' of grapes or wine, and the classification of quality in both remains largely subjective. However, the scientific quest for descriptive, measurable parameters to describe 'quality' is ongoing. Some simple measures of grape juice characteristics have been used for many years to predict wine quality, namely juice content of soluble solids (°Baumé or °Brix), pH and titratable acidity. The measurement of grape soluble solids provides an assessment of grape ripeness in terms of sugar content. General observations have been that higher levels of total soluble solids (TSS) in grape juice have been associated with increases in wine quality (Carroll *et al.* 1978). However, these improvements in wine quality are most likely to be indirectly due to other changes in fruit composition associated with advanced ripeness, rather than due to increases in sugar content alone. The pH of grape juice is also an important criterion for juice and wine quality and largely determines the pH of wine after fermentation (Boulton, 1980). This is of particular importance in winegrowing regions where the pre-fermentation addition of tartaric acid to wine musts is prohibited. High acid levels not only affect the palatability of table grapes, but are also associated with low sugar concentrations, resulting in poor wine quality (Ruffner, 1982). On the other hand, low acid levels in harvested grapes can accompany either high or low sugar levels but produce unbalanced and flat wines (Ruffner, 1982).

It is now generally recognised that these measures of grape composition alone do not give an accurate assessment of potential wine quality. This is because there are a variety of environmental and viticultural factors which affect grape berry ripening and final sugar levels at harvest. In winegrowing regions where the season is short, over-ripeness seldom occurs, and TSS is an accurate indicator of other quality components associated with quality e.g. optimum levels of colour (red varieties) and flavour. However in warmer, low rainfall regions, TSS may not reflect potential wine quality as a rapid ripening rate may lead to very high °Baumé levels, producing high alcohol wines in which other sensory

properties are masked (Winkler *et al.* 1974). In these regions, the maturity at which fruit is harvested must be assessed together with other parameters which define 'quality'. Furthermore, viticultural practices such as irrigation, pruning, canopy management, genotype (grapevine variety or clone), use of fertilisers and pesticides; and environmental factors such as temperature, rainfall, humidity and sun-exposure can further impact the composition of fruit at harvest (Jackson and Lombard, 1993). In recent years, there has been a move within the wine industry toward a more objective definition of 'grape quality' in terms of composition parameters other than sugar and acidity which affect wine properties. Essentially, this has centered around the study of secondary metabolic pathways in grape berries, which are responsible for the biosynthesis of many of the compounds responsible for the sensory properties of wine.

1.2 Secondary metabolites as an indicator of grape and wine quality

The term 'secondary metabolism' refers collectively to biochemical pathways which derive from primary carbon metabolism in plants, but which have no direct function in plant growth and development (Taiz and Zeiger, 1998; Edwards and Gatehouse, 1999). Secondary metabolism differs from primary metabolism which governs the assimilation of carbon, nitrogen and sulphur in the flux through the respective pathways. Essentially, secondary metabolism only accounts for trace quantities of metabolites within the plant (Edwards and Gatehouse, 1999). Furthermore, whereas primary metabolic pathway are governed through the complex regulation of many allosteric enzymes, secondary metabolic pathways appear to be under complex regulation only at the branch point between primary and secondary metabolism (Edwards and Gatehouse, 1999). The secondary metabolites which are of importance to the viticulturist and winemaker can be broadly classed into two groups: the phenols and the terpenes (Figure 1.1). The phenols contribute to wine colour, bitterness and astringency in the case of red grape varieties, and the terpenes to flavour and aroma in both white and red grape varieties (Singleton, 1988).

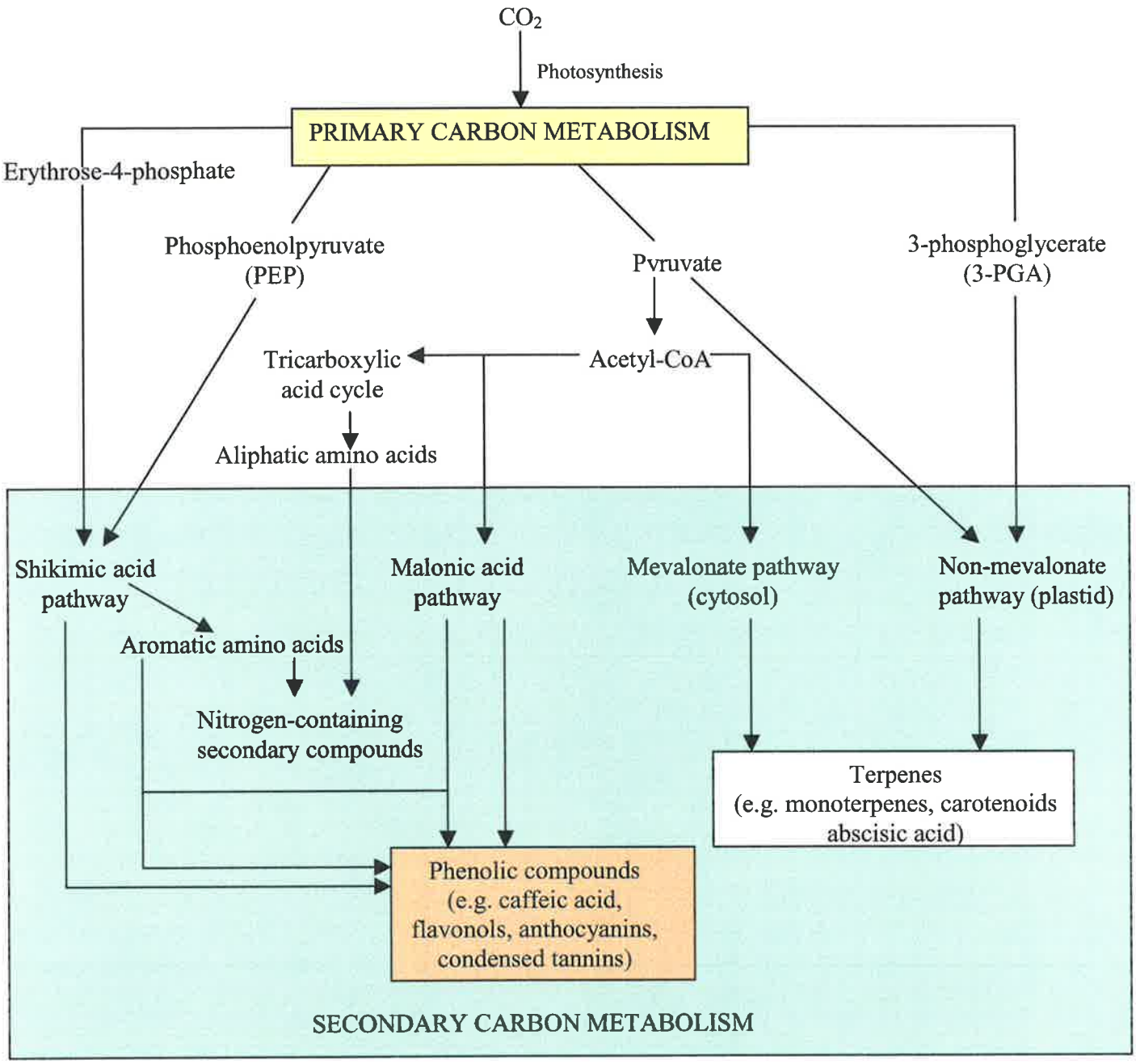


Figure 1.1: A simplified scheme of the major pathways of secondary carbon metabolism in higher plants (adapted from Taiz and Zeiger, 1998)

1.2.1 The phenols

Phenolic compounds are distinct from other metabolites by the presence of a phenol group, which is an hydroxylated aromatic ring (Figure 1.2)

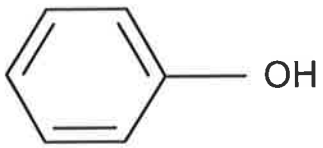


Figure 1.2: Phenol group containing an hydroxyl functional group on an aromatic ring

In grapes and wines, the primary phenolic compounds are simple phenolics e.g. caffeic and caftaric acid, and flavonoids e.g. anthocyanins, quercetin, condensed tannins (proanthocyanidins) and flavan-3-ols (Singleton, 1988). These are originally derived from a single source in the shikimic acid pathway. This pathway initially converts the precursors erythrose-4-phosphate and phosphoenolpyruvic acid from the pentose-phosphate pathway and glycolysis respectively into shikimic acid, and from thence to phenylalanine through a series of enzymatic steps. It occurs in the plastids of higher plants (Seigler, 1998). Most phenolics have phenylalanine as a precursor, which is converted to cinnamic acid via the enzyme phenylalanine ammonia lyase through removal of an ammonia molecule (Figure 1.3). Cinnamic acid undergoes further conversions to form the flavonoids (Figure 1.3). In plant tissues, most flavonoids are present in the form of glycosides, that is, bonded to a glucose molecule (Seigler, 1998). Flavonoids are sub-divided into fourteen classes on the basis of the oxidation level of the central pyran ring denoted C (Figure 1.3). In the case of anthocyanin and proanthocyanidin formation, dihydroflavanol precursors undergo a series of enzyme-mediated reduction steps. The proanthocyanidins are formed through polymerisation of oligomeric flavonoid units, linked between flavonoid units with the addition of other phenolics e.g. gallic acid (Seigler, 1998). These condensed tannins yield anthocyanidins when heated in the presence of acid, hence the term 'proanthocyanidin.' They are found in both the skins and seeds of grape berries (Downey *et al.* 2003), and contribute to the perception of astringency in wines, and are potential copigments enhancing wine colour (Boulton, 2001).

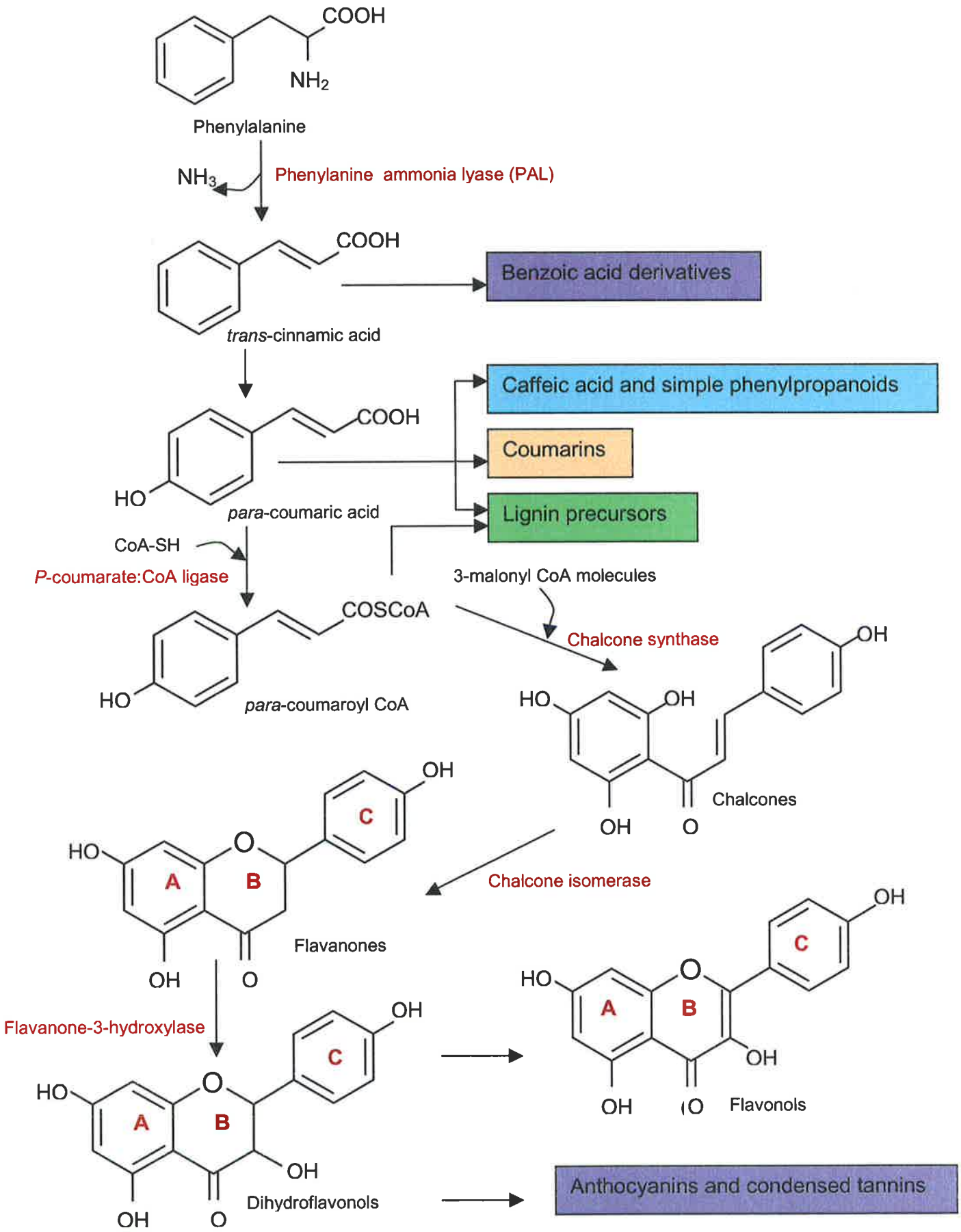


Figure 1.3: Phenolic biosynthesis from phenylalanine showing the branch points for the synthesis of the major groups of phenolic compounds (adapted from Taiz and Zeiger (1998) and Seigler (1998))

Anthocyanins are glycosides of anthocyanidins, and are the main pigments responsible for the red, blue and violet colours of flowers and fruits (Seigler, 1998). In the grape berry, they are located in the firmer tissues of the skin, and skin maceration is therefore necessary to ensure optimal extraction of coloured pigments into wine (Somers and Vérete, 1988; Singleton, 1988). The colour of these flavonoids is determined by a number of factors including: pH, hydroxylation of the aglycone, glycosylation pattern, complexing with metals and or other compounds e.g. tannins and phenols (Seigler, 1998).

In the determination of red wine colour, the stability of anthocyanins is as important as the amount of anthocyanin present. This is largely dependent upon the composition and diversity of phenolic compounds present in the wine (Somers and Vérete, 1988). During winemaking, an early decline in monomeric anthocyanins has been noted, which is due to their progressive transition from the monomeric form, through oligomers to polymeric pigments (Somers and Vérete, 1988). This increasing complexation of wine anthocyanins enables stabilisation of wine colour and confers resistance to oxidative degradation. The presence of phenolic compounds other than anthocyanins is necessary to facilitate this process (Boulton, 2001) and thus a higher proportion of total phenolics to total anthocyanin is important in determining wine quality. A measure of anthocyanin content of grapes or wines is therefore not an accurate measure of final wine colour and stability during ageing. Thus, according to Somers and Vérete (1988) wine can be classed into two categories based on an index (absorbance units (a.u.) at 280 nm) of total phenolics. Firstly, light red wines have a low phenolic index (20-30 a.u.) and although they may display good colour, are usually susceptible to oxidative damage within the first few months after bottling. Secondly, robust red wines have the potential to be classed as 'premium' and thus higher quality have high phenolic indices (>40 a.u.). These wines may initially be quite astringent due to a high phenolic index, but this is lost during ageing with a concomitant increase in wine flavour. It has been suggested that wines from the first category are the product of intensive irrigation where yield per hectare is high, whereas the second category is associated with low yields, but may also be dependent upon cultivar (Somers and Vérete, 1988).

1.2.2 The terpenes

Of the different classes of secondary metabolites, the largest group is the terpenes. All the terpenes are formed from linked 5-carbon units, termed isoprene units (Figure 1.4):

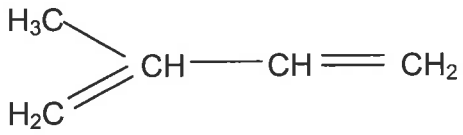


Figure 1.4: Basic structural unit of the terpenes: the 5-carbon molecule isoprene

Due to their common structural unit, the terpenes are commonly called isoprenoids. This group of compounds are classified on the basis of the number of isoprene units they contain, and include the 10-carbon monoterpenes, the 40-carbon tetraterpenes or carotenoids, and polyterpenoids which contain greater than ten 5-carbon units (Taiz and Zeiger, 1998). This group also contains the important plant hormone, abscisic acid (ABA). The isoprenoids are derived from primary metabolism via two possible pathways: the cytosolic mevalonate pathway from acetyl CoA or a non-mevalonate pathway via glycolytic intermediates (Figure 1.1). The latter non-mevalonate pathway was only recently discovered as operating within chloroplasts or plastids by Lichtenthaler *et al.* (1997a,b), and still remains to be completely elucidated. Later work indicated that the non-mevalonate pathway was the pathway primarily responsible for the bulk of isoprenoid synthesis, namely the monoterpenes, diterpenes and tetraterpenes, whereas the classical mevalonate pathway gives rise to phytosterols and some sesiquiterpenes (Figure 1.5) (Lichtenthaler, 1999). However, as both pathways have isopentenyl pyrophosphate (IPP) as an intermediate, the potential exists that some interchange between pathways may occur (Figure 1.5). A recent review of ABA biosynthesis has indicated that it is likely to be formed through either the mevalonate or non-mevalonate pathway, but that alterations in plant stress may activate one or the other of the pathways (Cowan, 2001). It appears that under stress conditions, that the non-mevalonate pathway is activated, and ABA is formed from carotenoids via this route (Cowan, 2001). The potential therefore exists that other intermediates of the non-mevalonate may also be increased under stress conditions, due to increased flux within the pathway toward ABA.

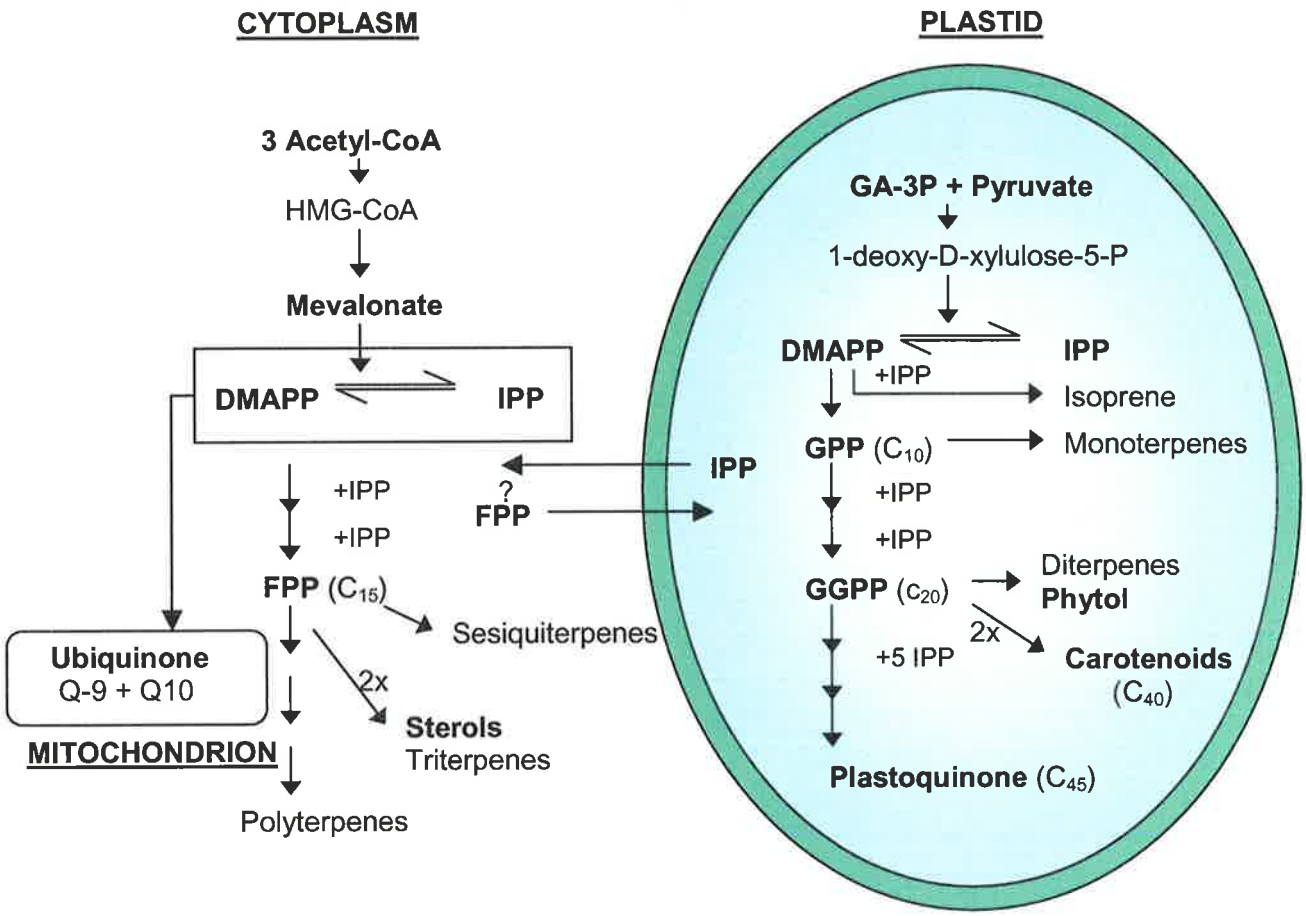


Figure 1.5: Compartmentation of the mevalonate (cytoplasmic) and non-mevalonate (plastidic) pathways of isoprenoid biosynthesis. Abbreviations used: DMAPP, dimethylallyl diphosphate; GPP, geranyl diphosphate; FPP, farnesyl disphosphate; GGPP, geranylgeranyl diphosphate; IPP, isopentenyl pyrophosphate. Diagram from Lichtenthaler (1999).

In many plant species, the monoterpenes are the major odiferous compounds of flowers and fruit (Seigler, 1998). Many studies have indicated that monoterpenes derived from grapes form a strong basis for the aroma of wines, and can be used to classify wines by variety (Rapp, 1988). Examples of monoterpene isoprenoids in grapes and wine are linalool, geraniol, nerol and citronellol which are present as alcohols (Rapp, 1988). However, due to strong varietal differences, monoterpenes may not always be present in appreciable quantities in grapes and wines of certain winegrape varieties. In red varieties such as Cabernet Sauvignon and Shiraz, the contribution of the monoterpene isoprenoids to wine aroma is small relative to other volatile compounds (Abbott *et al.* 1989). In these varieties, it appears that another class of isoprenoid compounds contribute more strongly toward grape aroma: the C_{13}-norisoprenoids (Abbott *et al.* 1989; Sefton, 1998; Bureau *et al.* 2000). The C_{13}-norisoprenoids are derived from the carotenoids in grape tissues, and are usually present as glycosides (Baumes *et al.* 2002). Three well-known examples of grape and wine C_{13}-norisoprenoids are β-damascenone, β-ionone and TDN, which form the focus of the current study.

1.3 The environmental regulation of grape secondary metabolites

1.3.1 A definition of water deficit

Traditionally, grapevines have been grown in 'Mediterranean' climates, which have hot, dry summers and a distinct period of winter rainfall (Winkler *et al.* 1974). In many countries where grapes are currently grown, irrigation is minimal or completely avoided, and to a large extent the grapevine relies on soil water retained from the winter rainfall period (Van Zyl and Weber, 1981). However, in countries like Australia, the USA and other 'new world' countries, irrigation of grapevines during the period of shoot and fruit growth is commonly practiced (Smart and Coombe, 1983). When irrigation is practiced, the amount of water required by grapevines under the climatic conditions and soil type of the region becomes extremely important, in terms of water conservation but more importantly to avoid a possible loss of quality in fruit by excess water application (Smart and Coombe, 1983).

For many irrigated crops, including vineyard systems, there has been an attempt to predict water requirements according to the prevailing environmental conditions of a particular site. For example, Doorenbos and Pruitt (1977) proposed a method for the prediction of crop water use under various climatic and agronomic conditions, using the Penman equation to estimate crop evapotranspiration (ET_0) from climatic data. This method also incorporated factors such as the stage of crop growth, growing season, prevailing weather, local soil water availability, salinity and irrigation method. That study provided a prediction for the potential water requirements of a variety of crops, including grapes. In the light of studies like that of Doorenbos and Pruitt (1977), the question arises as to whether the potential evapotranspiration of a crop can be predicted using climatic data, or whether plant systems like grapevines have the potential to tightly regulate their water use under conditions of high evaporative demand, thus reducing their water requirement. For example, a recent study of split-root pear trees showed a correlation between atmospheric vapour pressure deficit (VPD) and sap flow when the whole root system was watered (Davies *et al.* 2002). However, when water was withheld from one half of the root system, an uncoupling of VPD and sap flow occurred (Davies *et al.*

2002). This demonstrated that where water supply to the root system is plentiful, plant water use is determined by the prevailing environmental conditions. However, when water availability is reduced, the plant has an inbuilt mechanism which reduces water use, irrespective of the atmospheric demand for water. This finding brings into question the definition of water deficit. Given the ability of plants to control their water use irrespective of atmospheric conditions, under what conditions does a plant actually enter 'water stress' *per se*? This definition is further complicated by genotypic differences in the way in which plants respond to drought. Plants can show either strong or little regulation of stomatal aperture in response to water deficit, termed isohydric or anisohydric responses respectively (Schultz, 2003). For grapevines, it has been shown for the variety Grenache, that the former showed the ability to maintain leaf water potential even when are subjected to drought, an isohydric response (Schultz, 2003). On the other hand, leaf water potential of Shiraz grapevines was significantly decreased in water-stressed plants relative to watered plants, an anisohydric response (Schultz, 2003). Thus, under similar environmental and atmospheric conditions, different grapevine genotypes may show variable physiological responses.

For the purpose of the current study, 'water deficit' or 'water stress' is defined according to physiological responses within the grapevine, rather than the amount of water applied for the prevailing climatic and agronomic conditions. Thus, a grapevine is defined as entering 'water deficit' or being 'water stressed' when there is evidence of an hydraulic response to a reduction in irrigation water supplied, that is, a reduction in leaf water potential relative to a well-watered control. This definition is further complicated by the discovery of so-called 'non-hydraulic' responses by some plants, including grapevines, to a reduction in water supply. It has been shown that signals produced in drying roots can bring about a decrease in stomatal conductance, independently of leaf water status (Davies *et al.* 1986; Schulze, 1986; Dry and Loveys, 1999). This 'non-hydraulic' response has formed the basis for experimentation with partial rootzone drying (PRD) on grapevines, which is a central theme in the current study and will be described in detail in the chapters which follow (Dry, 1997; Stoll, 2000; Stoll *et al.* 2000). To facilitate discussion of this topic, an attempt has been made to distinguish between the

physiological effects of a 'non-hydraulic' response on grapevines (PRD), and the effects of water stress, which has been assumed to include both 'non-hydraulic' and 'hydraulic' responses to a reduction in soil water availability.

1.3.2 The concept of oxidative stress

During the essential plant processes of photosynthesis and respiration, the potential exists that conditions of excess energy in the form of excited electrons may arise. Such conditions might give rise to reactive oxygen species, (ROS) which form through electron transfer to molecular, or other forms of oxygen. As a result of their biochemical properties, these ROS will have detrimental consequences on plant metabolism (Kiraly *et al.* 1993). For example, the transfer of one electron to molecular oxygen will yield the superoxide radical (O_2^-). This radical can then behave as a weak base, forming hydrogen peroxide (H_2O_2). Furthermore, the decomposition of H_2O_2 in the presence of metal ions yield the hydroxyl radical (OH^-) (Cadenas, 1995). These radicals can form in all aerobically respiring cells. A further route for the formation of radicals in aerobic biological systems is that of lipid peroxidation, which leads to the formation of peroxyl radicals, containing a carbon functional group. ROS have the potential to attack and cause oxidation of all cell components e.g. lipids, nucleic acids, proteins and carbohydrates (Cadenas, 1995).

In plant systems, the process of photosynthesis operates in a high-energy environment, where light is absorbed in the presence of a relatively high concentration of oxygen. In plants experiencing water stress, the use of this light energy in the processes of photosynthesis and photorespiration may not be sufficient to cope with this excess energy (Chaves *et al.* 2003). This can result in the transfer of electrons to molecular oxygen, with resultant formation of ROS which can damage the photosynthetic apparatus and possibly other metabolic systems (Smirnoff, 1998; Niyogi, 1999). Inherent within plant systems is the ability to scavenge these ROS. Well-studied examples of such systems are the superoxide dismutases (SOD), catalases (CAT) and the ascorbate-glutathione cycle (Smirnoff, 1998; Chaves *et al.* 2003). Generally, it has been observed that under water stress, these antioxidant systems show enhanced enzyme activity (Smirnoff, 1998).

1.3.3 Secondary metabolites as protectants against oxidative stress

Apart from enzyme-mediated removal of ROS, plants may prevent the formation of ROS through other mechanisms. An important mechanism for the dissipation of light energy is through the xanthophyll cycle, which forms part of the terpenoid class secondary metabolites. This occurs in photosynthetic tissues under environmental stresses such as high light intensity, heat or water deficit and has been shown to dissipate up to 75% of the photons absorbed by leaves (Niyogi, 1999). This cycle involves the conversion of the carotenoid violaxanthin (**V**) to the de-epoxidised carotenoids antheraxanthin (**A**) and then to zeaxanthin (**Z**), at the expense of **V**. This allows for a substantial amount of the excitation energy derived from light to be converted to heat energy, and thus dissipated. When the stress conditions which initiated the cycle return to normal, the **A** and **Z** are converted back to **V** (Taiz and Zeiger, 1998).

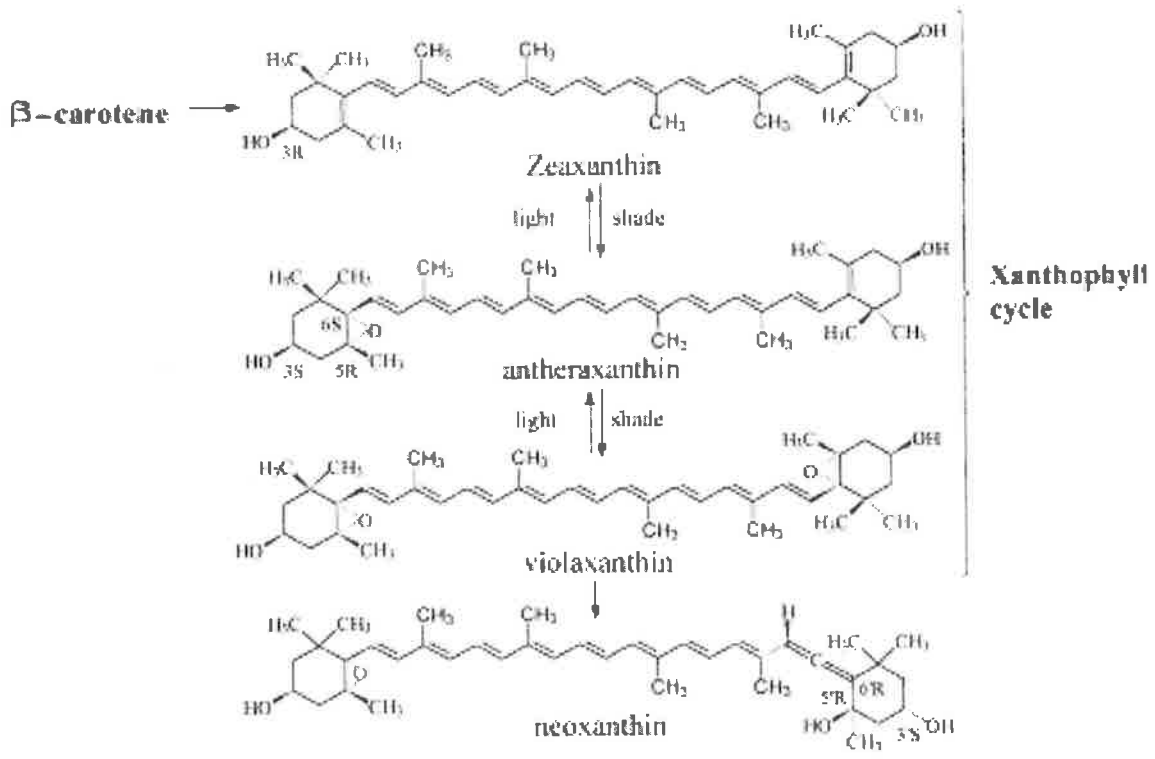


Figure 1.6: The operation of the xanthophyll cycle under conditions of light and shade, showing the conversion of carotenoid intermediates (from Baumes *et al.* 2002).

It has been found that leaves grown under high light contain higher levels of xanthophyll cycle intermediates than shade-grown leaves (Demmig-Adams *et al.* 1996). The xanthophyll cycle has been shown to occur in grape berries, and operates at higher capacity in sun-exposed fruit than in shaded fruit (Düring and Davytan, 2002). Furthermore, increased levels of total carotenoids, including xanthophyll intermediates have been found in grape berries under water deficit (Oliveira *et al.* 2003). Presumably, this response indicates an increase in the capacity of the fruit to dissipate excess light energy under stress conditions. In the case of products of the phenylpropanoid pathway of secondary metabolism, the anthocyanins and other flavonoids have been shown to have a photoprotectant function. Some plants may produce anthocyanins in their leaves under conditions of stress e.g. high light, low temperature or drought, and it has been proposed that anthocyanins serve as photoprotectants, due to their ability to strongly absorb light and as to act as antioxidants (Rice-Evans *et al.* 1997; Wang *et al.* 1997; Steyn *et al.* 2002). A study on photosynthesising leaves of *Rosa* sp. and *Riccinus communis* L. showed that the development of anthocyanins was correlated to lowered carotenoid concentration, and thus a low photodissipative potential via the xanthophyll cycle (Manetas *et al.* 2002). The presence of anthocyanins were proposed to function to increase photoprotection, allowing for the dissipation of excess light energy.

In grape berries, the fruit is green and photosynthetic pre-veraison, but at veraison an increase in anthocyanin in berry skins occurs, along with a decline in chlorophyll and carotenoid concentration (Razungles *et al.* 1988; Razungles *et al.* 1996; Düring and Davtyan, 2002). As in leaves of some plant species, it is possible that anthocyanins develop at this stage to prevent oxidative damage by light, to compensate for degradation of the photosynthetic apparatus at this stage. In grape berries, increases in light have been associated with higher concentration of anthocyanins in the skin of the fruit, although extremes in light intensity can result in the degradation of anthocyanins (Kliewer, 1970, 1972; Smart *et al.* 1985; Bergqvist *et al.* 2001). Furthermore, conditions of water stress have been shown to increase the anthocyanin content of berry skins (Matthews and Anderson, 1988; Esteban *et al.* 2001).

It is conceivable that this occurs as a response to increased potential for oxidative damage where water is limiting, which is exacerbated by high light intensities. However, other biochemical changes in fruit may also account for this response. In grape berries placed under water deficit, increases in the content of the plant signal ABA have been found in berry juice (Coombe and Monk, 1979), whole berries (Antolin *et al.* 2003) and extracted into resultant wines (Coombe and Forrest, 1979). The application of ABA to grape berries is known to increase anthocyanin production, by upregulating the expression of the enzyme chalcone-flavone isomerase (Hiratsuka *et al.* 2001), and by promoting the formation of anthocyanoplasts (anthocyanin-forming cells) (Kim *et al.* 1997). It is therefore possible that increases in fruit ABA under water deficit will bring about an increase in the production of anthocyanin in grape berry skins, and may not require the presence of high light intensities in order to elicit the response.

1.3.4 The potential to manipulate secondary metabolism with deficit irrigation

The by-products of the xanthophyll cycle intermediates, the C_{13}-norisoprenoid volatiles together with the products of the phenylpropanoid pathway, the flavonoids e.g. anthocyanins, are important determinants of grape and wine quality. Higher levels of both aroma compounds and phenolic compounds have the potential to increase wine quality. As has already been discussed, exposure of grapevines to water stress has been shown to alter these pathways of secondary metabolism in grape berries. Through the application of threshold levels of water stress, without causing damage to plant function, it may be possible to increase flux though these pathways, altering fruit composition, and potentially improving wine quality. The aim of the current study is to use the irrigation technique partial rootzone drying (PRD) as a tool to manipulate these pathways. Grapevines have been found to respond to partial drying of the root system through increased production of the plant signal ABA, while part of the root system receives adequate water (Stoll *et al.* 2000). When half of the root system is subjected to drying, it has been shown that the production of ABA in these roots increases initially in response to the drought, but it is only a transient response (Loveys *et al.* 2000; Stoll *et al.* 2000). Thus, continuous alternation of the 'wet' and 'dry' sides of the plant allows for the sustained production of these chemical signals. The movement of water from 'wet' to

'dry' roots and its subsequent transpiration also facilitates the movement of these signals from roots to shoots (Stoll *et al.* 2000). Theoretically, the developing fruit should respond to stress signals from the roots such as ABA, which may bring about a response in secondary metabolism. However, as little is yet known about the regulation of many secondary pathways (Edwards and Gatehouse, 1999) the response of either the phenylpropanoid or isoprenoid pathways of metabolism to this treatment is not guaranteed, although the PRD technique has been shown to bring about improvements in both wine colour and aroma (Stoll, 2000).

1.4 Current research focus within the Australian wine industry

The boom of the Australian wine industry in the early 1990s has resulted in rapid growth of an international export market, and facilitated continuing expansion of the local wine industry. However, the major challenge ahead is to ensure continued profitability and sustainability of this growth. The current study addresses two main issues within the Australian wine industry which have recently become a research focus. Firstly, that of water use efficiency in Australian vineyards and secondly, the need for improved wine quality to maintain the current rate of industry growth. It is well known that irrigation of grapevines can increase yield, but potentially reduces grape and wine quality (Smart and Coombe, 1983). This means that many countries, particularly in Europe, have discouraged the use of irrigation within the wine industry (Smart and Coombe, 1983). In other 'new world' countries, for example Australia, New Zealand, South Africa, Argentina, Chile and the USA irrigation has been unrestricted, but in recent years there has been a move toward gaining a greater understanding of the yield:quality relationship which is implied in European viticultural practice. Thus, in order to improve grape and wine quality in irrigated vineyards, as well as to improve their water use efficiency, a restriction in irrigation may be required.

Within Australia, two so-called 'deficit' irrigation strategies have been developed. Regulated deficit irrigation (RDI) was developed from experiments on peach orchards during the 1980s, which aimed at reducing vegetative growth (Boland *et al.* 1993). In the late 1980s this technique was applied to vineyards. RDI aims to place the grapevine

under water deficit at selected times within the growing season. Through application of this technique in experiments, it was found that water deficit between flowering and fruit set should be avoided, as this leads to marked reductions in bunch and berry weight (Goodwin and Jerie, 1992). Pre-veraison and post-veraison application of water deficit appeared to provide the greatest benefit, both reducing vegetative growth and improving colour but without a significant yield penalty (Goodwin and Jerie, 1992). However, limitations of this technique are incurred due to an inability to precisely measure the amount of water stress applied. Thus, where soil or irrigation water is saline, or seasons where temperatures have a high frequency over 40°C there are real dangers that if the level of water deficit applied is too high, a yield penalty or actual stress-induced damage to fruit e.g. sunburn could result (Possingham, 2001).

A technique was developed which could potentially alleviate the risks associated with RDI, partial rootzone drying (PRD). This technique has already been mentioned in this chapter, in terms of potential gains in fruit quality. This technique does not rely on the application of water deficit at specific stages of development but applies as little as 50% of the water of conventional irrigation through continuously alternating the side of the root system under irrigation, termed the 'wet' and 'dry' sides of the grapevine, placing the vine under a continuous 'physiological stress.' Theoretically, and according to early experiments with PRD, the technique should allow plant hormones like ABA to transmit a signal from roots in drying soil to the apical parts of the plant, while the 'wet' side of the root system provides adequate water (Dry and Loveys, 1998; Stoll *et al.* 2000). This should bring about the changes associated with stress-signalling under water stress, such as reduced canopy and shoot growth and reduced stomatal conductance, but without causing the deficit-induced reduction in leaf water potential and yield. It has been found that the stress signals are only induced when the root system is in drying soil, and that when soil is dry, i.e. when soil water content is at a minimum, there is a recovery of shoot growth and stomatal conductance (Dry and Loveys, 1999). Thus, the PRD technique requires continual cycling between the 'wet' and 'dry' sides of the root system in order to facilitate continuing production of root-derived signals which bring about the desired response in terms of canopy growth and water use.

In practice, however, large-scale field trials and commercial trials of the PRD irrigation technique have shown that the technique is difficult to perfect, and has been associated with yield penalties relative to fully-irrigated vines, albeit small (M. McCarthy, pers comm.). This indicates that with reduced application of the irrigation volume, grapevines will have entered water deficit relative to standard-irrigated vines at certain key stages of fruit development. Where this has occurred, potential changes in fruit composition observed though the PRD technique must be viewed as due to water deficit, even if it is minor. Despite this observation, the PRD technique allows for a relatively 'safe' application of water stress, in the form of a continual, small deficit rather than long periods of severe deficit as under RDI. This allows for a potential gain in water use efficiency, without a significant yield penalty. Furthermore, as discussed previously, there is the opportunity to manipulate secondary metabolic pathways through the application of water stress.

1.5 Aims and objectives within the current study

The current study aimed to explore the potential effects of the PRD irrigation technique on two pathways of grape secondary metabolism. Firstly, the effect of PRD on the recovery of total phenolics in fruit and wines, with special reference to anthocyanins derived from grape berry skins and condensed tannins derived from the seeds. Secondly, the effect of PRD on fruit carotenoids and their C_{13}-norisoprenoid products was assessed in terms of their potential contribution to wine flavour and aroma.

Key issues addressed within the current study were:

1. ***The effect of PRD on berry size.*** Does a decrease in berry size increase the concentration of secondary metabolites, and in turn improve wine quality?
2. ***The separation of the effects of irrigation strategy and bunch exposure.*** Does an increase in sunlight incidence on developing fruit increase the final concentration of secondary metabolites?
3. ***The limitations of the PRD technique in terms of vine balance.*** Using the PRD technique is there a threshold in terms of node number per vine over which carbon available for partitioning to reproductive and vegetative growth becomes limiting?

CHAPTER 2: GENERAL MATERIALS AND METHODS

2.1 Field experiments

2.1.1 Nuriootpa Shiraz experiments

The vineyard site was at Nuriootpa, in the Barossa Valley, South Australia (34°48'S, 139°14'E, elevation 274 m). The general climate of the region is Mediterranean, warm, with a mean January temperature of 21.4 °C and 1817 biologically effective degree days. Rainfall is moderate (503 mm) with high summer evaporation and low relative humidity (Dry and Smart, 1988). The soil at the experimental site is classified as a Light Pass fine sandy loam (Northcote *et al.* 1954). This topsoil horizon 'A' was 30-40 cm deep and overlaid a red-brown earth clay soil horizon 'B' (M. McCarthy, pers comm).

2.1.1.1. Shiraz PRD and pruning experiment

The experiment was set up as a split-plot, fully randomised design of six treatments with five replicates of two-vine plots (Appendix A). The treatments were: three pruning levels determined by node number at winter pruning of 30, 60 and 120 nodes superimposed over PRD and standard irrigation strategies. A 60 node per vine pruning level is standard practice for the Barossa Valley region. The trellis type was a permanent bilateral cordon and the row and vine spacing was 3.0 m and 2.25 m respectively. Rows were oriented in an east-west direction. For the PRD and control treatments, two 4L/h drippers were set up 45 cm on either side of the vine trunk. The PRD used a specially designed dual dripline (Netafim) that allowed for the sides of the irrigation to be switched while preventing the shifting of the dripper position. For the PRD treatment, only one side of the vine's root system received water at any time, whereas for standard-irrigated vines both sides of the root system were watered at a given point. The time between PRD cycles was approximately 15 days, and the 'wet' side received an additional 'top up' irrigation mid-way through a cycle. In the seasons 2000/2001 and 2002/2003 the PRD treatment received half the irrigation water of the control. In 2000/2001 the total water applied was 1.0 ML/ha and 0.5 ML/ha for control and PRD respectively. In 2002/2003, the total water applied was 1.2 ML/ha and 0.6 ML/ha for control and PRD respectively. In 2001/2002, the same amount of irrigation water was applied to both treatments by doubling the time

period of irrigation applied to the PRD treatment relative to the control, and was 1.0 ML/ha. This decision was made in response to vine performance for 2000/2001, where PRD-treated vines appeared to enter water deficit relative to the controls. The decision was reversed in 2002/2003, as a relatively small response to the PRD treatment was observed in 2001/2002. In the 2001/2002 and 2002/2003 seasons an EnviroSCAN soil water continuous monitoring system (Sentek) was used to monitor soil moisture status and to schedule irrigation. The access tubes were installed 25 cm on either side of two vines pruned to 60 nodes, each under PRD or control irrigation respectively (Figure 2.1).

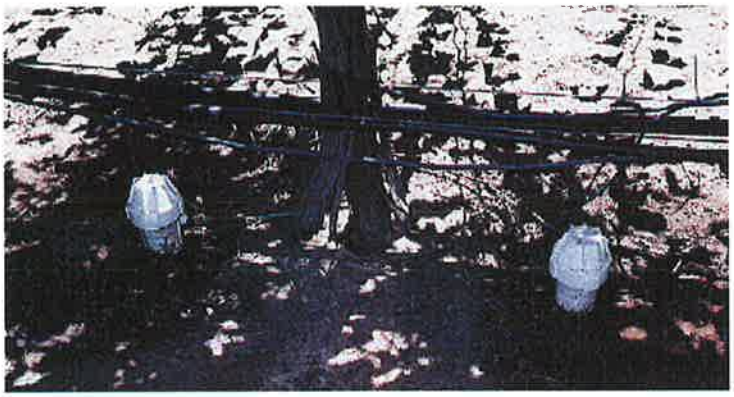


Figure 2.1: Position of access tubes for soil water monitoring using the EnviroSCAN system in Shiraz grapevines at Nuriootpa

2.1.1.2. Shiraz canopy experiment

The experiment was a randomised block design of five treatment replicates. The trellis system was a single-wire system, cordon trained and spur pruned. Row and vine spacing were 3.0 and 2.25 m respectively, with the rows oriented in an east-west direction. Vineyard practices were in accordance with restrictions for the region. For the 1999/2000 and 2000/2001 seasons, all vines received 1 ML/ha, which produced vigorous canopies. For the duration of the experimental period, an adjacent row received 0.5 ML/ha irrigation. This adjacent row was on an identical soil type, having vines of the same age, but of a different Shiraz clone to the remaining three treatments. A previous study had shown that the two clones BVRC12 and PT15N showed similar responses in terms of fruit composition when they were treated with identical vineyard practices (Appendix G). Under the conditions of the current study, a primary response to reduced irrigation was a reduction in vine vigour. On this basis, the row was included as an additional treatment.

For the vines (clone BVRC12) irrigated to 1 ML/ha, three canopy treatments were set up:

1. **Shaded treatment**. The canopies were wrapped in bird nets immediately after fruit set to constrict the canopy and create shaded conditions.
2. **Moderate treatment**. Canopies were allowed to sprawl, to produce an intermediate level of shading.
3. **Sun treatment.** High posts of 2.5 m were placed on the ends of the panels, with the addition of three rows of foliage wires 50 cm apart. Vine canopies were divided and shoots were trained upwards and downwards. Vertical positioning of shoots and leaf removal from bunches were carried out when required during the season, to ensure a maximal level of bunch exposure within the canopy was attained.

For the vines (clone PT15N) irrigated to 0.5 ML/ha a fourth treatment was set up:

4. **Reference treatment**. The vines had naturally open canopies with low canopy density and a relatively high bunch exposure.

Fruit and wine samples from this experiment were a kind donation by Ms Renata Ristic (Adelaide University) and further details can be found in Ristic (2004).

2.1.2 Langhorne Creek Cabernet Sauvignon experiment

This experiment was carried out over two seasons (2001/2002 and 2002/2003) as part of a commercial field experiment by Orlando-Wyndham, at Langhorne Creek, South Australia. The detailed temperature and rainfall data for these seasons are shown in Appendix D. It compared PRD and control irrigation treatments on 5-year old Cabernet Sauvignon vines pruned to 60 nodes with sprawling canopies. Three field replicates of PRD and standard irrigation treatments were set up, with seven two-vine sub-plots within each replicate (Appendix B). The soil type was determined from samples taken from four pits dug at four randomly selected positions at the experimental site (Figure 2.2). From this detailed analysis, a general description of the 'A' horizon of the soil at this site was a loamy sand up to approximately 60 cm depth. The 'B' horizon below this was comprised of soils with a higher clay content, either a clay loam or a medium clay (Figure 2.2). The row and vine spacing was 2.4 m and 1.8 m respectively. The control treatment was

irrigated with a standard dripline and the PRD treatment was irrigated using a dual dripline (Netafim). For both treatments, drippers were spaced 0.6 m apart and supplied water at a rate of 2L/h.

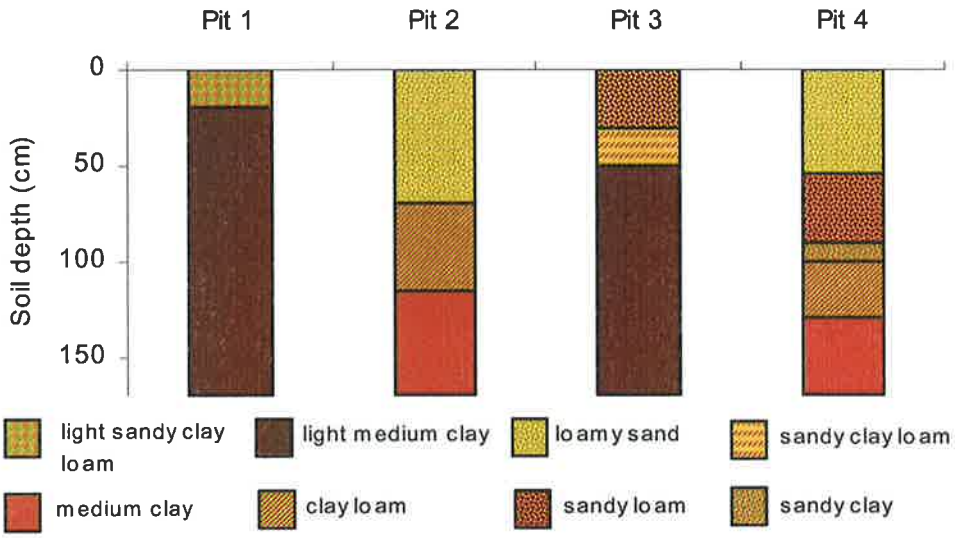


Figure 2.2: Soil profile for four soil pits dug at the Langhorne Creek Cabernet Sauvignon experimental site (data courtesy of M. Partridge, Orlando-Wyndham, Langhorne Creek).

For the PRD treatment, the spatial arrangement of the drippers between the dual driplines allowed for two consecutive drippers to be 'on' at any one time, with two drippers on each side of to be turned 'off' on the opposite dripline (Figure 2.3). This pattern was based on the assumption that prior to the setup of the PRD experiment, the vines had been irrigated by a single dripline, causing a shallow, spreading root system to develop close to the soil surface (J. Kennedy, pers comm.). Thus, clear separation of root systems between consecutive vines within a row was not possible. To counter this problem, PRD was set up so that at any one point in time, a substantial portion of a vine's root system would be in either a wetted or a drying zone of the soil (Figure 2.3). The time between PRD cycles was 7 – 8 days, and the 'wet' side of the PRD treatment received an additional irrigation mid-way through each cycle. In 2001/2002, the control and PRD treatments received 1.19 and 0.84 ML/ha respectively. In 2002/2003, the control and PRD treatments received 1.37 and 0.85 ML/ha respectively.

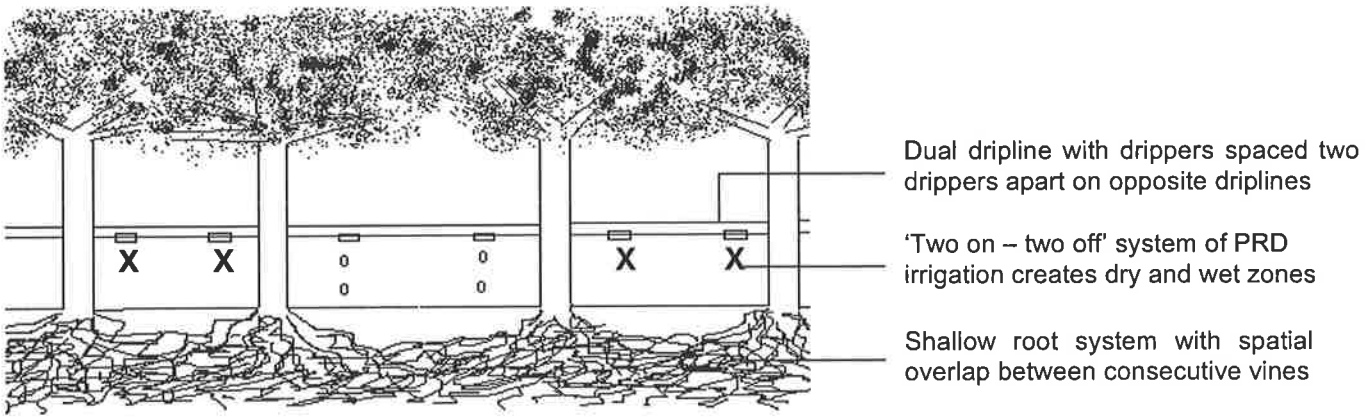


Figure 2.3: Spatial arrangement of drippers for the PRD treatment on Cabernet Sauvignon at Langhorne Creek for 2001/2002 and 2002/2003.

2.1.3 Shading experiments on Shiraz and Cabernet Sauvignon

For the PRD experiments at Nuriootpa and Langhorne Creek, a shading experiment was applied for the 2001/2002 and 2002/2003 seasons respectively. Boxes designed by Mark Downey (CSIRO Plant Industry, Adelaide, Australia) were placed over randomly-selected bunches, and secured to adjacent shoots using cable ties (Figure 2.4). The boxes were made from white polypropylene sheeting (0.6 mm) which was painted black on the box interior to prevent light penetration. The boxes were designed with an inner and outer compartment (Figure 2.5) to allow for air flow over the bunch, while preventing light entry through the open sides. For the 2001/2002 season of the Nuriootpa Shiraz PRD trial, boxes were applied to six bunches of five replicate plots of conventionally-irrigated 60-node vines. In 2003, in the Langhorne Creek Cabernet Sauvignon experiment, the shading treatment was superimposed over the entire experiment, with boxes placed over three bunches per plot for 3 x 7 replicates of PRD irrigation and 3 x 7 replicates of conventional irrigation. Temperature was monitored at certain stages of the season using thermistor probes positioned within the interior of bunches either within selected boxes or free within the canopy. The data were collected using a 12-channel macro 32 Starlog data logger (Measurement Engineering Australia). A comparison between the temperature of boxed and free bunches showed negligible differences at all points where temperature was measured (Appendix C).

Figure 2.4: Shade boxes placed over bunches of Shiraz vines grown at Nuriootpa (2001/2002 season)

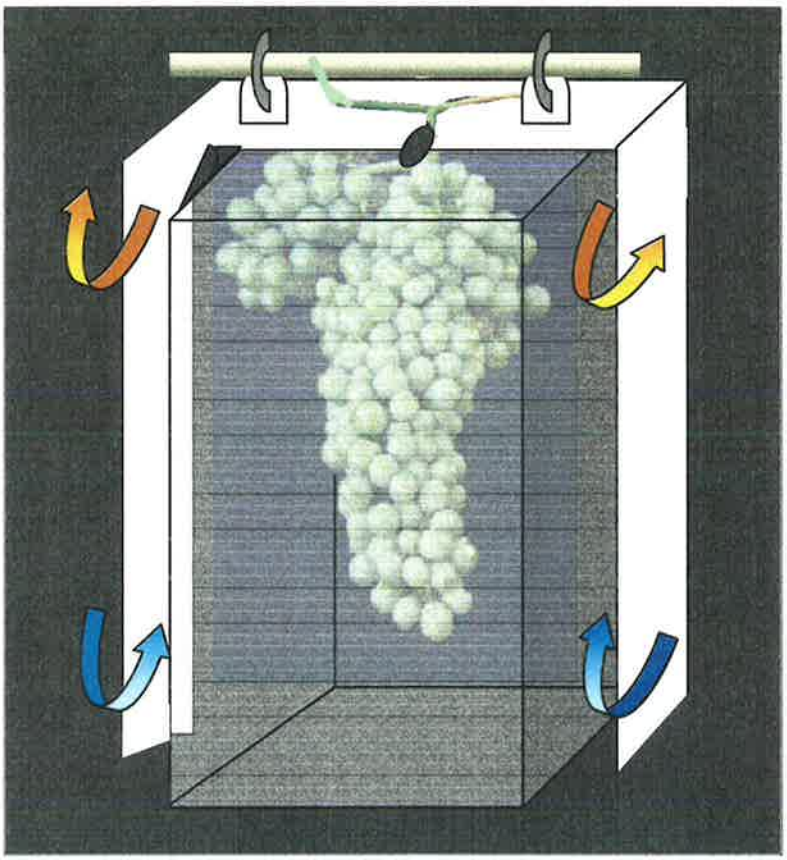


Figure 2.5: Design of the shade box by M. Downey, CSIRO. The bunch is positioned centrally and enclosed by two overlapping inner and outer compartments, which are attached to a shoot within the canopy. The design of the box creates a space between the inner and outer compartments which allowed for air flow in (blue arrow) and out (orange arrow) of the box interior.

2.1.4 Fruit sampling procedure

For the Nuriootpa Shiraz and the Langhorne Creek Cabernet Sauvignon PRD experiments, fruit sampling began at the time of colour change (veraison). Two sets of fifty-berry samples were collected from randomly selected bunches within the canopies of the two-vine plots of each treatment replicate for both experiments. One sample was immediately processed for measures of °Brix and pH, and the second was frozen at -20°C for later analysis of fruit secondary metabolites. For the Langhorne Creek experiment, a further thirty-berry sample was collected from one month pre-veraison to harvest and frozen at -80°C for later analysis of seeds. When the fruit reached 23-24 °Brix, six-bunch samples were removed from each vine plot and frozen for later analysis of yield components: berry number per bunch and berry weight. For the box-shading experiments, all shaded bunches were harvested when free fruit within the canopy reached a desirable °Brix level. This was 25 °Brix for the 2001/2002 Nuriootpa Shiraz experiment and 21.5 °Brix for the 2002/2003 Langhorne Creek Cabernet Sauvignon experiment. Two representative fifty-berry samples were collected from the bunches. One was immediately processed for measures of °Brix and pH, and the second was frozen at -20°C for later analysis of fruit secondary metabolites.

2.2 Cabernet Sauvignon pot experiment

Four-year-old split-rooted Cabernet Sauvignon vines were obtained from a previous experiment conducted by Stoll (2000). The root system of each vine was divided between two separate 250 mm pots, filled with pure sand. Two irrigation treatments of five replicates were set up comparing PRD and conventional irrigation for one season in 2002/2003, where the PRD treatment received half the irrigation level of the control treatment. The cycle between alternate PRD irrigations was 3-4 days. Irrigation was started in September 2002, but the actual treatments began on 15th January 2003. The daily irrigation was replaced by fertiliser solution (Tournament fomulation, T-link, South Australia) once a week until two weeks before harvest on 25 March 2003.

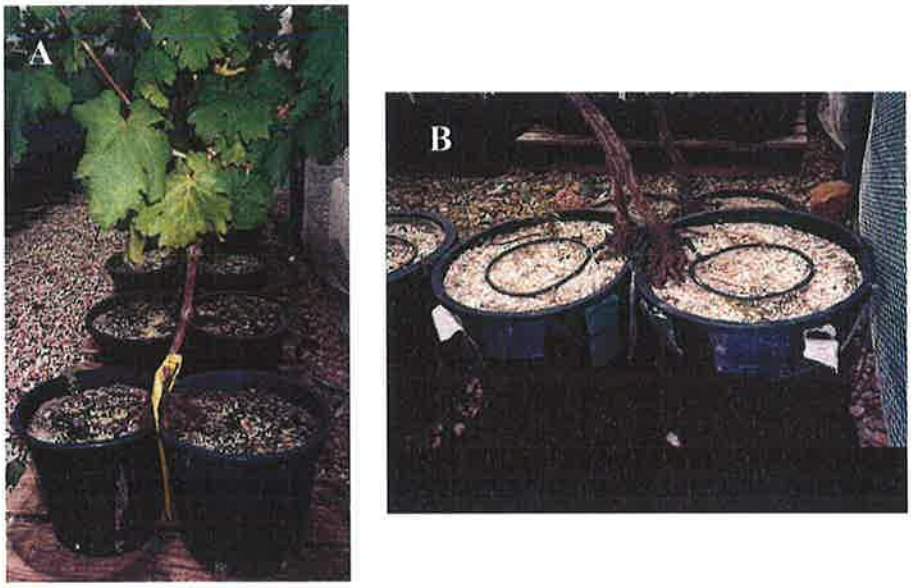


Figure 2.6: Pot experiment on split-rooted Cabernet Sauvignon grapevines (experiment conducted by G. du Toit, Adelaide University) **A.** Split-rooted grapevines, **B.** Irrigation of split-rooted grapevines

The potted vines were placed under shade net throughout the course of the experiment, so that vines received approximately 50% of ambient light. Daily stomatal conductance measurements were made by G. du Toit (Adelaide University) throughout the experimental period using a portable porometer (Delta-T AP4, Delta-T Devices, Cambridge, UK) and were made available for use in the current study. All fruit was harvested at one time when the fruit reached 25-26 °Brix. Because there was on average only two bunches produced per potted vine, five-berry samples were collected for later analysis, representing approximately 5% of all available fruit. These samples were retained and frozen at -40°C, while the remaining fresh fruit was used for an assessment of berry weight, °Brix and pH.

2.3 Mini-winemaking procedure

Wine was made in one vintage from fruit harvested from the Langhorne Creek Cabernet Sauvignon experiment in 2002. All fruit from three treatment replicates of each irrigation treatment was harvested at 23.5 °Brix, to give a total of six treatment replicates. Within each treatment replicate, fruit was divided to give two winemaking replicates, giving a total of twelve experimental wines. Approximately 30 kg of fruit was allocated to each winemaking replicate. Fruit was de-stemmed and crushed (Zambelli roller de-stemmer 1-

2 t/h) into 18 L fermenters and 4% SO_2 solution was added during crushing at 12 mL per 10 L of must (Figure 2.6 A). Following this, 8% DAP was added at 1 mL per L of must and pH was adjusted to 3.6 by addition of tartaric acid. The musts were then inoculated with yeast (EC 1118 *Saccharomyces cerevisiae*) at 0.25 g/L.

Wines were fermented at 15 °C on skins for 10 days, and plunged every 6 hours. When the levels of total soluble solids were reduced to approximately 10-12 °Brix, the wines were pressed and transferred to glass 12 L fermenters equipped with fermentation locks (Figure 2.6 B). Fermentation was continued at 20 °C for a further 11 days until reducing sugars were measured at below 0.25%. Wines were then racked from the lees to fresh 12 L glass fermenters and then malolactic culture (Viniflora, Australia) was added at 10 mg/L of wine. After three weeks, re-innoculation of the ferments with a further 10 mg/L of bacteria was required to initiate malolactic fermentation. Malolactic fermentation was continued at 20 °C for three months, until levels of malic acid in the ferments was below 0.1 g/L. Wines were then moved to a room at 0 °C for cold stabilisation for one month. Wines were then racked, pH was re-adjusted to 3.6 with tartaric acid. Then SO_2 was added to give a total SO_2 value of 60 ppm. Wines were bottled in 750 mL glass bottles and sealed with screw caps.

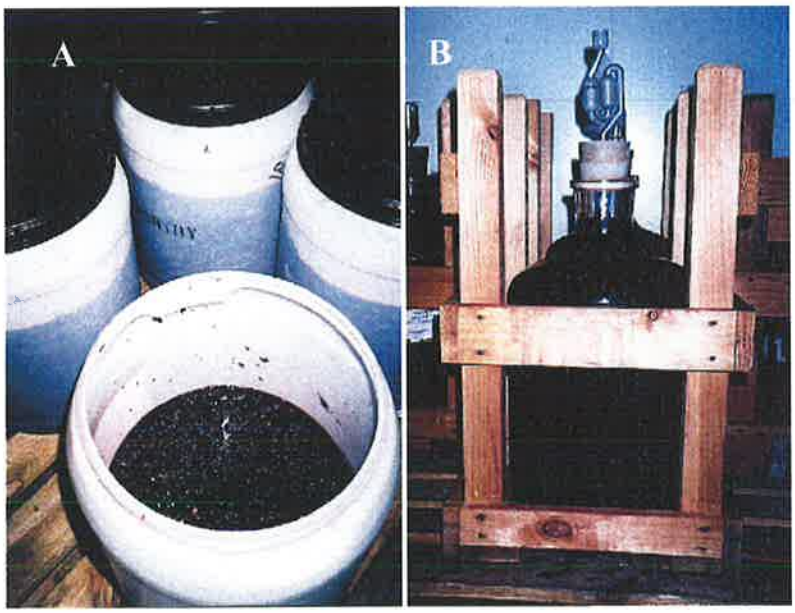


Figure 2.7: Stages of the mini-winemaking procedure **A**. Grape must following crushing and addition of SO_2 **B**. Pressed wine in 12L glass fermenting tank

2.4 Statistical analysis

Data from the Nuriootpa Shiraz experiment comparing PRD and pruning level were analysed using a split-plot ANOVA with Genstat 6.1 software. This analysis allowed for the comparison of the treatment means at three levels: irrigation treatment (T); pruning level (P), and the interactive effect of irrigation treatment by pruning level (T x P). For the Nuriootpa Shiraz canopy management experiment, data were analysed using a one-way ANOVA with the software package Instat 3. For a significant ANOVA result, Tukey's test was used to determine the mean separation between individual treatments. For the Langhorne Creek PRD experiment, a one-way ANOVA was performed using Gentat 6.1 software. In the chapters which follow, a detailed description of the type of statistical tests used is given where the analyses required differ from those described here.

CHAPTER 3: THE EFFECT OF PRD ON VINE PHYSIOLOGY, GROWTH AND YIELD COMPONENTS

3.1 Introduction

3.1.1 Irrigation of grapevines and the need for shoot vigour control

When vines are grown in arid regions irrigation is required, which may lead to increased vine vigour, in terms of vegetative growth. Both pruning weight and shoot growth rate have been shown to increase under irrigation (Smart and Coombe, 1983; Bravdo and Hepner, 1986). Furthermore, irrigation has been reported to increase average leaf area per shoot (Carbonneau and Casteran, 1979; Van Rooyen *et al*, 1980). If the overall shoot vigour is high, dense leaf canopies may develop, leading to a high level of within-canopy shading (Smart, 1974; Smart and Coombe, 1983). The detrimental effects of dense canopies are various, but a significant consequence is that of reduced fruit and wine quality. Highly shaded canopies have been reported to produce grapes with increased juice pH, and contents of K^+ and malic acid; and decreased content of sugars, tartaric acid, anthocyanins and phenolics (Smart, 1985). Wines produced from shaded grapes have also been reported to have lower scores for flavour (Smart, 1985; Dokoozolian and Kliewer, 1995).

An ongoing endeavour in viticultural research is the development of effective irrigation management strategies to improve grape and wine quality. To reduce shoot vigour in vines, a strategy that has been widely used is the application of water stress (Smart and Coombe, 1983; Dry and Loveys, 1998). This has been found to have significant effects on internode elongation and the duration of shoot growth, especially when the water deficit occurs early in the season (Williams and Grimes, 1987). However, in most experiments where shoot growth is reduced as a result of a water deficit, a concomitant reduction in yield has been observed (McCarthy and Staniford, 1984; Matthews and Anderson, 1988; Goodwin and Jerie, 1992; Poni *et al*, 1993). The use of regulated deficit irrigation (RDI) as an irrigation strategy applies a mild stress to the vines through the application of precisely controlled amounts of water at critical stages in the season (McCarthy, 1996; Goodwin and Jerie, 1992). However, where RDI treatments have

brought about a large reduction in vigour, it has often been accompanied by a significant penalty in terms of yield, resulting from reduced berry size (Dry and Loveys, 1998; Kriedemann and Goodwin, 2003).

3.1.2 The introduction of Partial Rootzone Drying (PRD)

3.1.2.1 The concept of PRD

Partial rootzone drying (PRD) was developed as an irrigation technique which reduces shoot growth in grapevines through partial drying of the root system, but maintains water relations by a supply of water from an hydrated part of the root system (Dry *et al*, 2000a,b). This is achieved through the use of vines in which the root system has been divided into two sections, termed 'split-root' vines. One-half of the root system is watered at a time, for a specified period, while the other half gradually becomes dry (Figure 3.1). The technique maintains the roots in the early stages of drying by transferring irrigation to the opposite half of the root system at intervals. Studies on other plant species showed that when part of the root system was dried there is a reduction in leaf conductance, without an apparent leaf water deficit (Blackman and Davies, 1985; Zhang *et al*, 1987; Saab and Sharp, 1989; Gowing *et al*, 1990). Currently, the PRD technique is carried out on field-grown vines, with one half of the root-system watered at a time, simulating the response found for split-rooted potted vines (Stoll, 2000).

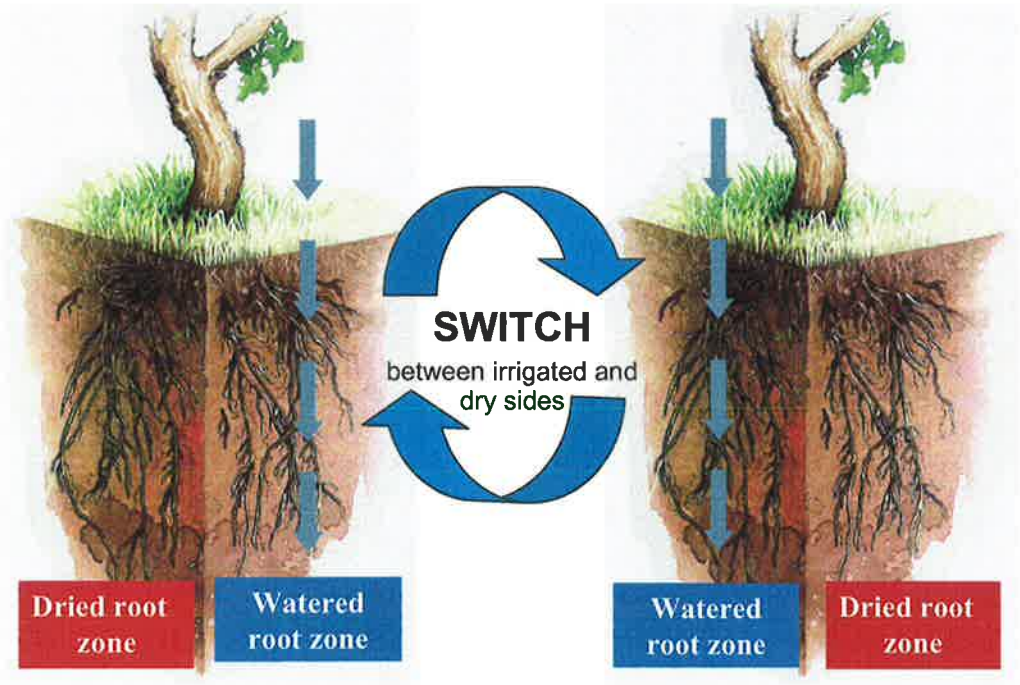


Figure 3.1: Schematic representation of the PRD irrigation technique

3.1.2.2 The effect of PRD on vine physiology

In grapevines, it was found that an initial decrease in gas exchange and shoot growth rate occurred in response to half-drying of the root system, which coincided with a decrease in the soil water content of the dried half of the root system (Dry *et al*, 2000a,b). However, a later full recovery of gas exchange in half-dried grapevines was found to occur without any further change in the soil water content of the dried half of the root system (Dry *et al*, 2000b). This recovery coincided with the point at which there was no further decrease in soil water content on the 'dry' side. In an attempt to maintain the response in terms of gas exchange and shoot growth, cycling between the 'wet' and 'dry' sides was introduced, so that one half of the root system was in the process of drying at any point in time. This allowed the response of reduced stomatal conductance and shoot growth rate to be sustained (Dry, 1997; Stoll, 2000; Stoll *et al.* 2000).

It was proposed that for half-dry plants, there is a movement of water from wet to dry roots which enables their survival (Dry *et al,* 2000b). This was later verified in a study by Stoll *et al.* (2000), which showed nocturnal redistribution of deuterium-labelled water from roots in wet soil to roots in drying soil in potted Cabernet Sauvignon vines. Despite the inhibition in stomatal conductance and shoot growth, PRD was reported to cause no change in leaf water potential as a result of half-drying (Dry *et al*, 2000a; Stoll *et al.*, 2000). This suggests the involvement of a non-hydraulic signal, originating from the roots in drying soil. Abscisic acid (ABA) is a possible candidate for a root signal in the grapevine, as ABA levels in roots and xylem sap closely follow the changes in stomatal conductance observed with PRD (Stoll *et al.*, 2000). However, reduced levels of the cytokinins zeatin and zeatin riboside were also reported in shoot tips, roots and buds of PRD-treated vines, indicating that an alteration in the ABA to cytokinin ratio may be responsible for the physiological responses associated with the treatment (Stoll *et al.*, 2000). The PRD strategy aims to induce sustained high levels of root and shoot ABA by maintaining the root system in the early stages of drying. This effectively maintains the hormonal signal to the leaf canopy, causing a reduction in shoot and canopy vigour (Dry and Loveys, 1998; Loveys *et al.*, 2000). In addition, light penetration into the fruiting zone of the vine canopy was found to be increased in response to PRD, primarily due to a

reduction in lateral shoot growth and lateral leaf area (Dry, 1997). As increased levels of bunch exposure to light have been associated with an improvement in secondary metabolite concentration in fruit (Bergqvist *et al.* 2001; Zoecklein *et al.* 1998) this was proposed to be a factor which could potentially increase secondary metabolites in PRD-treated berries (Dry, 1997; Dry *et al.* 2000c; Stoll, 2000).

3.1.2.3 The effect of PRD on yield components

Interestingly, early studies with PRD showed no change in berry size or yield as a result of partial drying of the root system in field-grown vines over three seasons (Dry, 1997) and in commercial trials (Dry *et al.*, 2000c). This holds significant implications for the application of this technique commercially, in that when correctly applied it will not cause a reduction in yield. However, a more recent report of PRD application in the field, showed a significant reduction in the yield of PRD-treated vines of cvs. Moscatel and Castelao (dos Santos *et al.,* 2003). This yield reduction was associated with an observed reduction in pre-dawn leaf water potential of PRD-treated vines relative to fully-irrigated, albeit intermediate between lower leaf water potentials observed in non-irrigated vines (dos Santos *et al.*, 2003).

This indicates that under some conditions, despite rigorous irrigation scheduling, the PRD treatment may result in vines experiencing a water deficit as defined by the vine's physiological response. This may be due to a lack of adequate refilling of deep soil layers after switching the sides of irrigation between PRD cycles (Dry *et al.*, 2000c). Additionally, depending upon the soil type where PRD is implemented, the 'dry' side may resist infiltration after the sides following the switch between PRD cycles, leading to vines experiencing water stress i.e. both sides of the root system become dry (Kriedemann and Goodwin, 2003). However, despite the yield reduction observed by dos Santos *et al.* (2003), PRD was reported to be more effective in the control of shoot vigour than a conventional deficit irrigation treatment at the same level of water applied. This indicates that maintenance of signals such as ABA from part of the root system in a constant state of partial-drying with PRD can potentially result in the effective control of shoot vigour.

3.1.3 The combined effect of pruning and irrigation on vine growth and yield components

The number of nodes per vine left at pruning has significant implications for both yield and vine vegetative growth. A study by Miller and Howell (1998) investigated the effect of increasing node number per vine from 20 to 160 nodes on field-grown vines, and it provides a model by which the effect of node number per vine on yield components and vine growth can be predicted. In that study, higher node number per vine at pruning increased the number of bunches per vine, leading to a higher average yield, although both berry weight and bunch weight were reduced as these factors increased. Berry number per bunch was reported to be slightly decreased with higher node number per vine. Vine vegetative growth in terms of winter pruning weight significantly decreased as node number per vine increased, although leaf area per shoot and leaf size showed the reverse effect, with both parameters increasing with higher node number per vine. However after veraison, total leaf area per vine was not significantly altered by pruning level, most likely due to an overall reduction in pruning weight with increasing node number.

The effect of the interaction of irrigation and pruning level on yield and vegetative growth has not been extensively studied. However, a series of studies by Freeman *et al.* (1979, 1980) explored the effect of non-irrigated and frequently irrigated treatments on Shiraz vines pruned to 20, 40, 80 and 160 nodes per vine. Interestingly, at lower node number per vine (20 and 40 nodes) there was no effect of the irrigation treatment on yield, while in the 160 node vines, irrigation increased yield by 127% relative to the non-irrigated treatment. Clearly, at higher bunch number per vine there was an increased sensitivity to water deficit, which resulted in decreased yield. In that study, pruning level did not significantly affect winter pruning weight, but shoot weight was significantly decreased as node number increased. Irrigation increased pruning weight and shoot weight for all pruning levels, but the greatest difference was observed for the 160 node vines. This again demonstrated the increased sensitivity of vines to water deficit at higher node numbers. As the 160 node vines had a higher shoot and bunch number, this would have created an increased demand for fixed photosynthate, and its partitioning between vegetative and reproductive growth. Clearly, this resource became limiting as node

number per vine was increased, and was exacerbated by the additional water stress in the non-irrigated treatment.

A further study by Bravdo *et al.* (1985) compared the effect of regulated deficit irrigation and standard irrigation on three cropping levels which were determined by bunch thinning, rather than node number at pruning. In that study, the effect of bunch number on yield was highly significant as would be expected, but the additional deficit irrigation treatment had little effect on yield. Bunch-thinning significantly increased winter pruning weight in each of the irrigation treatments. Although pruning weight was lowered in each of the deficit-irrigation treatments relative to the most highly-irrigated treatment, there was no clear interactive effect between cropping level and irrigation strategy on pruning weight. This result is different from that found by Freeman *et al.* (1979, 1980) and thus, the question remains as to the effect of deficit irrigation in altering the balance between vegetative and reproductive growth in vines of different node number. As irrigation of grapevines can result in excessive vigour over reproductive growth (Coombe and Smart, 1983), the potential exists that there is a crop-load threshold over which vines will not have the capacity to support a vigorous canopy. This effect could be exacerbated by an additional water stress, but the literature indicates that this will depend on the magnitude of the water deficit experienced by vines (Freeman *et al.* 1979; Bravdo *et al.* 1985)

The PRD irrigation strategy can create more open canopies through a reduction in shoot growth rate and canopy development (Dry, 1997; Stoll, 2000), although at higher bunch numbers there may be a limit to which the balance between vegetative and reproductive growth can be exploited (Howell, 1999). Therefore, it is possible that at higher node number per vine, PRD may result in insufficient vegetative growth to enable ripening of a larger crop. The aim of the current study was to explore the effect of PRD on yield components, vegetative growth and vine physiology in both commercial and experimental applications of the irrigation treatment. Within this, the interactive effect of PRD and pruning level at 30, 60 and 120 nodes per vine was assessed, in order to determine the limitations of PRD in terms of vine balance between vegetative and reproductive growth, while aiming to maintain or improve grape quality.

3.2 Materials and Methods

3.2.1 Experimental

The analyses which follow refer to the Cabernet Sauvignon experiment at Langhorne Creek (Section 2.1.2, Chapter 2) or to the Shiraz experiment at Nuriootpa (Section 2.1.1.1, Chapter 2). The latter experiment compared three different pruning levels, of node number 30, 60 and 120 nodes superimposed over PRD and standard irrigation strategies.

3.2.2 Shoot growth rate

Six main shoots were randomly selected from each experimental replicate. A reference node was tagged 7 nodes below the shoot tip and the distance from that node to the shoot tip was measured at weekly intervals from October to January of 2000/2001 and 2001/2002. Shoot growth rate was calculated on a weekly basis as the average increase in shoot length since the previous measurement (cm/week). When individual shoots ceased growing, they were excluded from the sample set. Data points for excluded shoots were given a value of zero, and included in the average shoot growth rate at later stages of the season, together with the remaining growing shoots. In cases where shoots stopped growing due to damage, rather than a physiological cessation of growth, samples were substituted with replacement shoots.

3.2.3 Gas exchange measurements

Stomatal conductance of leaves was determined using a portable porometer (Delta-T AP4, Delta-T Devices, Cambridge, UK). On cloudless days, measurements were made during two intervals, 'morning' from 9:30 to 11 am or 'midday' from 12 – 1:30 pm. Due to excessive cloud coverage in the morning in all seasons of the study, generally only 'midday' readings were possible. Where possible, diurnal stomatal conductance measurements were made from 9 am to 4 pm. In all experiments, six reference leaves were randomly selected for each treatment replicate. Selected leaves were sun-exposed leaves of similar maturity, approximately the fifth leaf from the shoot apex, were selected for measurement. For measurement, the terminal part of the main lobe was placed into

the cup on the head unit which was positioned normal to the sun. The porometer was calibrated prior to each use, and was re-calibrated within the daily period subject to changes in environmental conditions e.g. relative humidity or temperature.

3.2.4 Leaf and Stem water potential

Leaf (Ψ_L) and stem (Ψ_S) water potentials were measured with a manual pump-up pressure chamber (PMS Instrument Co). Measurements were made at midday (12:30 to 1:30 pm) on leaves of similar maturity to those selected for gas exchange measurements. For the measurement of stem water potential, clear plastic bags were placed over two selected leaves per treatment replicate at 9 am, followed by a second opaque bag. The opaque bags were specially constructed from plastic that was black on the interior and white on the exterior to prevent light penetration to the leaf and minimise leaf heating. These leaves were left to equilibrate until readings were taken at midday. Selected leaves were detached from the shoot by cutting through the base of the petiole, and for the Ψ_L measurements an additional two leaves per treatment replicate were immediately transferred to a plastic bag, and measured within one minute. Water potential pressure readings were recorded when sap was first observed to exude from the cut end of the petiole.

3.2.5 Pruning weight (PW)

This is defined as the mass of mature, one year-old shoots (canes) removed from the vine at pruning time in winter. The Langhorne Creek Cabernet Sauvignon vines were pruned to 60 nodes, and the three pruning treatments in the Nuriootpa Shiraz experiment were pruned to 30, 60 and 120 nodes. All the shoots removed from a single vine were bundled together and weighed in the field with a spring balance. Pruning weight was expressed as kg/vine.

3.2.6 Yield components

All bunches were removed at harvest, placed into buckets or large plastic bags, and weighed using a spring balance in the field to give a total harvest weight in kg/vine. Bunch number was counted as the fruit was harvested (bunch number per vine). The

value for final fruit weight was adjusted from values of berry weight obtained at 23-24 °Brix, to enable comparison of yield figures at similar °Brix. An estimate of fruit weight removed during sampling was also made, and used to adjust the final fruit weight value. Mean bunch weight was calculated from this adjusted value (fruit weight/bunch number). From 6-10 randomly selected bunch samples removed at harvest, yield components were estimated to derive berry number per bunch and mean berry weight (g) by ignoring the weight of the bunch rachis. Fruit weight/pruning weight FW/PW = fruit weight (kg/vine)/pruning weight (kg/vine) was calculated from the adjusted yield value per vine. Yield was also estimated in terms of tonnes/ha and water use efficiency (WUE) in terms of tonnes per ML of irrigation applied.

3.2.7 Soil water measurement

A set of capacitance soil water sensors (EnviroSCAN soil water continuous monitoring system, Sentek Environmental Solutions, Adelaide, South Australia) was installed at the Nuriootpa Shiraz trial site in winter 2001. PVC access tubes were installed in undisturbed soil, one approximately 20 cm on either side of the trunks of two vines pruned to 60 nodes, under either PRD or standard irrigation respectively. Sensors were placed at 10, 20, 30, 40, 60, 70, and 100 cm in each access tube. Readings of soil water were logged at hourly intervals. Summed sensor data (10 to 60 cm) was subsequently used for irrigation scheduling, as this was the region of greatest water infiltration into the soil and use by vine roots.

3.2.8 Canopy measurements of solar radiation

Solar radiation within the vine canopy was determined by measurement of PAR with a ceptometer (model SF-80, Decagon Devices, Cambridge, UK) inserted horizontally within the bunch zone, parallel to the planting line. Readings were taken at solar noon, at angles 0°, 45°N, 90°N, 45°S and 90°S within the canopy. Ambient solar radiation was measured at half-hourly intervals during the sampling period. PAR measurements within the vine canopy were subsequently expressed as a percentage of ambient solar radiation.

3.2.9 Statistical analysis

Data from the Nuriootpa Shiraz experiment were analysed statistically with the Genstat6 software package, using a split-plot ANOVA to separate the effects of irrigation and pruning type. For shoot growth analysis, sample sets were separated into pruning level categories, and statistical differences were compared over time within the entire data set using a one-way ANOVA. For the Langhorne Creek Cabernet Sauvignon experiment, a one-way ANOVA was used to compare treatment differences, using Genstat6 software. For the 2002/2003 season, stomatal conductance and leaf water potential measurements were taken on a sub-set of representative vines, which were analysed using a Student's T-test with Microsoft Excel software. For shoot growth rate analysis in Cabernet Sauvignon, treatment differences were compared over time using a one-way ANOVA, as for the Shiraz experiment.

3.3 Results and Discussion:

3.3.1 The effect of PRD on leaf gas exchange and water status

3.3.1.1 The Langhorne Creek Cabernet Sauvignon experiment

In a commercial experiment on Cabernet Sauvignon, the effect of PRD on stomatal conductance was determined over two vintages, and midday measurements of stem and leaf water potential were determined for one out of the two seasons of the study. In the first vintage, diurnal measurements of stomatal conductance were taken at two stages of the developmental season, termed 'early' and 'advanced' corresponding to pre- and post-veraison respectively (Figure 3.2 A,B). Both readings were taken on days corresponding the end of the 7-day PRD cycle period, that is, when the soil on the 'dry' side of the PRD vines was at its driest prior to switching of the 'dry' and 'wet' sides for the following irrigation cycle. It would be expected that at this stage of the PRD cycle, stomatal conductance differences between PRD and control treatments would be greatest. The diurnal pattern of stomatal conductance was as expected for grapevines (Loveys and Düring, 1984), with the highest stomatal conductance measures recorded in the morning, dropping to low levels at midday, and then increasing again toward the late afternoon.

For both the 'early' and 'advanced' stages, PRD significantly reduced stomatal conductance at certain times of the day. The differences between the PRD and control treatments were greater in the morning, up until approximately 11 a.m., which is in agreement with findings by Stoll (2000). At the 'advanced' stage of the season, the midday stomatal conductance levels were lower than those recorded during the 'early' part of the season for both the PRD and control treatments. As ambient temperature was 6°C higher on the second sampling date (Appendix D) this could account for the lower midday stomatal conductance measurements. Leaf and stem water potential measurements were not taken in that season, so differences in plant water status were not compared between the two sampling dates. Thus, it is unclear whether the observed decrease in stomatal conductance in the PRD treatment relative to the control was due to a water deficit, or solely due to non-hydraulic signals produced by the PRD treatment alone.

In the second season of the study (2002/2003), stomatal conductance measurements were compared to measures of φ_L and φ_S in order to assess the effects of the treatment on plant water status. The readings were taken for four consecutive PRD cycles, termed cycle I to cycle IV for discussion purposes. It must be noted that these terms do not indicate the start and finish of the actual irrigation period, which began on 5/11/02 and ended on 12/03/03. Midday measurements of stomatal conductance were used for comparison, even though this time of day had previously shown the least significant response to the PRD treatment in the previous season. This was done due to morning cloud cover in the Langhorne Creek region during most of the 2002/2003 season, which prevented analysis of stomatal conductance until later in the day. At the end of cycle I in the 2002/2003 season, a clear PRD effect was observed. Midday stomatal conductance was reduced by the PRD treatment, with no significant effect on either φ_L or φ_S (Figure 3.3). As φ_S represents an equilibration of the covered leaf with shoot, root and soil water status, this is a sensitive indicator of actual plant water status. φ_S was significantly less negative than φ_L at all stages of the season (Figure 3.3 B,C) as would be expected. The results for stomatal conductance, φ_L and φ_S indicate that a non-hydraulic signal was operating to bring about the observed reduction in stomatal conductance, independent of plant water

status for cycle I. This indicates a classic PRD effect, according to original work done by Dry and Loveys (1999), where there was a reduction in stomatal conductance without any change in shoot water status in response to half-drying of the root system in Chardonnay and Shiraz grapevines.

However, later measures of stomatal conductance, φ_L and φ_S taken at the end of cycles III and IV indicated that the PRD vines had experienced water deficit relative to the control vines, suggesting that an hydraulic effect on stomatal conductance was occurring (Figure 3.3). This was evident from reduced midday stomatal conductance readings, which corresponded to significantly more negative measures of both φ_L and φ_S for PRD. At both stages, φ_S was more sensitive than φ_L to changes in soil water status, with greater, and more significant differences between the PRD and control treatments (Figure 3.3 C,D). The strong effect of the PRD irrigation on φ_S indicates that there was most likely inadequate soil water in the PRD treatment on both the 'wet' and 'dry' sides of the plant, which caused the vines to experience a water deficit relative to the control treatment.

Measurements were also taken during the 'switch' period of the PRD cycle, when the 'wet' and 'dry' sides of the irrigation pattern were alternated. This theoretically represents a period where both sides of the vine would have been wet, and measures of vine water status in PRD-treated vines should not have differed significantly to those observed for the control treatment. During the 'switch' periods, stomatal conductance was higher on average than at other stages of the irrigation cycles (Figure 3.3 A), and measures of φ_L and φ_S were less negative. Generally, there was no observed effect of the PRD treatment on stomatal conductance or plant water status during the switch period (Figure 3.3). Some small, significant changes in these measures were observed during the switch in some instances (Figure 3.3), but these were minor and most likely reflect differences in equilibration of the soil water profile between treatments, following irrigation.

The current data show that despite rigorous irrigation scheduling and monitoring of plant water status, the PRD irrigation technique may be difficult to implement under some field conditions. There are a number of potential reasons for this. A primary factor which determines the suitability of a location to PRD is soil type. Depending upon soil structure, and soil water-holding capacity certain sites may lend themselves better to a PRD-type irrigation strategy than others. A recent review of both published and unpublished data from PRD experiments in various localities and on different varieties reported that soils with a high clay content can limit the effectiveness of PRD (Kriedemann and Goodwin, 2003). On the 'dry' side of the PRD-treated vine, retention of soil water in soils of a high clay content can slow down the rate of soil drying and water uptake by the roots, effectively inhibiting the production of root signals which govern the PRD response. On the other hand, clay soils have also shown a slower rate of re-wetting after the drying period of a PRD cycle, effectively placing roots on the 'wet' side of the vine under water deficit immediately following the switch between PRD cycles (Kriedemann and Goodwin, 2003). By contrast, sandy soils such as in the current study have been shown to be more favourable to the implementation of PRD (Kriedemann and Goodwin, 2003). However, a potential problem in such soils is that the time interval between the switch-over of PRD cycles can be too long, such that soil water on the 'dry' side of the vine is depleted and roots no longer produce signals which mediate the PRD response (Dry *et al.* 2000b). At this point, the PRD-treated vines could enter an hydraulically-mediated water deficit relative to the control treatment, which is a possible explanation for the results seen in the current study.

The results of the Langhorne Creek Cabernet Sauvignon experiment are different to those observed for small-scale potted and field trials of the technique (Dry and Loveys, 1999; Stoll *et al*, 2000). It is clear that, under the conditions of the current study, a measure of water deficit was experienced by the vines, at least for part of the season. Depending on the timing of this deficit, in terms of fruit development, such a deficit could cause a reduction in berry size and the resulting yield (McCarthy, 2000).

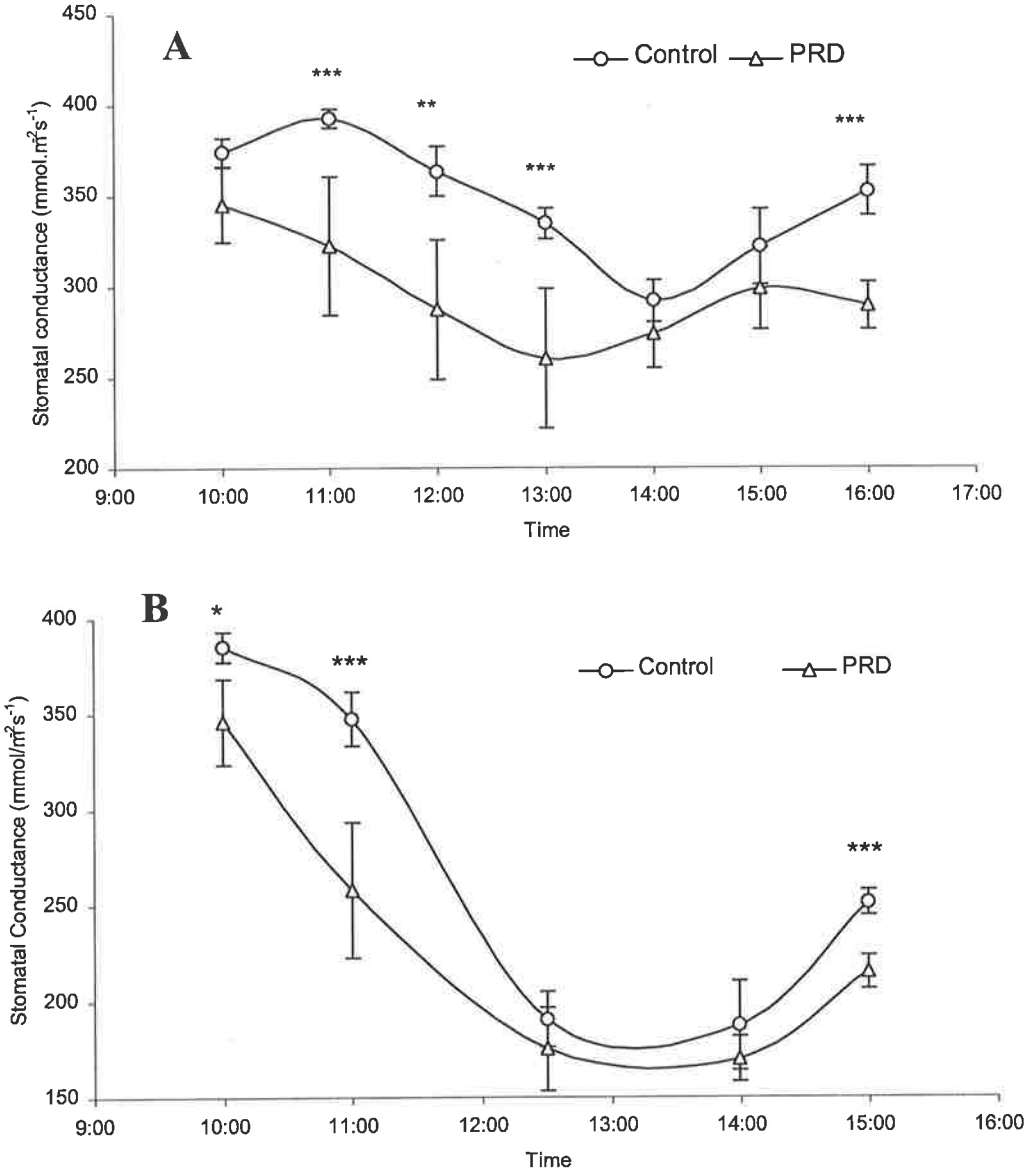


Figure 3.2: Effect of PRD on diurnal changes in stomatal conductance of Cabernet Sauvignon vines at two stages of the growing season **A.** 'Early' 31/01/2002 **B.** 'Advanced' 24/02/2002. (ANOVA; A: n=36; B: n=30; * = P<0.05; ** = P<0.01; *** = P<0.001)

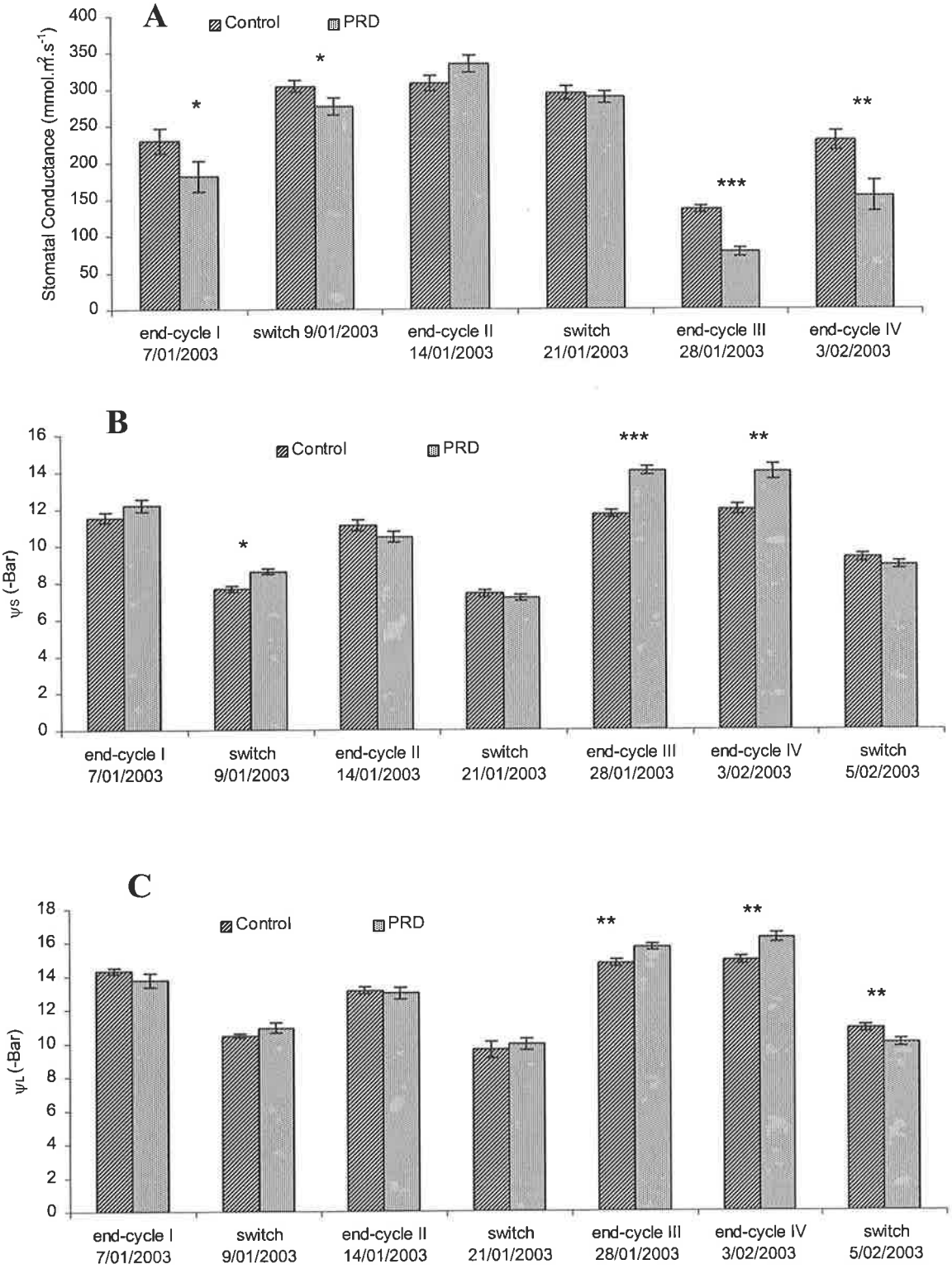


Figure 3.3: Effect of PRD irrigation on **A.** stomatal conductance, **B.** stem water potential (φ_S) and **C.** leaf water potential (φ_L) in Cabernet Sauvignon vines at Langhorne Creek, 2002/2003. (Students T-test; (**A**: n=42, **B,C**: n=28), * = $P<0.05$, ** = $P<0.01$, *** = $P<0.001$).

3.3.1.2 The Nuriootpa Shiraz experiment

This experiment was carried out over three consecutive seasons. In the first (2000/2001) and third (2002/2003) seasons, the PRD treatments received half the volume of irrigation water of the control, at approximately 0.5 ML/ha for the PRD and 1.0 ML/ha for the control. In the 2001/2002 season, PRD and control received equal irrigation, at 1 ML/ha. In that season, where both treatments received the same irrigation level, stomatal conductance was reduced by the PRD treatment at the end of the PRD cycle, despite no difference in irrigation amount (Figure 3.4 A). This is most likely to be a non-hydraulic response of stomata to root-derived signals, e.g. ABA from roots in drying soil, on the 'dry' side of the PRD-treated vines.

The stomatal conductance of the PRD-treated vines was restored to similar levels as the control during the morning after the irrigation 'switch' (Figure 3.4 B). At this stage, both the 'wet' and 'dry' sides of the PRD would have been at their highest soil water content. Unexpectedly, during the latter part of the day, stomatal conductance was higher in PRD-treated than standard irrigated vines. A similar result was found by Stoll (2000) for PRD- and control-treated Cabernet Sauvignon vines where both treatments received the same level of irrigation. In that study, the stomatal conductance of PRD vines exceeded readings for the control treatment on one day, toward the end of the experimental period, when the vines were entering their fourth 10-day PRD-cycle. The suggestion was made that this reflected a reduction in the depth of soil water penetration in the control treatment relative to PRD. In the current study, the PRD-treatment would have received the same irrigation amount as the controls, on one side of the root system only. A comparison of summed soil moisture data at this stage showed similar soil water content for both the 'wet' and 'dry' side of the PRD treatment, but one side of the control treatment was relatively drier than the other (results not shown), which could indicate inadequate refilling of the soil profile. This may have allowed the PRD vines to maintain higher stomatal conductance levels later in the day when the vines were under greatest environmental stress due to lowered relative humidity and higher temperature.

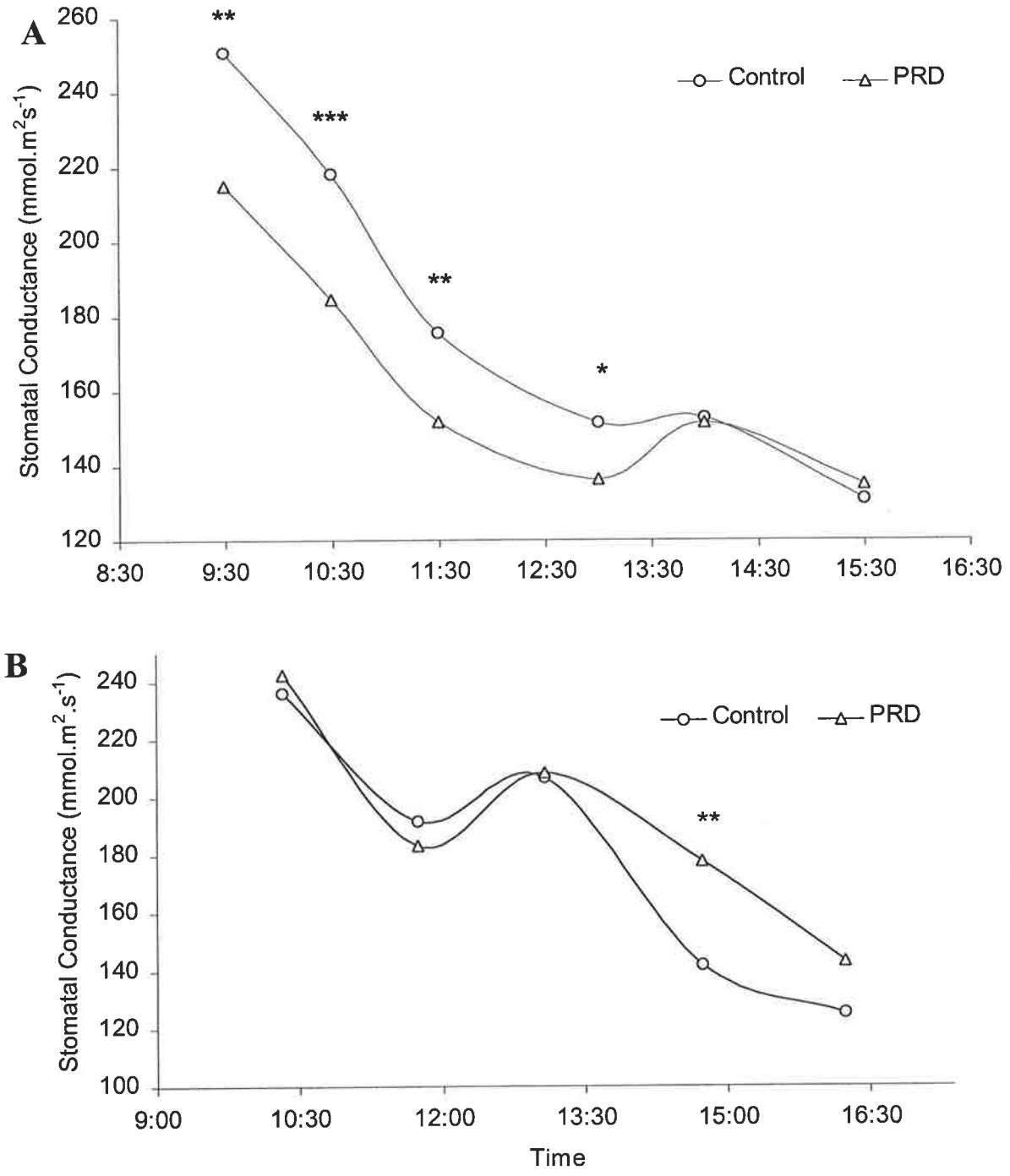


Figure 3.4: Diurnal stomatal conductance in PRD-irrigated and standard irrigated Shiraz vines where both treatments received the same irrigation level in 2001/2002. **A.** end of cycle and **B.** switch (ANOVA; n=60; *=P<0.05; **=P<0.01; ***=P<0.001).

For the 2000/2001 and 2002/2003 seasons, where the PRD treatment was irrigated to half the level of the control treatment, stomatal conductance was significantly reduced for both morning (9-11 am) and midday (12-1:30 pm) measures taken at the end of the PRD-cycle, where the soil on the 'dry' side of PRD-treated vines was at its lowest (Table 3.1 and 3.2). Mid-cycle in 2000/2001, shortly after the switching period between PRD cycles and during the switch in 2002/2003, morning stomatal conductance readings were similar for PRD- and control-treated vines (Table 3.1 and 3.2). However, a midday measurement of stomatal conductance taken during the switch period in 2003 showed a significant decrease in the PRD-treated vines relative to the control. Leaf and stem water potential were not determined at this stage of the cycle, but the measurement of soil water content (Figure 3.5) indicated that there was already a very rapid decrease of soil moisture on the 'dry' side of the PRD treatment at this point, which could have caused the reduction in stomatal conductance.

Table 3.1: Stomatal conductance in PRD- and standard-irrigated Shiraz vines pruned to different node numbers in 2000/2001 where the PRD treatment was irrigated to half the level of the control (ANOVA, n=30, *=P<0.05; **=P<0.001; ns = not significant; T = irrigation treatment, P = pruning; T x P = interactive effect).

		Morning ($mmol.m^{-1} s^{-2}$)			
Node number		30	60	120	Average
20/01/01 end-cycle	Control	169	151	132	151
	PRD	107	109	80	99
11/03/01 end-cycle	Control	129	128	121	126
	PRD	65	105	64	78
14/03/01 mid-cycle	Control	124	134	148	135
	PRD	150	133	158	147
15/03/01 mid-cycle	Control	98	98	99	98
	PRD	70	82	84	79
Probability		T	P	T x P	
20/01/01		**	ns	ns	
11/03/01		**	ns	ns	
14/03/01		ns	ns	ns	
15/03/01		*	ns	ns	

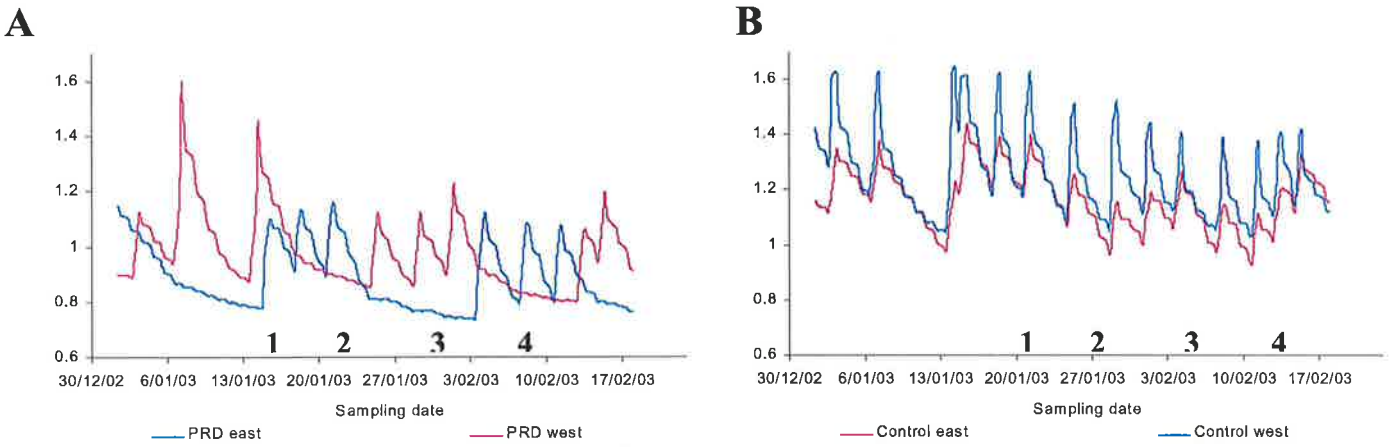


Figure 3.5: Soil water profile of east and west sides of **A**. PRD-irrigated and **B**. standard-irrigated vines, summed between 20 and 60 cm depth in 2002/2003. Soil water is expressed as the ratio of soil moisture to an average soil water value for one month prior to the onset of irrigation. Numbers 1-4 indicate points at which physiological data were collected.

Table 3.2: Stomatal conductance in PRD- and standard-irrigated Shiraz vines pruned to different node numbers in 2002/2003 where the PRD treatment was irrigated to half the level of the control (ANOVA, n=30, *=P<0.05; **=P<0.01 ns = not significant; nd = not determined; T = irrigation treatment, P = pruning; T x P = interactive effect).

		Morning ($mmol.m^{-1} s^{-2}$)				Mid-day ($mmol.m^{-1} s^{-2}$)			
Node number		30	60	120	Average	30	60	120	Average
16/01/03	Control	257	295	257	269	264	241	263	256
switch	PRD	233	261	261	251	198	188	199	196
22/01/03	Control	241	251	256	249	254	236	242	244
end-cycle	PRD	202	177	198	192	167	174	180	173
31/01/03	Control	nd	nd	nd	nd	189	228	210	209
end-cycle	PRD	nd	nd	nd	nd	154	146	133	144
6/02/03	Control	231	247	212	230	199	208	205	204
mid-cycle	PRD	195	191	178	188	137	118	149	135
Probability		T	P	T x P		T	P	T x P	
16/01/03		ns	ns	ns		**	ns	ns	
22/01/03		**	ns	ns		**	ns	ns	
31/01/03		nd	nd	nd		**	ns	ns	
6/02/03		*	ns	ns		**	ns	ns	

Table 3.3: Midday stem (φ_S) and leaf (φ_L) water potential in PRD- and standard-irrigated Shiraz vines pruned to different node numbers in 2002/2003 where the PRD treatment was irrigated to half the level of the control (ANOVA, n=30, **=P<0.01; T = irrigation treatment, P = pruning; T x P = interactive effect).

		Stem ψ (-Bar)				Leaf ψ (-Bar)			
Node number		30	60	120	Average	30	60	120	Average
22/01/03	Control	7.4	7.5	7.9	7.6	10.4	9.9	9.6	10.0
end-cycle	PRD	10.8	10.5	11.3	10.9	12.6	12.0	13.2	12.6
31/01/03	Control	8.6	9.0	9.1	8.9	10.2	10.8	10.9	10.6
end-cycle	PRD	12.9	12.6	12.8	12.8	13.8	12.3	14.7	13.6
6/02/03	Control	10.8	11.1	11.3	11.1	12.4	12.6	12.7	12.6
mid-cycle	PRD	13.6	12.3	13.4	13.1	15.2	14.1	15.7	15.0
Probability		T	P	T x P		T	P	T x P	
22/01/03		**	ns	ns		**	ns	ns	
31/01/03		**	ns	ns		**	ns	ns	
6/02/03		**	ns	ns		**	ns	ns	

In the 2002/2003 season, measures of plant water status were determined in order to further explain some of the changes in stomatal conductance observed in previous seasons. It was found that where PRD received half the irrigation water of the control, that this resulted in a significant decrease in both φ_S and φ_L at the mid-cycle and end-cycle stages of the PRD treatment (Table 3.3). At all stages, φ_S was less negative than φ_L as it is representative of whole-vine, root and soil water equilibrium (Table 3.3). The effect of the irrigation treatment on both φ_S and φ_L was independent of node number per vine, and is thus primarily a reflection of changes in soil water status. As with the PRD experiment on Cabernet Sauvignon, these data show that under the conditions of the current study, PRD-treated vines experienced an actual water deficit relative to the controls. The changes in stomatal conductance with PRD do not therefore reflect solely a response to partial drying of the root system, but a whole-vine water deficit. Irrigation scheduling was carried out using the soil water measurements shown in Figure 3.5, such that the PRD cycle was switched at the point at which the soil water profile on the dry side had started to level out, indicating that there were no further roots in the stage of

drying out. However, it is possible that under the conditions at the Nuriootpa site that the length of the period between cycles was too long. From the soil moisture data in Figure 3.5, the rapid decrease in soil water immediately following irrigation could be as short as 5 days, followed by an extended drying period of up to 10 days, during which the rate of soil moisture decrease was very slow. During this time, the minimum levels of relative soil water content reached in the PRD treatment exceeded those reached by the control treatment (Figure 3.5). It is likely that at this stage, the PRD-treated vines experienced water deficit relative to the control treatment. In certain regions of Australia where PRD is currently being trialled, the cycle time has been markedly reduced to as little as four days due to soil and climatic conditions (M. McCarthy, pers. comm.). In the current study, the cycle time was between ten and fifteen days, and may need to be reduced to prevent the vines experiencing water deficit under PRD irrigation in this region.

3.3.2 Effect of PRD and node number on canopy growth and yield components

3.3.2.1 The Langhorne Creek Cabernet Sauvignon experiment

In the Cabernet Sauvignon experiment at Langhorne Creek, the effect of the PRD treatment on growth rate of main shoots was determined over one growing season (2001/2002). The results were analysed as the cumulative increase in main shoot length over time. A significant difference in shoot growth rate was found, such that the PRD treatment decreased the shoot growth rate in that season. This is the expected result for PRD on shoot growth rate (Dry and Loveys, 1998,1999; Dry *et al.* 2000a) although this is also known to be a response of grapevines to water stress (Smart and Coombe, 1983). The reduction in shoot growth with PRD, although showing a significant response over the growing season, was small. Thus, the resultant average shoot length determined at harvest was only slightly reduced in the 2001/2002 season, and was not significant (Table 3.4). Similarly, pruning weight was only slightly reduced by PRD, and was significant only at the 10% confidence level (Table 3.4). In the following season, in 2002/2003, shoot growth rate was not determined. However, the PRD treatment had a greater effect on canopy development in that season as estimated by harvest shoot length, winter pruning weight and weight per shoot, all of which were significantly reduced (Table 3.4).

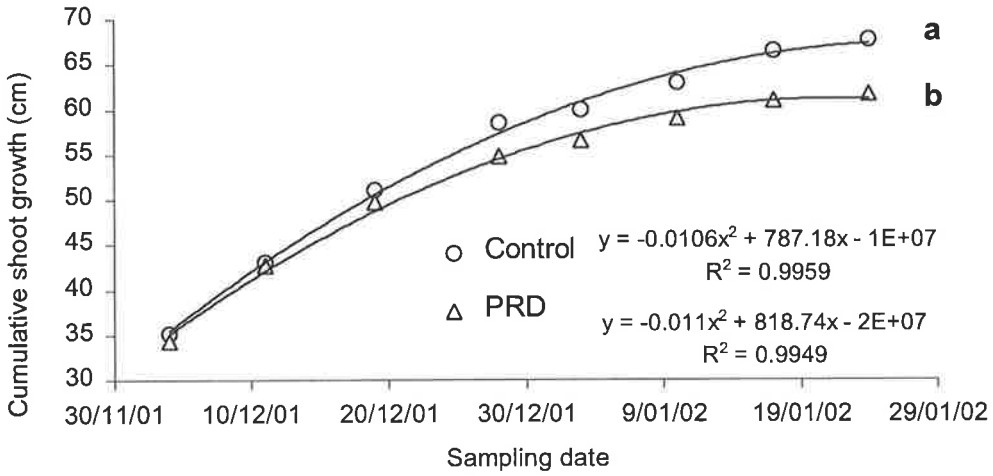


Figure 3.6: Effect of PRD on cumulative shoot growth in Cabernet Sauvignon grown at Langhorne Creek in 2001/2002, where PRD received 60% of the irrigation water of the control. (**a,b** = indicates significant difference P<0.01, ANOVA, n = 126 shoots over time x = 8 sampling dates)

Table 3.4: Effect of PRD on canopy growth and yield components for Cabernet Sauvignon grown at Langhorne Creek for the 2001/2002 and 2002/2003 vintages (ANOVA, n=42, ns = not significant).

	2001/2002 Vintage				2002/2003 Vintage			
	Control	PRD	% PRD> Control	P	Control	PRD	% PRD> Control	P
Berry no. per bunch	93.7	95.7	2	ns	81.15	80.97	-0.22	ns
Berry wt (g)	0.94	0.84	-12	<0.001	0.87	0.79	-10.39	<0.05
Bunch no. per vine	57.6	66.9	14	<0.01	58.20	53.60	-8.58	ns
Bunch wt (g)	92.9	86.0	-8	ns	74.99	66.76	-12.32	<0.01
Yield (kg/vine)	5.24	4.83	-8.49	ns	3.78	3.04	-24.34	<0.001
Yield (tonnes/ha)	12.04	11.19	-8.49	ns	8.74	7.03	-24.34	<0.001
WUE (tonnes/ML)	10.07	13.16	30.70	<0.001	7.34	8.40	14.44	<0.05
PW (kg/vine)	1.34	1.17	-14.53	<0.1	1.18	0.86	-37.21	<0.01
Yield/PW ratio	4.14	4.19	1.19	ns	3.35	4.19	20.05	ns
Shoot no. per vine	41.4	39.9	-3.76	ns	35.86	34.45	-4.09	ns
Shoot wt. (g/shoot)	33.10	29.30	-12.97	ns	32.39	24.35	-33.02	<0.001
Shoot length (cm)	104.8	97.8	-7.16	0.1	88.6	66.8	-32.63	<0.001

Differences in yield were observed in response to PRD over the two growing seasons of the study. In 2001/2002, yield was slightly but not significantly reduced by PRD, and was reduced significantly (-24%) by PRD in 2002/2003 (Table 3.4). In both seasons, berry weight determined at similar °Brix was significantly decreased at harvest, but in 2001/2002 this did not have a significant effect on yield due to a slightly higher bunch number per vine in the PRD treatment (Table 3.4). This increase in bunch number per vine, although significant, is more likely to be a random effect than a treatment effect as there was no PRD or other deficit irrigation carried out in the previous season, so that there was little chance that a deficit-induced reduction in inflorescence primordia initiation had occurred (Buttrose, 1974; McCarthy, 1996). In 2003, there was no change in bunch number with the PRD treatment, and the reduction in berry weight was the primary factor leading to decreases in bunch weight and yield in response to PRD. The berry weight decrease is most likely due to the water deficit which the vines experienced later in the growing season, coinciding approximately with one week prior to veraison.

Water deficit in the earlier stages of fruit development can lead to a reduction in berry size (Ojeda *et al.*, 2001). Previously, PRD has been shown to cause no change in berry size or yield as a result of partial drying of the root system in field-grown vines of a number of grape varieties (De la Hera Orts *et al.* 2002; Dry, 1997; Stoll, 2000) and in many commercial trials (Dry *et al.*, 2000c). Presumably, this is only possible under conditions where PRD causes an alteration in stress signalling to apical parts of the plant leading to decreased stomatal conductance and shoot growth rate, without causing an alteration in leaf or stem water potential. However, in some cases there have been reports of PRD causing a small reduction in berry weight and yield, within the range reported in the current study (Dry et al. 2000c; dos Santos *et al.* 2003; Kriedemann and Goodwin, 2003). In these cases, presumably the response of berry growth to water deficit is due an actual limitation in water availability, i.e. an hydraulic response, rather than due to non-hydraulic plant signals reaching the fruit, e.g. increased ABA or reduced cytokinin, as would be expected with PRD (Stoll *et al.*, 2000).

In 2002/2003 there was a large difference in the yield to pruning weight ratio (Yield/PW) caused by scatter within the data set, but this was not statistically significant, such that in both seasons there was no significant change in Yield/PW with PRD (Table 3.4). This is likely to be due to the fact that changes in PW by the PRD treatment were accompanied by corresponding changes in yield. In 2001/2002 and 2002/2003, the PRD treatment had approximately 60% of the irrigation volume of the control treatment, such that in both seasons PRD resulted in an increase in water use efficiency (WUE) in terms of tonnes of fruit produced per ML of irrigation water applied (Table 3.4). In 2002/2003, however, the significant reduction in yield with PRD reduced WUE to half that seen in the previous season.

3.3.2.2 The Nuriootpa Shiraz experiment

The combined effect of altered node number per vine and PRD on vine growth and yield components was determined for Shiraz vines grown at Nuriootpa. Shoot growth was compared between PRD and control treatments within the different pruning levels of the study for the 2000/2001 vintage, where PRD was irrigated to half the level of the control treatment; and in 2001/2002, where PRD and control treatments received the same irrigation volume. The incremental rate of shoot length increase was determined over consecutive weeks, and the response was similar for vines pruned to 30 and 60 nodes (results not shown). For these two pruning treatments, shoot growth rate was not affected by the PRD treatment in either of the growing seasons (Figure 3.7 A, C). Rather, independent of the amount of irrigation water applied, PRD caused a significant reduction in cumulative shoot growth over time in the 120 node vines (Figure 3.7 B, D).

Despite these negligible or small differences in shoot growth rate, average shoot length at harvest was decreased by PRD in 2000/2001, independent of node number per vine (Table 3.5). In 2001/2002 there was no significant effect of PRD on average shoot length, although there was a significant response for node number per vine, such that the 120 node vines had shorter shoots than the other pruning levels (Table 3.7). This would be expected, as shoot vigour is inversely proportional to shoot number per vine, which is determined by node number per vine at pruning (Clingeleffer and Sommer, 1995).

Although there was not a significant interactive effect for shoot length and node number for either season, average shoot length for 2000/2001 and 2002/2003 was decreased to a greater extent by PRD in the 120 node vines than the other pruning treatments. Shoot length was reduced 22% and 16% by PRD in the 120 node vines in 2000/2001 and 2001/2002 respectively, compared to 13% and 5% in the 30 node vines.

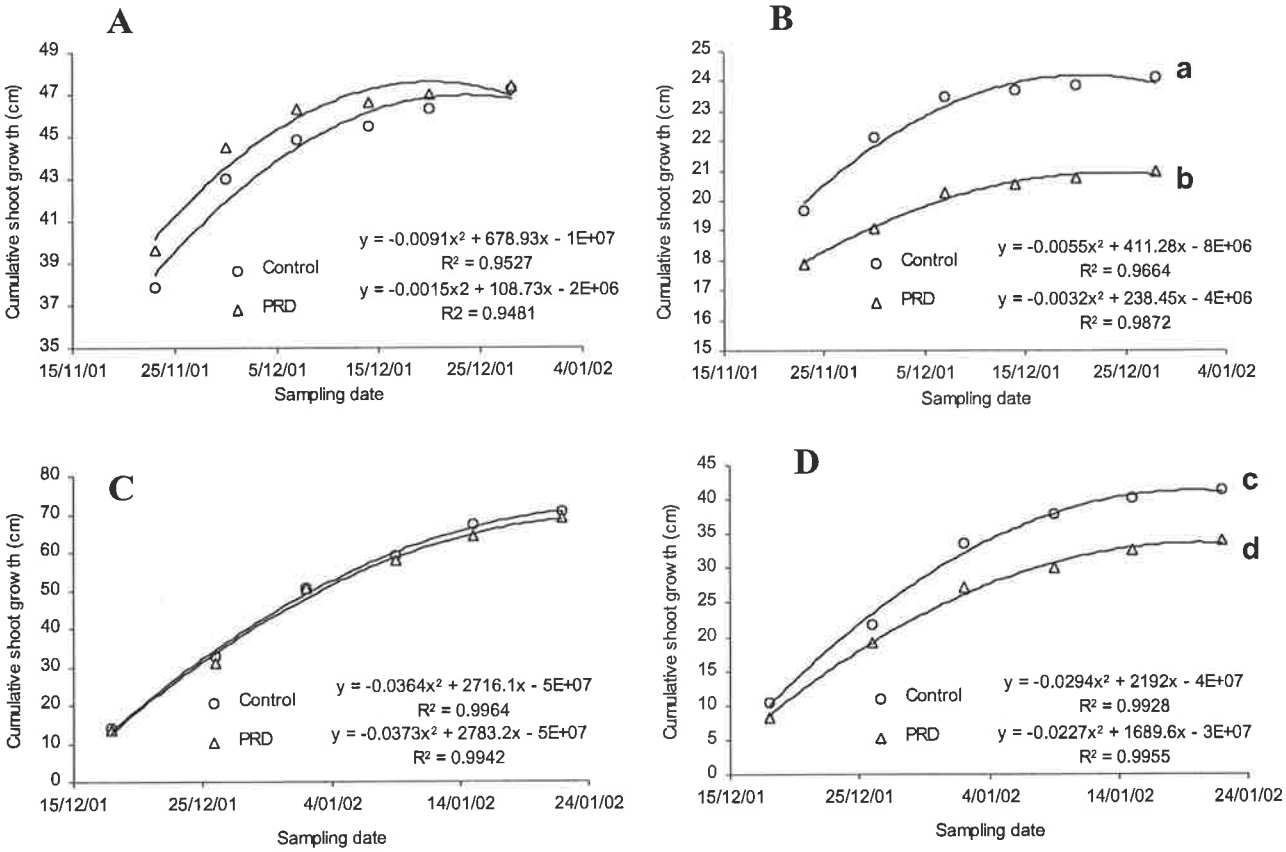


Figure 3.7: Effect of PRD on cumulative shoot growth in Shiraz grown at Nuriootpa where PRD received 50% of the irrigation of the control treatment in 2000/2001 **A.** 30 node 2000/2001; **B.** 120 node 2000/2001; or 100% of the irrigation of the control treatment in 2001/2002 **C.** 30 node 2002; **D.** 120 node 2001/2002 (ANOVA n × x; n = 30 shoots over time x = 6 sampling dates; **a,b** = indicates significant difference $P<0.05$; **c,d** = $P<0.01$).

This may indicate that for those two vintages of the study, that the 120 node vines were more sensitive to the PRD treatment than the other two pruning levels. This may be due to a limitation in available photosynthate for partitioning between vegetative and reproductive growth, where bunch number was highest in the 120 node vines (Tables 3.5 and 3.7). This may have come under further limitation where stomatal conductance, and

presumably photosythesis, was restricted by PRD in those seasons (Table 3.1, Figure 3.4A). Shoot growth rate was not determined in 2002/2003, where PRD was irrigated to half the level of the control treatment, as in 2000/2001. In the 2002/2003 vintage, average shoot length was significantly affected by both node number per vine and PRD (Table 3.6). In that season, PRD caused a decrease in shoot length for all the pruning treatments, with increased node number per vine causing an additional reduction in shoot length. The degree to which PRD decreased average shoot length was similar for all the pruning treatments, which differs from the results of the previous two seasons of the study.

Table 3.5: Vine growth and yield components for Shiraz vines pruned to different node numbers in the 2000/2001 season where PRD received half the irrigation water of the control treatment (ANOVA; n=30; ns = not significant; T = irrigation treatment, P = pruning; T x P = interactive effect).

Yield component		30 node	60 node	120 node	P (T)	P (P)	P (TxP)
Berry number/ bunch	Control	92.6	77.5	68.7	ns	<0.001	ns
	PRD	89.6	82.4	71.8			
Berry Weight (g)	Control	0.83	0.80	0.67	ns	<0.001	ns
	PRD	0.83	0.77	0.56			
Bunch number	Control	65.9	124.5	208.1	ns	<0.001	ns
	PRD	67.7	111.8	188.5			
Bunch weight (g)	Control	82.3	67.5	50.2	ns	<0.001	ns
	PRD	79.7	67.4	44.6			
Yield (kg/vine)	Control	6.26	8.84	11.06	ns	<0.001	ns
	PRD	6.19	8.17	8.89			
Yield (tonnes/Ha)	Control	7.94	11.22	14.04	ns	<0.001	ns
	PRD	7.86	10.37	11.28			
WUE (tonnes/ML)	Control	7.94	11.22	14.04	<0.001	<0.001	ns
	PRD	15.72	20.74	22.56			
PW (kg)	Control	2.42	2.41	2.02	ns	0.001	ns
	PRD	2.40	2.49	1.6			
Yield/PW ratio	Control	2.59	3.64	5.55	ns	<0.001	ns
	PRD	2.60	3.29	5.58			
Shoot no. per vine	Control	50.0	66.4	82.8	ns	<0.001	ns
	PRD	52.2	68.2	84.2			
Weight per shoot (g)	Control	48.4	36.2	24.0	ns	<0.001	ns
	PRD	45.6	36.4	19.0			
Shoot length (cm)	Control	148.5	143.4	137.8	<0.05	ns	ns
	PRD	131.5	131.0	112.7			

Table 3.6: Vine growth and yield components for Shiraz vines pruned to different node numbers in the 2002/2003 season where PRD received half the irrigation water of the control treatment (ANOVA; n=30; ns = not significant; T = irrigation treatment, P = pruning; T x P = interactive effect).

Yield component		30 node	60 node	120 node	P (T)	P (P)	P (TxP)
Berry number/bunch	Control	149.9	128.6	103.8	<0.05	<0.05	<0.05
	PRD	113.4	118.6	113.2			
Berry Weight (g)	Control	0.89	0.77	0.71	ns	<0.01	ns
	PRD	0.78	0.82	0.60			
Bunch number	Control	71.0	117.5	192.0	ns	<0.001	ns
	PRD	73.6	113.2	160.1			
Bunch weight (g)	Control	142.9	105.2	76.6	<0.01	<0.001	<0.01
	PRD	92.9	103.7	72.4			
Yield (kg/vine)	Control	10.14	12.39	14.48	<0.05	0.001	ns
	PRD	6.8	11.75	11.57			
Yield (tonnes/ha)	Control	12.87	15.72	18.38	<0.05	0.001	ns
	PRD	8.63	14.91	14.68			
WUE (tonnes/ML)	Control	12.87	15.72	18.38	<0.001	<0.001	ns
	PRD	17.26	29.82	29.36			
PW (kg)	Control	2.80	2.10	1.77	ns	0.001	ns
	PRD	2.36	2.06	1.31			
Yield/PW ratio	Control	3.83	5.93	8.73	ns	<0.001	ns
	PRD	2.95	5.88	8.97			
Shoot no. per vine	Control	45.3	62.3	93.6	ns	<0.001	ns
	PRD	45.2	65.1	89.3			
Weight per shoot (g)	Control	61.6	33.6	19.0	ns	<0.001	ns
	PRD	52.7	31.6	14.7			
Shoot length (cm)	Control	164.4	142.1	105.3	<0.001	<0.001	ns
	PRD	129.3	111.5	91.5			

The effect of PRD and node number on other vine growth parameters and yield components was compared between the 2000/2001 and 2002/2003 seasons. In both vintages, shoot number per vine, yield and bunch number were significantly increased as node number increased (Tables 3.5, 3.6). Pruning weight, shoot weight, bunch weight and berry weight were significantly decreased as node number per vine increased. In 2000/2001, berry number per bunch was decreased as node number per vine increased (Table 3.5), but in 2002/2003 there was no clear effect of node number per vine on berry number per bunch (Table 3.6). With higher node number per vine, the yield/PW ratio increased, such that larger yields were supported by reduced vegetative growth. In 2000/2001, yield was not significantly affected by PRD, but in 2002/2003 the irrigation

treatment brought about a yield reduction. Closer examination of the yield components showed that in that season, berry number per bunch was significantly reduced by PRD in the 30 node per vine treatment (Table 3.6). This resulted in a PRD-induced reduction in both bunch number and yield in that pruning level category which was greater than that for the other pruning treatments. Furthermore, the reduction in berry number per bunch was greater than the small reduction in berry size due to PRD, which would be expected to be the yield component most sensitive to an immediate water deficit. Water stress at flowering has been shown to cause a yield reduction, due to a reduction in berry set and a resultant cluster thinning effect (Alexander, 1965; Hardie and Considine, 1976). As the period of irrigation in the current study was started post-set, it is not likely that this was a result of the PRD treatment in the 2002/2003 season. It is also possible that water stress was experienced by the 120 node PRD vines in the previous growing season, which could have caused a decrease in inflorescence number and size in the year which followed (Buttrose, 1974). Again, this is unlikely as both the PRD and control treatments received equivalent irrigation in the 2001/2002 season.

Although there was no significant interactive effect of pruning level and PRD on berry weight or pruning weight in 2000/2001 or 2002/2003, the results for these parameters are similar to those for shoot length (Tables 3.5, 3.6). In 2000/2001, berry weight was reduced 4% by PRD in the 60 node vines compared with 20% in the 120 node vines (Table 3.5). Similarly in 2002/2003, berry weight was increased 6% by PRD in the 60 node vines and reduced 18% in the 120 node vines (Table 3.6). Pruning weight was changed only slightly by PRD in the 60 node vines in 2000/2001 or 2002/2003, but was reduced 35% and 25% respectively in the 120 node vines. Ignoring the effect of reduced berry number per bunch in the 30 node vines in 2002/2003, PRD also caused the greatest yield reduction due to reduced berry weight in the 120 node vines in both seasons where irrigation was halved for PRD. WUE in terms of tonnes of fruit produced per ML of irrigation water applied was improved with PRD in both 2000/2001 and 2002/2003 (Tables 3.5, 3.6), but this improvement was limited where PRD reduced yield for a given season or pruning level.

From the shoot growth, pruning weight, yield and berry weight data, there appears to be an interactive effect such that higher node number results in a higher yield/PW ratio, which increased the sensitivity of these vines to water deficit under PRD. From the stomatal conductance (Tables 3.1, 3.2) and vine water status (Table 3.3) measurements taken in those seasons, there was no apparent effect of pruning level on either component. Thus, it is not likely that net assimilation was lowered in the 120 node vines relative to the other pruning treatments. Rather, this effect is more likely to be due to a limitation in available carbon from photosynthesis in response to PRD, which was required for greater distribution between vegetative and reproductive sinks in the 120 node vines.

The 2001/2002 season, where the PRD and control treatments received the same level of irrigation, was characterised by high rainfall and cool temperatures (Appendix E) which led to excessive vigour relative to that seen in the preceding or following seasons. Increases in pruning weight in 2001/2002 were between 30 and 200% of the weights seen in 2001 or 2003 (Tables 3.5, 3.6 and 3.7). The relative increase in pruning weight in 2001/2002 was greatest in the 120 node vines, which removed the restriction in the yield/PW ratio which was observed where water was limiting in the other two seasons of the study. Thus, there was no significant effect on berry weight due to pruning level, despite significantly higher bunch numbers per vine as node number increased (Table 3.7). Consequently, yield was significantly increased as node number increased due to increasing bunch number alone, without the restriction in berry weight at higher node numbers observed in other seasons. There was no significant effect of PRD on any vine growth or yield component observed in 2001/2002, although berry weight was slightly increased under PRD in the 30 and 60 node treatments (Table 3.7). However, this did not result in an alteration in final yield. Thus, there was no improvement in WUE due to the PRD irrigation in 2002, as both control and PRD treatments received the same irrigation amount. However, due to the seasonal conditions of the 2001/2002 vintage, these data cannot be used to hypothesise as to the effect of this irrigation strategy in a season where vines are placed under environmentally stressful conditions.

Table 3.7 Vine growth and yield components for Shiraz vines pruned to different node numbers in the 2001/2002 season where PRD received the same amount of irrigation water as the control treatment (ANOVA; n=30; ns = not significant; T = irrigation treatment, P = pruning; T x P = interactive effect).

Yield component		30 node	60 node	120 node	P (T)	P (P)	P (TxP)
Berry number per bunch	Control	125.9	123.0	105.2	ns	ns	ns
	PRD	109.8	99.7	123.1			
Berry Weight (g)	Control	1.09	1.04	1.07	ns	ns	ns
	PRD	1.21	1.12	1.00			
Bunch number	Control	58.1	80.6	133.2	ns	<0.001	ns
	PRD	56.8	81.8	124.0			
Bunch weight (g)	Control	136.6	128.1	105.6	ns	ns	ns
	PRD	131.4	111.8	121.1			
Yield (kg)	Control	8.07	10.27	13.80	ns	<0.01	ns
	PRD	7.53	9.34	15.37			
PW (kg)	Control	3.68	3.98	4.10	ns	ns	ns
	PRD	3.64	3.66	4.06			
Yield/PW ratio	Control	2.59	2.66	3.57	ns	<0.05	ns
	PRD	2.10	2.58	3.85			
Shoot length (cm)	Control	162.1	152.3	129.6	ns	<0.001	ns
	PRD	154.0	153.5	112.1			

A comparison of the data from all three seasons of the study allow for some new considerations in the current understanding of 'vine balance.' The concept of 'vine balance' was first described by Partridge (1926), and proposed a relationship between photosynthetic carbon availability and its distribution among storage organs and sinks within the vine. Theoretically, there is an ideal balance between carbon distribution between vegetative/reproductive/root sinks, such that if allocation to one component occurs at the expense of the others, the 'balance' of that vine will be disturbed. At higher node numbers per vine, the potential exists that there is a higher demand for allocation to the reproductive sink early in the growing season, which will cause a restriction in allocation to vegetative growth. This could be seen in the 120 node vines in the 2000/2001 and 2002/2003 vintages. A higher yield/PW ratio could result in an inability of that vine to mature a large number of bunches, due to a relatively reduced photosynthetic capacity of a smaller canopy, such that final yield, fruit weight and degree of ripeness would be restricted under these conditions. This concept of 'vine balance' may account for the greater sensitivity of the 120 node vines to water deficit under PRD,

as was seen in the results for vine vegetative growth and yield components in the 2000/2001 and 2002/2003 vintages, and in shoot growth rate in the 2001/2002 season. These results reflect the response observed by Freeman *et al.* (1979) in the study on non-irrigated and irrigated Shiraz vines pruned to four different node numbers (20 – 160 nodes). In that study, the 160 node vines showed greater sensitivity to water deficit than the other pruning levels. This was shown in significantly decreased yield, pruning weight and shoot weight. Furthermore, Freeman *et al.* (1979) showed that at lower pruning levels (20 and 40 nodes) there was no change in yield despite small decreases in pruning weight and shoot weight in response to the non-irrigated treatment. This may also account for the lack of differences in yield observed by Bravdo *et al.* (1985) for fully-irrigated and deficit-irrigated treatments on three different crop loads produced by bunch thinning. In that study, the bunch number (20, 40 and 60-70 bunches) may not have been high enough to bring about a limitation in carbon available for partitioning to reproductive growth. Although the results are not statistically significant, the current study has shown that the lower pruning levels (30 and 60 nodes) had a greater capacity to 'buffer' water deficit responses of vine physiology to the PRD treatment than did the 120 node treatment. Clearly, there is a bunch number or yield threshold over which PRD will cause a restriction in canopy development, resulting in a limitation in photosynthetic carbon availability, and a reduction in yield.

3.3.3 The effect of PRD on light penetration within the vine canopy

In most irrigated vineyards, the situation arises where there is an excess of carbon allocation to vegetative rather than reproductive growth, such that dense, shaded canopies occur with a concomitant reduction in fruit quality (Smart and Coombe, 1983; Dry and Loveys, 1998). The implementation of deficit irrigation strategies such as RDI and PRD has allowed for a reduced vegetative vigour, with increased light penetration to the fruiting zone of irrigated vineyards (Dry, 1997; Stoll, 2000; Kriedemann and Goodwin, 2003). For the current study, only winter pruning weight was used as a measure of canopy size. For the 2000/2001 and 2001/2002 seasons, a non-destructive measure of leaf area per vine was made (results not shown), but this did not provide accurate results. This was primarily due to a large variation in shoot size within the treatments, particularly in

the Nuriootpa Shiraz site, and an inadequate sample size incorporating this range of shoot sizes within each treatment. However, reduced pruning weight has been strongly correlated with reduced leaf area per vine at harvest with PRD (Dry, 1997). Furthermore, low density canopies in terms of leaf area have been associated with lowered pruning weight, and higher light penetration within the canopy as estimated by photosynthetic photon flux density (PPFD) (Dokoozlian and Kliewer, 1995). Thus, for the purposes of the current study, winter pruning weight will be used as a measure of vine canopy size, and used as a reference point for the discussion of light penetration within the canopy. As the results for pruning weight in the Cabernet Sauvignon and Shiraz experiments have been discussed in 3.3.2.1 and 3.3.2.2, they will not be reiterated.

In the two seasons of the Cabernet Sauvignon experiment at Langhorne Creek, bunch exposure measured at different angles within the fruiting zone was increased within the canopies of PRD-treated relative to control-treated vines (Figure 3.8). The effect of PRD to increase solar radiation within the vine canopy was greatest at veraison, and less by harvest (Figure 3.8). The reduction in this effect of PRD by harvest was primarily due to the onset of leaf abscission by this stage, leading to more open canopies in both PRD- and control-treated vines (results not shown).

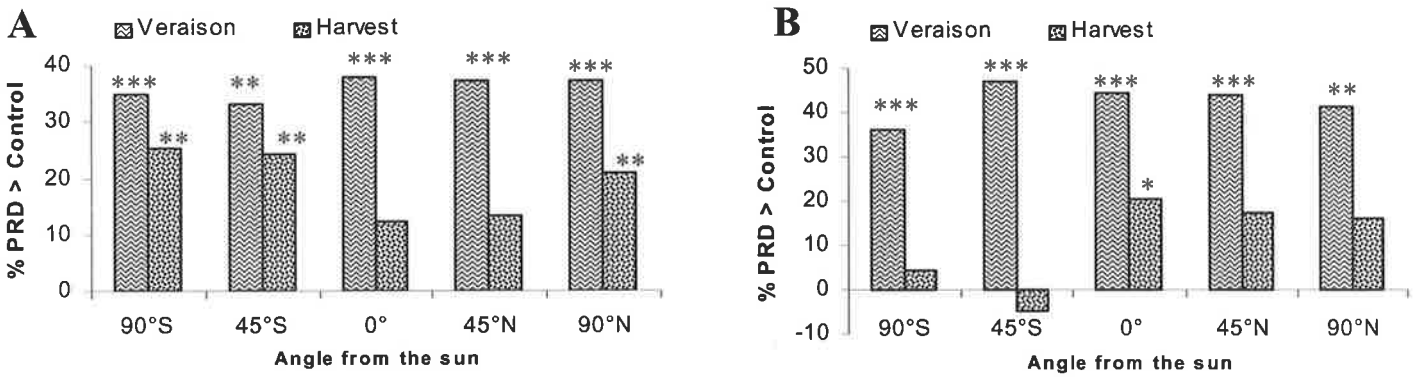
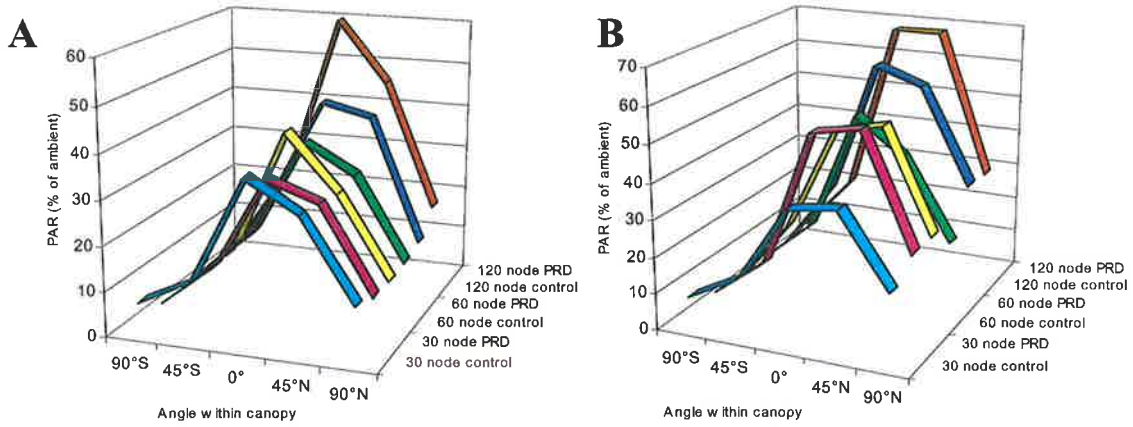


Figure 3.8: The effect of PRD on PAR (% of ambient $\mu mol.m^{-2}.s^{-1}$) measured at different angles within the fruiting zone at different stages of the season in Cabernet Sauvignon vines grown at Langhorne Creek in **A.** 2001/2002 and **B.** 2002/2003. (ANOVA, n=42, * = P<0.05, ** = P<0.01, *** = P<0.001; *=veraison; *=harvest).

It must be noted that similar results were obtained for both seasons of the study. However, canopy size as estimated by winter pruning weight was slightly, but not significantly reduced by PRD in 2001/2002 although it was significantly reduced in 2002/2003 (Table 3.4). Dry (1997) and Stoll (2000) reported large increases in bunch exposure with PRD, which were associated with a relatively smaller decrease in pruning weight or total leaf area per vine. However, it must be noted that in both of those studies, it was found that lateral shoot growth and lateral leaf area responded more strongly to the PRD treatment, and were reduced to a greater extent than the other components of canopy size. As lateral shoots form only a small component of total pruning weight, it is conceivable that a reduction in their growth in response to PRD will only cause a small change in pruning weight, but could potentially cause a large change in light penetration within the canopy (P. Dry, pers. comm.).

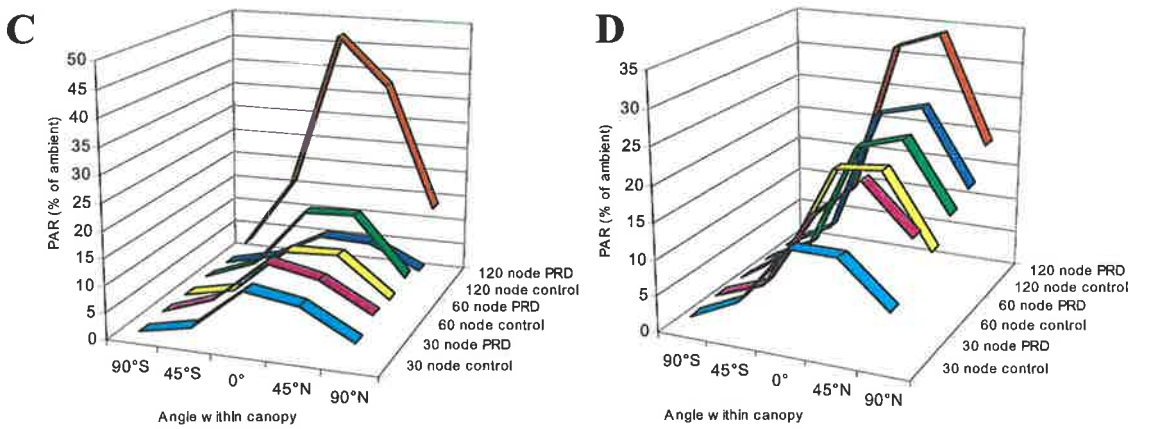
In the Shiraz experiment at Nuriootpa, the effect of differing node number per vine in conjunction with the effect of PRD irrigation on bunch exposure was investigated in two seasons where PRD vines were irrigated to half the level of the control treatment: 2000/2001 and 2002/2003. The angles 0° and 45°N were the points of greatest light penetration within the canopy (Figure 3.9). Pruning level had the most significant effect on bunch exposure at these two angles, and was greatest in the 120 node vines at both veraison and harvest in both seasons of the study (Figure 3.9), with the exception of the 120 node control treatment at veraison in 2002/2003 (Figure 3.9C). Unlike the Cabernet Sauvignon experiment at Langhorne Creek, Shiraz fruit exposure at harvest was not increased relative to that at veraison, as leaf abscission occurred much later, only three to four weeks after harvest. The effect of pruning level on the veraison and harvest measures of fruit exposure closely reflected the changes seen in pruning level for both seasons (Tables 3.5, 3.6). There was no significant effect of PRD across all pruning levels for both seasons of the study, except at certain angles within the canopy (Figure 3.9 A, D). However, these angles do not represent the primary source of light penetration within the fruiting zone. Rather, an irrigation (PRD and control) by pruning (node number) (T x P) effect on the veraison measure of bunch exposure was found at 0° in

both 2000/2001 and 2002/2003 (Figure 3.9 A, C). The strong PRD effect on bunch exposure detected at veraison in 2002/2003 (Figure 3.9 C) was due to this interactive (T x P) effect, caused by a very high level of light penetration within the canopies of the 120 node PRD treatment in both seasons, relative to all the other treatment categories (Figure 3.9 C). At harvest, the (T x P) effect was no longer statistically significant, but the average light penetration in the 120 node PRD vines was still clearly greater than that of the other treatments.



ANOVA	90°S	45°S	0°	45°N	90°N
T	*	ns	ns	ns	ns
P	ns	ns	*	*	*
T x P	ns	ns	*	ns	*

ANOVA	90°S	45°S	0°	45°N	90°N
T	ns	ns	ns	ns	ns
P	ns	ns	*	*	ns
T x P	ns	ns	ns	ns	ns



ANOVA	90°S	45°S	0°	45°N	90°N
T	***	***	***	**	**
P	***	***	**	*	ns
T x P	**	***	***	*	*

ANOVA	90°S	45°S	0°	45°N	90°N
T	*	ns	ns	ns	*
P	*	ns	*	*	ns
T x P	ns	ns	ns	ns	ns

Figure 3.9: The effect of PRD and node number on bunch exposure within the canopy of Shiraz vines grown at Nuriootpa **A**. Veraison 2000/2001; **B**. Harvest 2000/2001; **C**. Veraison 2002/2003; **D**. Harvest 2002/2003 (ANOVA; n=30; T = irrigation treatment; P = pruning; TxP = interaction; * = P<0.05; ** = P<0.01; ***=P<0.001).

From the results for pruning weights obtained in 2000/2001 and 2002/2003, there was no statistically strong interactive effect between PRD and node number (Tables 3.5, 3.6). However, as discussed previously, the 120 node PRD treatment showed consistently lower pruning weights than the other treatments in both seasons. This small, non-significant change in canopy size in the 120 node PRD treatment is the most likely cause of the large changes in light exposure of the fruiting zone. It appears that factors such as shoot growth and pruning weight were most sensitive to the PRD treatment in this pruning level category, which resulted in corresponding changes in bunch exposure. Again, it must be noted that the greatest increase in light penetration to the vine canopy in response to PRD is likely to be due to decreased lateral leaf area and shoot growth (Dry, 1997; Stoll, 2000). Although the increase in bunch exposure in the 120 node PRD treatment is likely to be due to reduced lateral leaf area, it was not determined in the current study.

Shiraz is described as a 'high vigour' variety, which can show extremely high levels of shoot growth when pruning is severe and environmental factors are not limiting (Dry and Loveys, 1998). This was demonstrated in the 2001/2002 season, where water was not limiting due to a wet spring in 2001 (Appendix E), and pruning weights were far higher than those of the other seasons of the study. The current data suggest that for Shiraz grown in the Barossa Valley, lower node number per vine (30 and 60 nodes) at pruning will lead to a high shoot vigour, where application of the PRD irrigation strategy may not necessarily curb canopy growth as measured by winter pruning weight and light penetration within the canopy. Furthermore, due to high rates of shoot growth in spring, canopy size may largely be established by the time soil water is depleted in early summer, and irrigation is required. Therefore, in this region, the application of deficit irrigation strategies such as PRD late in the growing season may be insufficient to control vine vigour. However, in vines pruned to a higher node number, i.e. 120 nodes or minimal pruning, a larger yield may lead to a restriction in carbon partitioning to vegetative growth. In this case, it may be possible to increase canopy openness and light penetration within the canopy using the PRD technique.

The implications of these results primarily concern berry development, as increased light penetration within the fruiting zone has been associated with higher concentrations of sugars, anthocyanins and phenolics; and lowered malic acid levels, juice pH and potassium compared with fruit from more shaded canopies (Smart, 1985; Dokoozlian and Kliewer, 1995). The question to be addressed in the current study is whether these changes in fruit exposure are large enough to bring about these changes in fruit composition, and will be considered in detail in the chapters which follow.

3.4 Conclusions

Under the conditions of the current study, a 'classic' PRD response was not attained throughout the growing season in either the Cabernet Sauvignon or Shiraz experiments. Thus, for both experiments, PRD-treated vines experienced a water deficit at certain stages of the relative to the control treatment. This resulted in reduced midday readings of stomatal conductance, φ_S and φ_L in PRD-treated vines relative to the controls at the end of a PRD cycle. The reductions in φ_S and φ_L in the PRD vines were indicative of an hydraulically-mediated response to the irrigation treatment rather than a solely non-hydraulic response due to signals produced in drying roots. During the switch between cycles when the soil water profile of both the 'wet' and 'dry' sides of the PRD-treated vines was refilled, stomatal conductance, φ_S and φ_L were restored to the same levels recorded for the control experiment, indicating that the water deficit was not sustained throughout the growing season.

Depending on the timing of the water deficit, this led to a reduction in final berry weight, and thus in resulting yield at harvest, which was more apparent in Cabernet Sauvignon vines pruned to 60 nodes per vines than in Shiraz vines at the same node number. For Shiraz vines, increasing node number per vine had the effect of reducing berry size and winter pruning weight; and increasing yield and the sensitivity of the vines to the PRD treatment. The effect of PRD in terms of decreased yield, berry weight, canopy size, shoot growth rate and increased bunch exposure, although not statistically significant in all cases, had the greatest effect in vines pruned to 120 nodes compared to those with 30 or 60 nodes. Thus, increased node number per vine in Shiraz may lead to a higher

yield/pruning weight ratio, which alters the 'balance' of the vine in the presence of a water deficit. This indicates that implementing PRD at higher node numbers per vine may lead to a greater loss in yield than at lower node numbers, but may potentially increase quality due to improved bunch exposure.

The responses to the PRD irrigation strategy shown in the current study bring to light some important questions surrounding the application of PRD as a deficit irrigation strategy. Firstly, despite rigorous irrigation scheduling according to the measurement of soil water content, the PRD treatment resulted in an hydraulic water deficit relative to the control treatment. Recent evidence has shown that the effectiveness of PRD as an irrigation strategy may be dependent upon soil type, with light textured soils providing the most advantageous medium for the treatment (Kriedemann and Goodwin, 2003). As the current study was performed on sandy loam topsoils in both experiments, the question which needs to be addressed is the length of the PRD cycle. It appears that a primary limitation to the application of PRD in such soils is a more rapid rate of soil drying, which may result in vines experiencing water deficit if the timing between PRD cycles is too long (Kriedemann and Goodwin, 2003). This has the potential to reduce yield and berry size in addition to vine canopy size. This implies that despite the potential gain in the WUE of vines with PRD, if there is a significant yield penalty associated with the treatment, there would be no net gain in WUE. However, although there are potential limitations surrounding the application of PRD in different regions and soil types (Kriedemann and Goodwin, 2003), the technique still has the advantage of providing a continuous and mild water stress to vines, and additionally has the potential to improve fruit quality (Dry *et al.* 2003c). Using PRD would allow growers to avoid the potential deleterious effect of other deficit irrigation strategies such as RDI, which could result in a devastating yield loss if incorrectly managed.

CHAPTER 4: THE EFFECT OF PRD ON GRAPE BERRY RIPENING

4.1 Introduction

4.1.1 Primary metabolic processes associated with grape berry ripening

Grapes are a non-climacteric fruit, which means that the ripening process is not associated with a burst of respiratory activity (Biale, 1964). During the initial phases of fruit development prior to the onset of ripening, grape berries accumulate organic acids, namely malate and tartrate (Amerine, 1956; Kliewer, 1965), but there is little storage of non-structural carbohydrate. The onset of the ripening process in grapes is termed 'veraison' and is associated with physiological and morphological changes in fruit composition including increases in fruit deformability and volume (Coombe, 1984).

The biochemical changes associated with veraison are the onset of a steady decline in organic acid content from the fruit pericarp and a sharp rise in the total content of hexose sugars (Kliewer, 1965; Hawker, 1969; Coombe, 1984; Iland and Coombe, 1988). An early study by Hawker (1969) on Sultana fruit showed that the increase in hexoses at veraison was preceded by a peak in invertase activity, an enzyme which cleaves sucrose into its component hexoses: glucose and fructose. Sucrose synthase, another enzyme which mediates the breakdown of sucrose, increased in activity at veraison, but its activity was low relative to that of invertase. Later studies have focused on invertase as the primary enzyme associated with sugar accumulation in grape berries, and have shown that it is most likely associated with the vacuole of grape berry cells (Nakanishi *et al.* 1991; Davies and Robinson, 1996). Changes in the timing and degree of increase in the activity of this enzyme associated with veraison may significantly impact the rate of berry ripening.

It was originally thought that the primary source of malic and tartaric acid in grapes was via their synthesis in vine leaves and subsequent translocation to the berry (Amerine, 1956). However, Hale (1962) showed that $^{14}CO_2$ feeding to vine leaves, followed by shoot girdling to eliminate phloem transport, resulted in a rise in radioactivity in berry

organic acids with time after girdling. Although this study does not eliminate the possibility that berry organic acids are derived from translocated photosynthate, it demonstrated that the fruit is also an important site of photosynthetic carbon synthesis. The decline in the concentration of organic acids at veraison is primarily through respiration (Ruffner, 1982b; Ruffner and Hawker, 1977). In addition to this, Ruffner *et al.* (1975) showed that feeding grape berries with fumaric acid-2,3-^{14}C led to the formation of glucose carrying more label in carbon positions 1 and 2, or 5 and 6, than in the central carbon atoms, which indicated that the formation of sugars can occur from organic acids by a reversal of glycolysis (gluconeogenesis). The decline in organic acids during ripening may therefore not only be via the respiratory pathway but also in sugar formation, although this flux is small.

Malic acid has been shown to decline at a faster rate than tartaric acid during maturation, leaving higher levels of tartaric acid present in grape berry tissue at harvest (Amerine, 1956; Kliewer, 1965). In some cases, tartaric acid levels have been found to remain unchanged in fruit tissues post-veraison (Iland and Coombe, 1988). This limited metabolism of tartrate in grape berries was initially thought to be because this acid is chemically inert in grape tissue, but its metabolism to $^{14}CO_2$ was later demonstrated in mature grape berries following feeding of bunches with ^{14}C-tartrate (Takimoto *et al.* 1976). A study by Gutierrez-Granda and Morrison (1992) showed that the metabolism of tartaric acid varies between tissue types in the grape berry, such that a rapid decline in tartrate is observed in the outer mesocarp post-veraison, while in the skin, inner mesocarp and core of the fruit its rate of decline was reduced. This may account for some of the variation in the data relating to tartrate metabolism to date, which has primarily looked at whole fruit, flesh or skins (Ruffner, 1982a; Iland and Coombe, 1988).

A similar compartmentation of metabolism has also been observed for malic acid within different grape berry tissues (Ruffner, 1982b). Feeding of green grape berries with ^{14}C-sucrose through the pedicel led to the formation of labelled malate predominantly in the peripheral areas of the fruit (Coombe and Matille, 1980). In a similar experiment, the more active metabolism of malic acid in ripening fruit was demonstrated in peripheral

fruit tissue, following feeding with ^{14}C-malate, whereas the core tissue of the fruit appeared to have a more inactive malate pool (Steffan and Rapp, 1979). A study of the concentration of malate in different tissues of the berry later showed the lowest content in the vascular bundles, with downward gradients toward these zones (Coombe, 1987). This suggests that the rapid metabolism of malic acid at the vascular bundles caused a concentration gradient to be established, thereby causing malate movement toward these zones, primarly in the periphery of the berry (Coombe, 1987). An investigation of malic enzyme activity showed higher levels in the outer mesocarp and skin, which increased at the time of veraison (Gutierrez-Granda and Morrison, 1992). However, no correlation between malic acid content and malic enzyme activity was established (Gutierrez-Granda and Morrison, 1992). Rather, a more significant relationship was shown for the enzyme malate dehydrogenase, which had a strong negative correlation in activity with post-veraison malic acid content (Taureilles-Saurel *et al.* 1995). This may indicate participation of this enzyme in the regulation of malate metabolism during ripening.

4.1.2 The effect of irrigation and berry microclimate on grape berry ripening

4.1.2.1 Irrigation

The application of irrigation to grapevines has generally been found to cause a delay in ripening, as measured by the accumulation of sugars (Neja *et al.* 1977; Kliewer *et al.* 1983; Bravdo *et al.* 1985). This was proposed to be due to the dilution effect of sugars in an increased fruit volume under irrigation, which is often associated with an increase in fruit weight (Esteban *et al.* 2002). However, the rate of ripening is not only determined by the rate of sugar accumulation, but also characterised by the rate of decline in organic acids. At harvest, irrigated vines have been shown to have higher acid levels when berries are harvested on the same date as those taken from unirrigated vines (Bravdo *et al.* 1985; McCarthy and Coombe. 1985). This initial observation was assumed to be primarily due to the delay in ripening induced in vines by irrigation, but was found to persist even when acid levels of irrigated and unirrigated vines were compared at similar sugar levels (Bravdo and Hepner, 1986). The effect was found be due to a decrease in malic acid in unirrigated vines, whereas tartrate was unaffected by the irrigation treatment (Bravdo *et*

al. 1985). Therefore, the effect of irrigation on the ripening process may not simply be due to an increase in berry volume, but may be the result of an alteration in plant water status, which in turn impacts the regulation of pathways governing carbon metabolism within the fruit.

A recent study by Esteban *et al.* (2002) investigated the effect of a non-irrigated treatment versus an irrigated treatment of Tempranillo, comparing pH, titratable acidity (TA), malate, tartrate and potassium in the fruit. Both pH and TA were affected by the non-irrigated treatment, with an increase in pH relative to the irrigated treatment observed throughout fruit development, corresponding to a decreased TA. Malic and tartaric acid levels in the fruit indicated that this response was due to a consistent decrease in both acids from early in fruit development in non-irrigated berries compared with irrigated berries. A consistently significant relationship between potassium and pH was not demonstrated, indicating that the changes in pH and TA may be primarily due to changes in the free acids alone.

Apart from studies comparing non-irrigated and irrigated vines, the effect of a controlled water deficit has also been found to affect the ripening response. A study of early deficit (pre-veraison) on vine water status has been associated with a reduction in malate concentration, whereas a late deficit (post-veraison) water stress was found to have no effect on berry acidity (Matthews and Anderson, 1988). A variable response has been obtained with PRD irrigation strategies. In expressed juice from Cabernet Sauvignon fruit at similar sugar concentration, there was no change in pH or TA despite a 50% reduction in irrigation water applied (Stoll, 2000). This may indicate that, under the controlled conditions of the PRD treatment, the plant is not placed under actual water deficit, such that there is no effect on primary metabolism in the fruit. However, other studies have indicated that TA can be increased (Dry, 2000) or decreased (dos Santos *et al.* 2003) where PRD was run at 50% the irrigation level of fully irrigated fruit, even when bunch weight and sugar content was unaffected. Further research is therefore needed to determine the effect of PRD on fruit acid composition.

4.1.2.2 Bunch exposure and temperature

Many studies have shown that a primary factor regulating the concentration of malate post-veraison is temperature, with increased temperature enhancing its metabolism (for a review see Ruffner, 1982b). Grape berries have been shown to have two isoforms of malate dehydrogenase, which are localised to the cytosol and the mitochondria respectively, which differ in their temperature sensitivity (Taureilles-Saurel *et al.* 1995). The mitochondrial isoform is more easily activated at temperatures exceeding 32°C, which may indicate its role in metabolising malate in response to summer growth, under conditions of thermic stress (Taureilles-Saurel *et al.* 1995). However, research to separate the effects of temperature and radiation on grape acidity has not been attempted. In shaded canopies of Shiraz, Smart *et al.* (1985) showed a higher malic acid content and pH in grape musts, associated with a lower pH in wine. Furthermore, in Cardinal and Pinot Noir vines, low light intensity on bunches resulted in berries with reduced total acidity and increased malate content (Kliewer and Lider, 1970). Dokoozlian and Kliewer (1996) found similar results for mature sun-exposed and shaded fruit of cv. Cabernet Sauvignon and Pinot Noir fruit, where temperatures in the light and shade were the same. This indicates that low malate levels in fruit may be caused by increased radiation alone. A further study exploring organic acid levels throughout berry development indicated that in exposed versus shaded berries, malate was lower throughout development post-veraison, and was proposed to be due to a higher rate of malate metabolism (Ruffner *et al.* 1975).

In the separate experiments discussed, irrigation and bunch exposure have been found to affect the rate of sugar accumulation, and the organic acid levels in grape berries. It is known that the light environment of the grapevine canopy can be altered through water deficit, leading to more exposed fruit (Dry, 1997; Stoll, 2000). In cases where water deficit has been shown to alter grape malic acid levels, this finding must clearly be separated from a possible effect of increased light interception within the canopy, which may cause accelerated metabolism of malic acid. The current study aims to investigate the effect of PRD on sugar accumulation, grape pH and TA during fruit development, and their final levels at harvest. Alterations in juice pH and TA at harvest will be compared

with alterations in malic and tartaric acid and potassium in fruit at the same sugar level. Complete shading of bunches has been superimposed over the PRD treatment in order to separate the effect of sun exposure and water-deficit on the fruit composition.

4.1.3 The effect of crop load on grape berry ripening

The concept of "vine balance" was first proposed by Partridge (1926), and describes the relationship between photosynthetic carbon availability and its distribution among storage organs and sinks within the vine. It may be possible for a vine of a certain size to mature a large crop load and still produce sufficient storage carbohydrate for shoot growth the following season. However, the potential risk with increasing the crop load on a vine is that restriction of carbon resources may prevent the crop ripening to full capacity, as well as reducing its capacity for growth from year to year. Increasing bunch number per vine has been reported to decrease the rate of ripening, and final sugar levels attained at harvest (Edson *et al.* 1993; Miller *et al.* 1993; Miller and Howell, 1998). The effect of increasing crop level on final pH and TA can be variable; either having no significant effect (Edson *et al.* 1993) or being correlated with lower pH or higher TA (Miller and Howell, 1998). This indicates that the potential effect of increasing crop level is a decreased ability of the fruit to reach maturity.

The effect of increased crop load on sugar accumulation can be exacerbated by a water stress imposed by reduced irrigation, according to a study by Bravdo *et al.* (1985). That study showed the results of a crop-thinning experiment, where there was little effect of crop load on total soluble solids where water supply was adequate, but where irrigation was reduced 35%, increased crop load caused a significant reduction in the rate of sugar accumulation. This was caused by an increased yield to pruning weight ratio under the deficit irrigation treatment where crop load was high, which shows that 'vine balance' between reproductive and vegetative sinks may have been disturbed by the irrigation treatment. A reduction in pruning weight is associated with a cutback in irrigation (McCarthy and Staniford, 1984; Matthews and Anderson, 1988, 1989; Goodwin and Jerie, 1992) and when this is combined with higher crop load will cause a significant change in the fruit weight to pruning weight ratio. PRD is a deficit irrigation strategy

which has been shown to reduce vegetative growth in vines as measured by pruning weight, shoot growth rate and leaf area without causing a significant change in fruit weight or sugar accumulation (Dry *et al.* 1996). However, the effectiveness of this treatment at higher cropping levels has not been determined. In the light of the effect of deficit irrigation on the ability of vines to mature fruit at higher cropping load (Bravdo *et al.* 1985), there may potentially be a limit of the PRD treatment, above which vines become 'unbalanced'.

The aim of the current study was to determine the effect of PRD irrigation at different cropping loads produced by the retention of varying node number per vine at pruning, in terms of sugar accumulation and fruit acidity.

4.2 Materials and Methods:

4.2.1 Experimental

Fruit at different stages of development was obtained from the Cabernet Sauvignon experiment at Langhorne Creek (Section 2.1.2, Chapter 2) and from the Shiraz experiment at Nuriootpa (Section 2.1.1.1, Chapter 2). The latter experiment compared three different pruning levels, of node number 30, 60 and 120 nodes superimposed over PRD and standard irrigation strategies. A further experiment was carried out within the Cabernet Sauvignon experiment at Langhorne Creek in 2002/2003, which compared totally shaded bunches grown in shade boxes from the stage of fruit set to bunches which were free within the canopy (Section 2.1.3, Chapter 2).

4.2.2 Determination of sugars, pH and titratable acidity (TA) in juice samples

Juice was extracted from a fresh 50 berry sample by gentle pressure. The juice samples were centrifuged at 10 000 g and the supernatant was retained. The pH and of the juice sample was determined immediately using an Activon pH meter. Total sugars were measured as total soluble solids (TSS) of the juice using an Erma BRX-242 digital refractometer. Five mL of the juice sample was then diluted 1:5 with deionised water and frozen at -20°C for later analysis. In immature samples with high levels of organic acids,

this dilution was 1:10. The TA of the defrosted juice samples was determined using a Crison autotitrator, with the end-point for the titration against 0.1 N NaOH set at a pH of 8.2.

4.2.3 Extraction and analysis of malic and tartaric acids

A further 50 berry sample was frozen at -20°C for later analysis. This sample was then defrosted for 30 min at room temperature and then ground to a slurry using an Ultra Turrax homogeniser. One gram of the berry homogenate was weighed into 10 mL centrifuge tubes and immediately extracted in 10ml 1N HCl (Mora and Rossello, 1992) to prevent the precipitation of insoluble tartrate salts. Extraction was carried out for one hour on a rotary shaker. Samples were then centrifuged 5 min at 10 000 g, and the supernatant collected. For tartaric acid analysis an adaptation of the HPLC method of Frayne (1986) was used. The HPLC system used was a Hewlett Packard HP 1100. Twenty µL of the extract was injected onto a Supelco Supelcogel C-610H HPLC column (30 cm x 7.8 mm) with a pre-column of the same material. The method used an isocratic flow of 0.5 mL/min 0.1% (v/v) phosphoric acid, monitored at 210.8 nm. The final run time was 30 min. Tartaric acid eluted at 16.57 min, and was quantitated according to a standard curve of tartaric acid showing linearity between 0 and 6 g/L with an R^2 value of 0.99993. For malic acid analysis, the HPLC method was inefficient due to co-elution of malic acid with an unknown sugar compound. Instead, malic acid was determined enzymatically according to the method of Bergmeyer (1974). Prior to the assay, 1 mL of the HCl extract was prepared by the addition of insoluble PVPP, and filtered to remove phenolic compounds.

4.2.4 Determination of the inorganic ion content of berry tissue

Analysis of the inorganic ion content of the grape homogenates was determined by Waite Analytical Services, University of Adelaide. A further 2 g of the berry homogenate was weighed into 50 mL centrifuge tubes and dried in an oven at <50 °C. Nine mL of conc. HNO_3 was added to the samples which were then cold-digested overnight. Following this, the samples were heated using a ramped temperature gradient over 5-6 hours with

the maximum temperature not exceeding 150 °C and subjected to atomic emission spectroscopy (ARL Inductively Coupled Plasma Optical Emission Spectrometer) to determine the content of inorganic ions.

4.3 Results and Discussion

4.3.1 The effect of PRD on grape berry ripening in Cabernet Sauvignon at Langhorne Creek

The results of the PRD experiment on Cabernet Sauvignon at Langhorne Creek (Section 2.1.2, Chapter 2) are shown in Figure 4.1 and Table 4.1. Throughout development in 2001/2002, the PRD treatment showed a more rapid rate of sugar accumulation (Figure 4.1 A) whereas in 2002/2003, the rate of sugar accumulation was relatively unaffected by PRD post-veraison, but slightly higher TSS measures were recorded on two sampling dates as the fruit approached maturity (Figure 4.1 B). The slight changes in TSS caused by the PRD treatment were accompanied by much larger changes in TA, with PRD having consistently lower TA levels in both 2001/2002 and 2002/2003. Immediately post-veraison TA was relatively unaltered by PRD in both seasons, but as the fruit matured the PRD treatment was characterised by an accelerated loss of TA. When these parameters were compared in juice extracts at harvest, TA was decreased and pH was increased by the PRD treatment in both seasons (Table 4.1). As this comparison was made where the TSS level was the same in both PRD and control samples, it is evident that the change in acidity was not due to an accelerated rate of ripening in the PRD fruit.

This result with PRD is similar to the results found by dos Santos *et al.* (2003), where TA was significantly lower over one season in response to PRD irrigation, in the Moscatel and Castelão varieties of *Vitis vinifera*. Another study where this effect was observed in response to deficit irrigation proposed that a change in the microclimatic conditions of the fruit was responsible for decreased TA and increased pH in deficit-irrigated fruit relative to fully irrigated fruit (Esteban *et al.* 2002). It has been suggested that alterations in organic acid metabolism would be a response to changes in sun exposure or temperature of the fruit, as a result of changes in vegetative growth in response to the

irrigation treatment (Smart *et al.* 1985; Esteban *et al.* 2002). The current results indicate that the PRD treatment may affect the primary processes of fruit metabolism, altering the activity of enzymes or the expression of genes associated with the organic acid and sugar pathways in the berry. These changes may be a direct response of the fruit to the irrigation treatment, and thus plant signals (Stoll *et al.* 2000) or an indirect response to changes in canopy architecture resulting from the PRD treatment. Thus, a simple study of the changes in organic acids throughout development cannot provide an explanation for the biochemical basis of the PRD response, even if alterations in fruit microclimate are closely monitored. A clear separation of the effects of sun exposure, temperature and the irrigation treatment is necessary. This could be determined where whole bunches of PRD-treated and control-treated fruit are completely shaded throughout development, under similar temperature conditions.

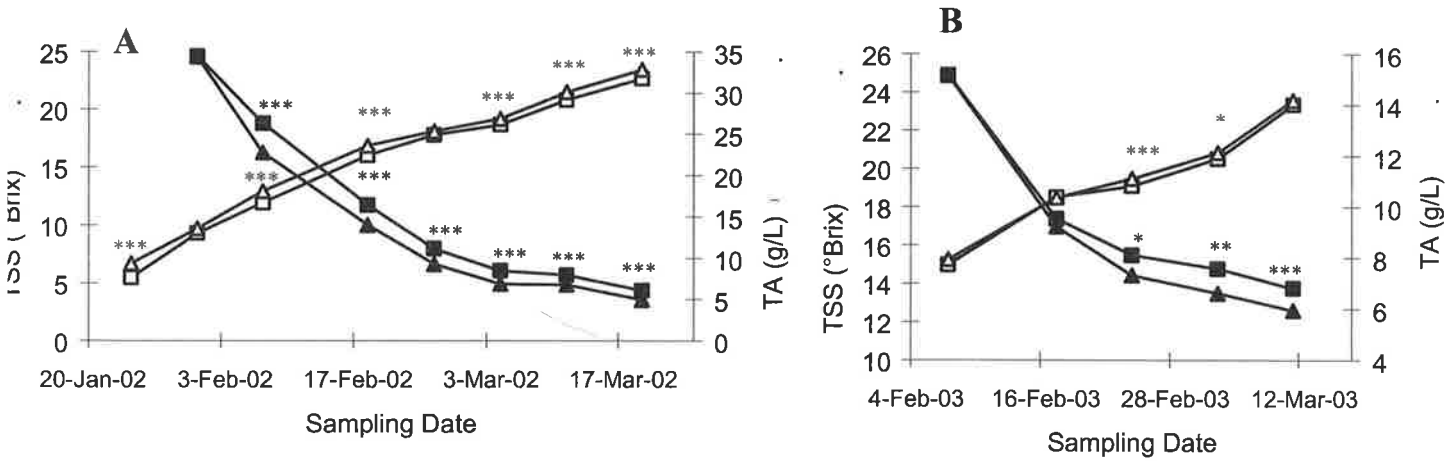


Figure 4.1: Effect of PRD on TSS and TA of juice in Cabernet Sauvignon **A.** 2001/2002 vintage; **B.** 2002/2003 vintage; □ TSS Control; ■ TA Control; △TSS PRD; ▲ TA PRD (ANOVA, n=42, *=P<0.05 **=P<0.01,***=P<0.001; * TSS; * TA)

Table 4.1: Effect of PRD on pH and TA of Cabernet Sauvignon juice at harvest in the 2001/2002 and 2002/2003 vintages at Langhorne Creek (ANOVA, n=42)

	2001/2002 Vintage				2002/2003 Vintage			
	Control	PRD	P	%	Control	PRD	P	%
TSS (°Brix)	23.44	23.47	ns	0	23.35	23.57	ns	1
pH	3.51	3.62	<0.001	3	3.44	3.55	<0.05	3
TA (g/L)	5.77	4.93	<0.001	-17	6.09	5.43	<0.001	-12

4.3.2 The effect of PRD on fruit acidity under shaded conditions

In order to investigate the effect of PRD on TA further, the PRD response was compared at similar TSS under exposed and artificially shaded conditions for one season in 2003 (Table 4.2). The shading treatment did not cause a change in the temperature of bunches within the boxes, relative to bunches under ambient conditions within the canopy (Appendix C). Total shading caused relatively large changes in TA, pH and the components of acidity: tartaric and malic acids. Total shading unexpectedly caused an increase in pH and a decrease in TA. This was due primarily to a large decrease in tartaric acid in the shading treatment, despite the fact that malic acid was increased relative to the sun-exposed fruit. A reduction in the rate of malic acid decline under shaded conditions has previously been reported (Kliewer and Lider, 1970; Dokoozlian and Kliewer, 1996) but there is no indication in the literature that changes in tartaric acid metabolism should occur due to shading alone. In fact, most examples of treatments where an increase in malate was observed, it was accompanied by a relative increase in tartaric acid (Coombe, 1987; Esteban *et al.* 2002) or no change (Bravdo and Hepner, 1986). It is probable that the reduction in tartaric acid in the shaded fruit at 21.5°Brix is due to decreased synthesis or early in fruit development rather than an increased rate of degradation post-veraison. This is the most likely cause, because the fixation of $^{14}CO_2$ into ^{14}C-tartaric acid in grape berries has been shown to be inhibited in the dark (Saito and Kasai, 1969).

High levels of tartaric acid accumulate during the initial phase of bloom, and change 200-600% within a short period from bloom through berry set (Kliewer, 1965). As the shading experiment would have been applied at fruit set, it may have caused a decrease in the accumulation rate early in fruit development, which would have affected final levels of tartrate at harvest. This hypothesis could not be tested in the current study, as shaded fruit was only sampled at one developmental stage; but it is unlikely that the shading treatment would have caused an accelerated decline in tartaric acid concentration, as temperature was unaltered by the shading boxes. The increase in pH caused by the shading treatment could also partly be attributed to significant increases in the concentration of both potassium and sodium ions, 13% and 11% respectively, relative to

the sun-exposed treatment (Table 4.3). It has been shown that the uptake of sodium and potassium ions by grape berries will lead to an increase in pH, for a given level of acidity (Boulton, 1980). These ions act to neutralise the acidic properties of both tartaric and malic acid in the juice, thereby affecting the juice pH (Hale, 1977). As temperature was constant in both the shaded and sun-exposed experiments, this response would be caused by the change in fruit exposure alone.

The relatively lower TA and higher pH which was measured over time for the PRD treatment on Cabernet Sauvignon (Figure 4.1) was also shown in the results of the totally shaded experiment (Table 4.2). Although tartaric acid was lower in the shaded treatment, its concentration was unaffected by PRD. In 2001/2002 and 2002/2003, the decrease in TA with PRD was 18% and 19% respectively, and the results indicated an equivalent decrease in malic acid for both seasons. The ions potassium and sodium were unaffected by the PRD treatment (Table 4.3) although the levels of other ions e.g. calcium and magnesium, were significantly increased in both the shaded and sun-exposed PRD fruit. However, as potassium and sodium are the primary ions affecting the pH of berry juice (Boulton, 1980), it is unlikely that changes in ion ratios were responsible for the observed change in pH. Thus, the decrease in pH and TA was primarily due to a decrease in malic acid, in both the sun-exposed and shaded PRD fruit. This finding is in agreement with other studies which indicate that changes in acidity in grape juice as a result of irrigation treatments and other environmental factors is primarily due to changes in malic acid (Boulton, 1980; Hrazdina *et al.* 1984; Esteban *et al.* 2002).

The current study additionally demonstrates that the changes in the rate of malic acid decrease caused by the effect of water deficit on respiration is independent of fruit exposure or temperature. Although the PRD canopies were more open, and fruit was 30% more exposed in both seasons (Figure 3.9), this is not the overriding effect influencing the levels of malic acid in the PRD-treated fruit. It has been shown that a primary physiological response to the PRD treatment is an increase in the levels of the plant hormone abscisic acid (ABA) in the xylem sap (Stoll *et al*, 2000). This finding does not negate the possibility that other plant signals may also be altered by the PRD treatment. It

is possible that alterations in the concentration of xylem sap ABA can influence ABA concentrations in both flowers and berries of grapevines, as a recent study has shown a strong positive relationship between xylem sap ABA and its concentration in flowers, immature and mature fruit (Antolín *et al.* 2003). Increasing endogenous levels of ABA in fruit through feeding of ABA to developing fruit prior to veraison is associated with an advance in the onset of ripening (Hale and Coombe, 1974; Coombe, 1984).

It is probable that a primary response to the PRD treatment in the current study is an increase in xylem sap ABA, which resulted in the decreases observed in leaf stomatal conductance (Figure 3.3). This may have resulted in increases in fruit ABA concentration, which may in part account for the accelerated accumulation of sugars with PRD, particularly in the 2001/2002 vintage (Figure 4.1; Table 4.1). To date, there is no report in the literature linking ABA concentration and the metabolism of organic acids in grape berries. The current data indicate that changes in berry microclimate, namely bunch exposure and temperature, are not the primary factors influencing the decrease in malic acid in PRD-treated fruit. Rather, it is a biochemically-mediated response to the PRD-treatment itself. In view of the research on PRD to date (Dry, 1997; Stoll *et al.* 2002) an ABA-mediated response is a strong possibility, although it is not likely to be the exclusive plant signal involved. Thus, to better account for the results of the current study, further research into the effect of ABA and deficit irrigation strategies on the metabolism of malic acid in grape berries is required.

Table 4.2 Effect of PRD and shading on components of acidity in Cabernet Sauvignon fruit compared at 20.5 – 21.0 °Brix for the 2002/2003 vintage (ANOVA, n=42).

Component	Sun: Control vs PRD				Shade: Control vs PRD				Sun vs Shade			
	Control	PRD	P	%	Control	PRD	P	%	Sun	Shade	P	%
Berry wt (g)	1.00	0.86	<0.001	-16	0.94	0.85	<0.01	-11	0.93	0.90	ns	-3
pH	3.27	3.33	<0.05	2	3.46	3.55	<0.001	3	3.30	3.51	<0.001	6
TA (g/L)	7.6	6.41	<0.001	-19	7.10	6.01	<0.001	-18	7.00	6.60	<0.05	-6
Malic acid (mg/g)	2.01	1.62	<0.001	-24	2.94	2.47	<0.001	-19	1.82	2.71	<0.001	33
Tartaric acid (mg/g)	5.09	5.08	ns	0	4.35	4.25	ns	-2	5.08	4.31	<0.001	-18
Ratio (Malic/Tartaric)	0.40	0.32	<0.001	-24	0.68	0.58	<0.05	-17	0.36	0.63	<0.001	43

Table 4.3 Effect of PRD and shading on inorganic ion content in Cabernet Sauvignon fruit compared at 20.5 – 21.0 °Brix for the 2002/2003 vintage (ANOVA, n=42).

Component (mg/kg)	Sun: Control vs PRD				Shade: Control vs PRD				Sun vs Shade			
	Control	PRD	P	%	Control	PRD	P	%	Shade	Sun	P	%
Cu	0.90	1.04	<0.01	16	1.06	1.24	<0.01	17	1.15	0.97	<0.001	-16
B	11.0	11.8	ns	7	12.0	13.3	<0.05	11	12.0	11.0	<0.05	-8
Ca	279	310	<0.01	11	241	259	<0.05	7	250	294	<0.001	18
Fe	3.20	3.50	ns	9	4.48	4.72	ns	5	4.60	3.34	<0.001	-27
K	2301	2338	ns	2	2662	2700	ns	1	2681	2320	<0.001	-13
Mg	109	117	<0.01	7	111	117	<0.01	5	114	114	ns	0
Mn	1.26	1.46	ns	16	1.29	1.34	ns	4	1.32	1.36	ns	3
Na	19.3	21.5	<0.05	11	22.1	23.8	ns	8	23.0	20.4	<0.001	-11
P	220	232	<0.05	5	235	251	<0.01	7	243	226	<0.001	-7
S	101	111	<0.001	10	104	114	<0.001	10	109	106	ns	-3
Zn	0.86	0.96	<0.01	12	0.89	0.98	<0.01	10	0.94	0.91	ns	-3

4.3.3 The effect of PRD and node number per vine on grape berry ripening in Shiraz at Nuriootpa

For this experiment, Shiraz vines at Nuriootpa were pruned to three different node numbers: 30, 60 and 120 nodes. These three pruning levels were superimposed over PRD and control irrigation treatments for three consecutive seasons. With increasing node number per vine, there was a corresponding increase in crop load (Tables 3.5 – 3.7). For the 2000/2001 season, from 30 to 120 nodes per vine, the crop load increased by 43% and 30% for control and PRD-treated respectively (Figure 4.2 A, Table 3.5). In 2002/2003, this was 30% and 41% for control and PRD treatments respectively (Figure 4.2 A; Table 3.6). However, from the 60 to the 120 node pruning level an increase in crop load was only observed for the control treatment for both the 2000/2001 and 2002/2003 seasons (Figure 4.2 A). This was because yield was reduced by the PRD treatment when bunch number per vine was higher, bringing the yield in the range of that obtained for the 60 node pruning level (Figure 4.2 A). For the 2000/2001 and 2002/2003 seasons, the irrigation level of the PRD treatment was 50% of the control, approximately 0.5 ML/ha and 1ML/ha for the PRD and control experiments respectively. In 2001/2002, PRD and control treatments received the same level of irrigation, approximately 1ML/ha. In that season, crop level increased in a similar manner from 30 to 120 nodes, by 41% and 51% for the control and PRD treatments respectively (Table 3.7).

The effect of increasing crop level on grape berry ripening differed between seasons, depending on the irrigation level applied. For the control treatments in the 2000/2001 and 2002/2003 seasons, there was a clear effect of crop load on the rate at which sugar accumulated (Figure 4.2 B-F). In this case, for increasing crop load per vine, the time taken to reach a certain level of ripeness was increased. However, in the PRD-treated vines, it appears that photosynthetic carbon availability became limiting in the 120 node treatment relative to the other pruning levels. Thus, the 120 node PRD treatment had yield within the range of the 60 node pruning level, but required an extended period to ripen that crop load (Figure 4.2 B-F).

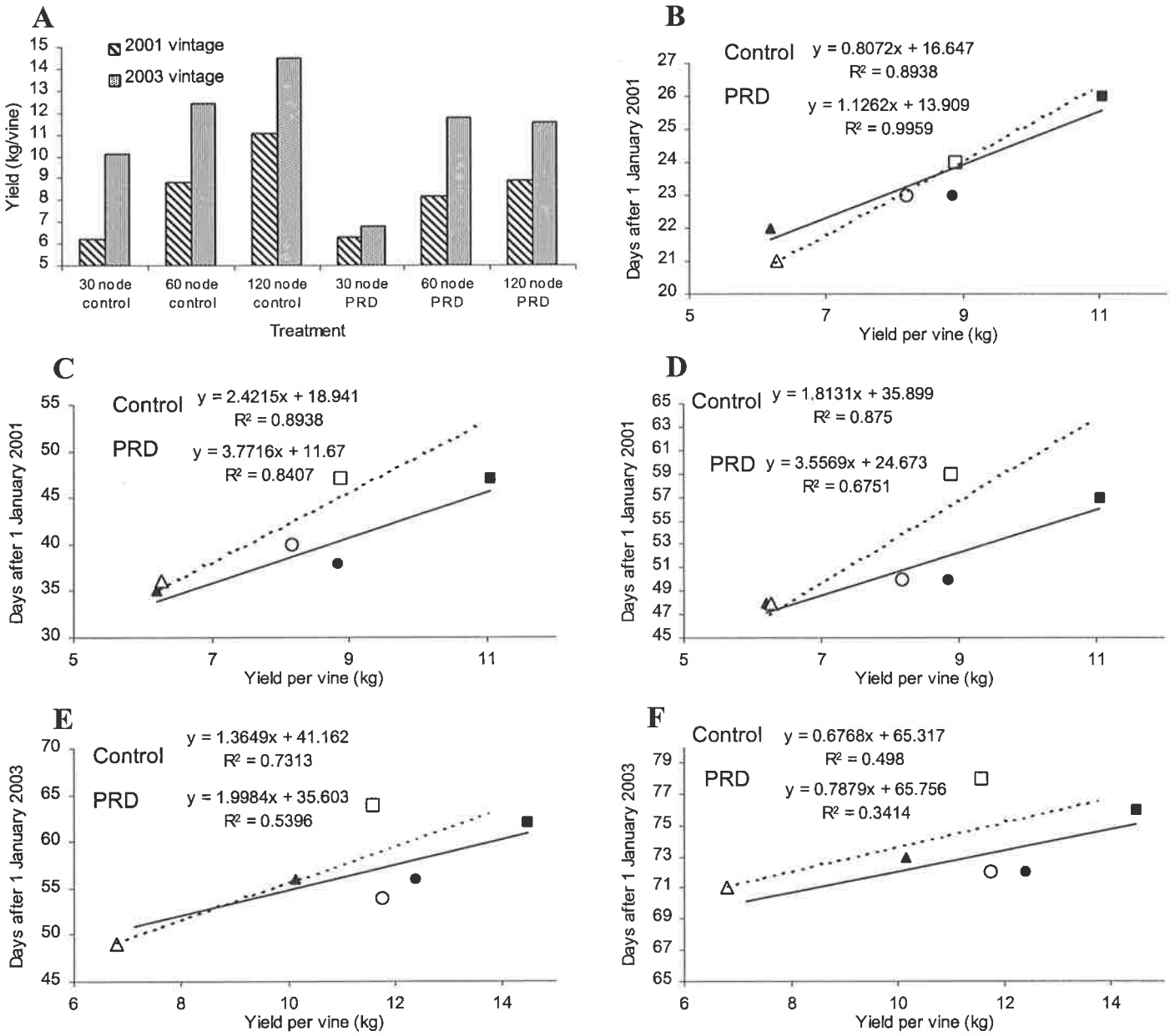
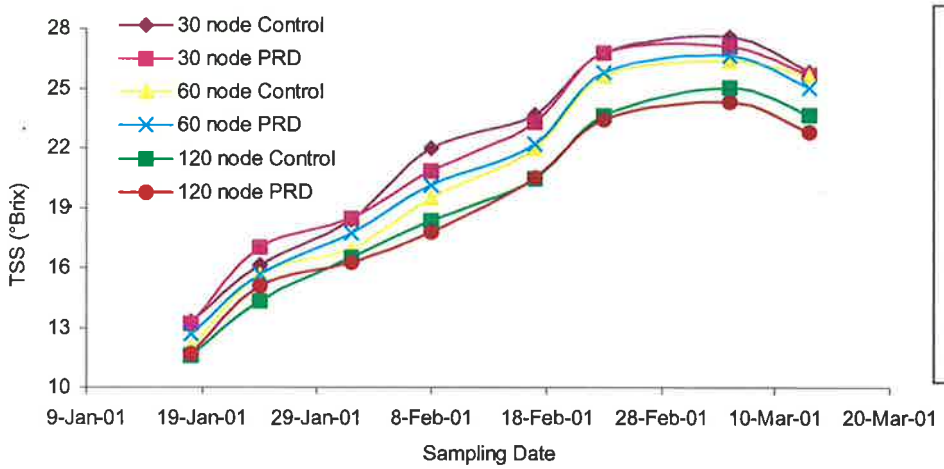


Figure 4.2: Effect of node number and PRD on crop load and rate of sugar accumulation in Shiraz vines grown at Nuriootpa for the 2000/2001 and 2002/2003 vintages. **A.** Yield per vine; **B-F** = Linear regression analysis of time taken to reach *x* °Brix: **B.** 15 °Brix 2000/2001, **C.** 20 °Brix 2000/2001; **D.** 24 °Brix 2000/2001; **E.** 21 °Brix 2002/2003; **F.** 25 °Brix 2002/2003.

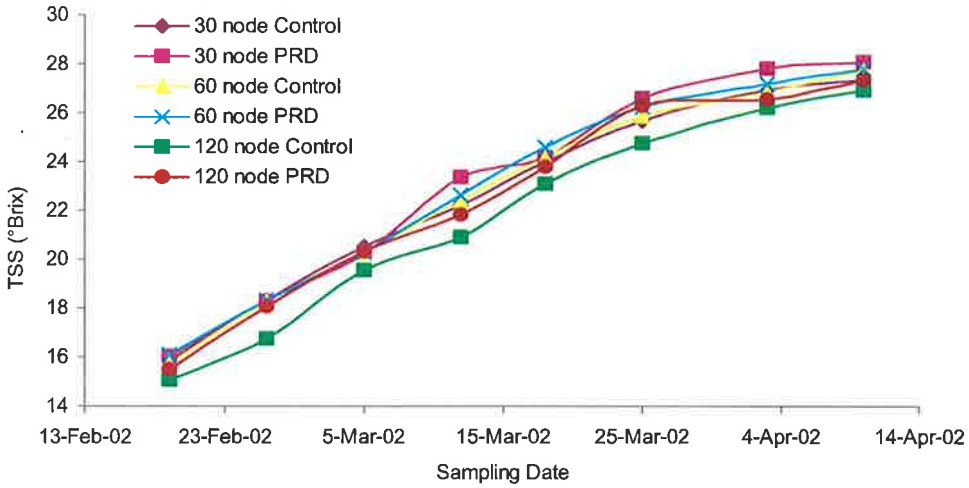
Independent of differences in yield, however, statistical analysis of the pattern of sugar accumulation (Figures 4.3, 4.5) and pH decline (Figure 4.6 and 4.8) showed no significant difference between the PRD and control treatments for the 2000/2001 and 2002/2003 seasons. Rather, a statistically significant difference was found between pruning levels. Therefore, with increasing node number and thus bunch number per vine (Tables 3.5 – 3.7) there was a decrease in the rate of sugar accumulation and pH decline in the fruit. It can be concluded that node number per vine had a stronger effect in determining the rate of ripening and the final TSS and pH levels reached in the fruit at maturity than did final yield or irrigation strategy. Thus, the effect of increasing node and bunch number not only alters the rate of sugar accumulation but also the final sugar level attainable by the vine, and is not dependent upon the time of harvest alone. This result is in agreement with previous work on the effect of crop load on fruit ripening, where higher crop loads resulted in a limitation in the final sugar level attainable in the fruit at harvest (Edson *et al.* 1993; Miller *et al.* 1993; Miller and Howell, 1998).

In 2001/2002, where both PRD- and control-treated vines received equivalent irrigation, there was not a clear effect of pruning level on sugar accumulation or juice pH, except a few points which showed a significant pruning interaction (Figures 4.4 and 4.7). However, these points of significance appear to be weighted by a consistently lower average sugar level for the 120 node control vines. When viewed across the developmental profile, it appears that the 120 node control vines showed a limit in the rate of TSS accumulation in the fruit (Figure 4.4) although this was not apparent with regards to juice pH (Figure 4.7). The 120 node PRD fruit showed an equivalent rate of TSS accumulation to the remaining pruning treatments, which was an unexpected result in the light of the data for 2000/2001 and 2002/2003. The difference in the results between the 2001/2002 vintage and the other seasons of the study appear to be a direct response to irrigation level applied between seasons. This demonstrates that at higher node numbers vines may have increased sensitivity to photosynthetic inhibition under water stress, thus producing a limiting effect on the rate of sugar accumulation (Figure 4.2 B-F). This phenomenon was also demonstrated by Bravdo *et al.* (1985), where water



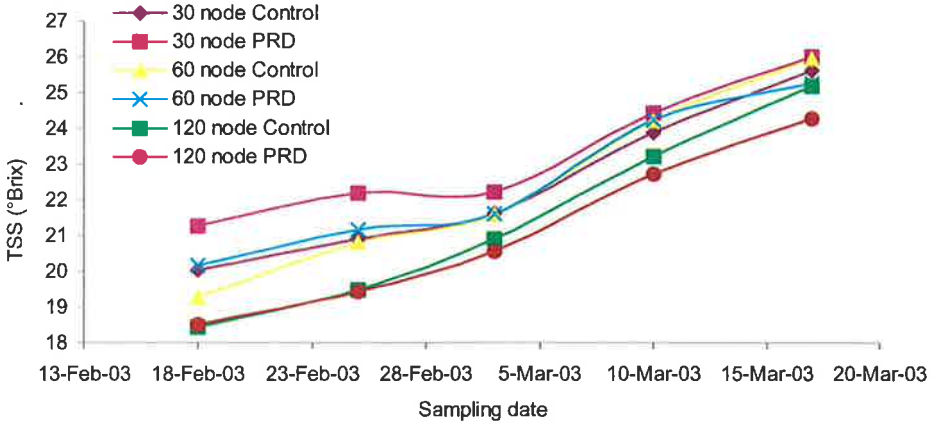
Date	Probability		
	T	P	T x P
18-Jan-01	ns	<0.001	ns
24-Jan-01	<0.05	<0.001	ns
1-Feb-01	ns	<0.001	ns
8-Feb-01	ns	<0.001	ns
17-Feb-01	ns	<0.001	ns
23-Feb-01	ns	<0.001	ns
6-Mar-01	ns	<0.001	ns
13-Mar-01	ns	<0.001	ns
23-Mar-01	ns	<0.001	ns

Figure 4.3: Effect of PRD and node number on juice TSS in developing Shiraz fruit for the 2000/2001 vintage where PRD irrigation was 50% of control (ANOVA, n=30, T= irrigation treatment, P = pruning/node number, TxP = interaction effect)



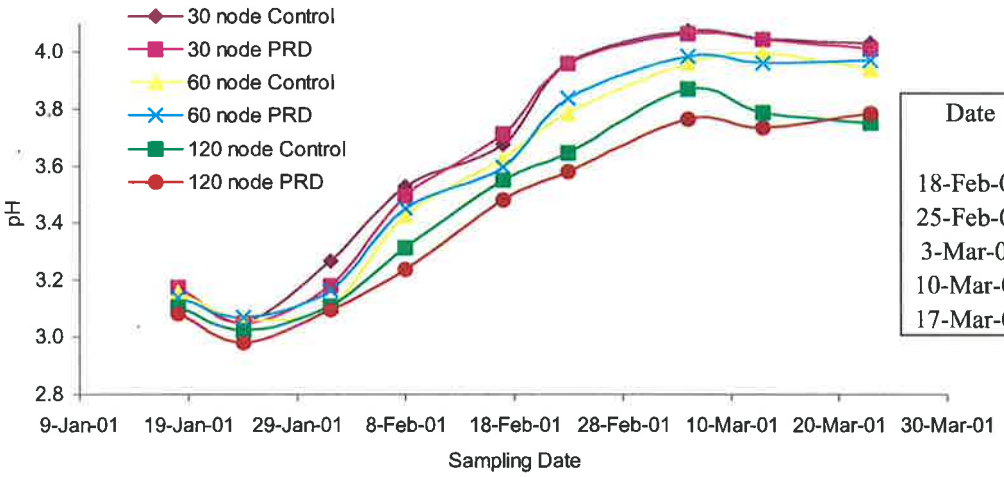
Date	Probability		
	T	P	T x P
19-Feb-02	ns	ns	ns
26-Feb-02	ns	<0.05	ns
5-Mar-02	ns	ns	ns
12-Mar-02	<0.05	0.001	ns
18-Mar-02	ns	ns	ns
25-Mar-02	<0.01	ns	ns
3-Apr-02	ns	<0.05	ns
10-Apr-02	ns	ns	ns

Figure 4.4: Effect of PRD and node number on juice TSS in developing Shiraz fruit for the 2001/2002 vintage where PRD irrigation was 100% of control (ANOVA, n=30, T= irrigation treatment, P = pruning/node number, TxP = interaction effect)



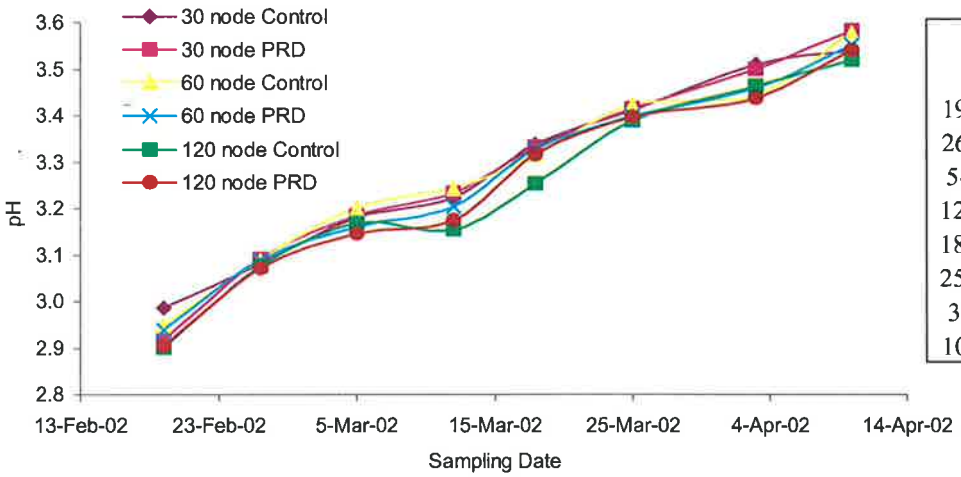
Date	Probability		
	T	P	T x P
18-Jan-01	ns	<0.001	ns
24-Jan-01	ns	ns	ns
1-Feb-01	ns	<0.001	ns
8-Feb-01	ns	<0.001	ns
17-Feb-01	ns	<0.001	ns
23-Feb-01	ns	<0.001	ns
6-Mar-01	ns	<0.001	ns
13-Mar-01	ns	<0.001	ns
23-Mar-01	ns	<0.001	ns

Figure 4.5: Effect of PRD and node number on juice TSS in developing Shiraz fruit for the 2002/2003 vintage where PRD irrigation was 50% of control (ANOVA, n=30, T= irrigation treatment, P = pruning/node number, TxP = interaction effect)



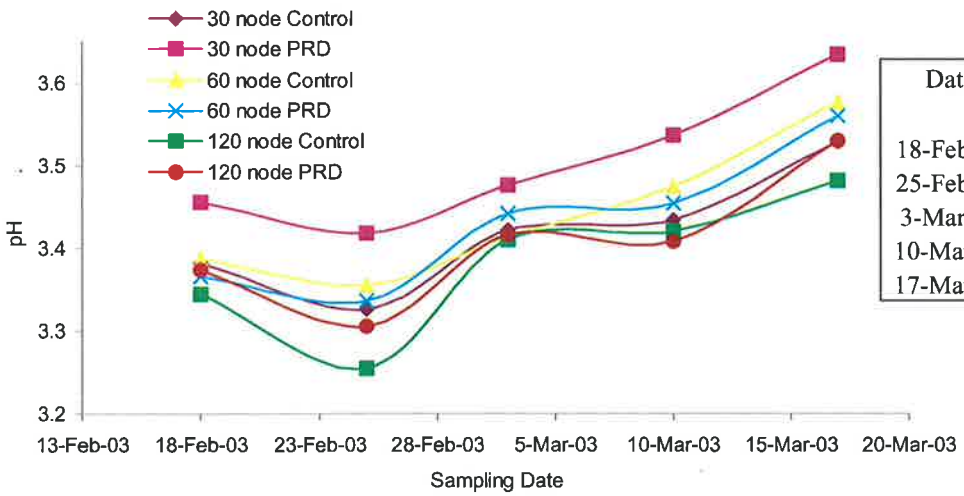
Date	Probability		
	T	P	T x P
18-Feb-03	<0.05	<0.001	ns
25-Feb-03	ns	<0.001	ns
3-Mar-03	ns	<0.01	ns
10-Mar-03	ns	<0.001	ns
17-Mar-03	ns	<0.05	ns

Figure 4.6: Effect of PRD and node number on juice pH in developing Shiraz fruit for the 2000/2001 vintage where PRD irrigation was 50% of control (ANOVA, n=30, T= irrigation treatment, P = pruning/node number, TxP = interaction effect)



Date	Probability		
	T	P	T x P
19-Feb-02	ns	0.05	ns
26-Feb-02	ns	ns	ns
5-Mar-02	ns	ns	ns
12-Mar-02	ns	0.01	ns
18-Mar-02	ns	<0.05	ns
25-Mar-02	ns	ns	ns
3-Apr-02	ns	<0.05	ns
10-Apr-02	ns	ns	ns

Figure 4.7: Effect of PRD and node number on juice pH in developing Shiraz fruit for the 2001/2002 vintage where PRD irrigation was 100% of control (ANOVA, n=30, T= irrigation treatment, P = pruning/node number, TxP = interaction effect)



Date	Probability		
	T	P	T x P
18-Feb-03	ns	<0.05	ns
25-Feb-03	<0.05	<0.001	0.05
3-Mar-03	ns	ns	ns
10-Mar-03	ns	<0.05	<0.05
17-Mar-03	ns	<0.05	ns

Figure 4.8: Effect of PRD and node number on juice pH in developing Shiraz fruit for the 2002/2003 vintage where PRD irrigation was 50% of control (ANOVA, n=30, T= irrigation treatment, P = pruning/node number, TxP = interaction effect)

stress combined with increased crop load led to a significant reduction in the rate of sugar accumulation. This was thought to be due to an increased yield to pruning weight ratio under the deficit irrigation treatment where crop load was high, leading to a reduction in photosynthate available from a limited leaf area for a large crop load. However, under PRD irrigation it appears that this response is minor, and would not lead to a significant change in the sugar level attainable by the fruit at harvest. This may be due to the increased ability of the vine to retain photosynthetic function under deficit with PRD, through maintenance of whole-vine water status (Stoll *et al.* 2000).

Some insight into these results can be gained by analysis of the yield to pruning weight ratios for each season. For the 2000/2001 and 2002/2003 seasons, the increased yield with increased node number was accompanied by a decrease in pruning weight (Tables 3.5 and 3.6). This resulted in a 53-67% higher yield to pruning weight ratio for the 120 node relative to the 30 node vines in both vintages. There were no significant differences observed in the yield to pruning weight ratios with PRD, even in 2002/2003 where yield was decreased with PRD (Table 3.6). In this instance, the yield reduction was accompanied by a decrease in pruning weight, which resulted in no change in the yield to pruning weight ratio relative to the control treatment. Thus, the primary response to increasing node number was an increase in the yield to pruning ratio. This might account for the observed limitation in sugar accumulation in the 120 node vines for both 2000/2001 and 2002/2003, where the increased partitioning of carbon to the higher crop load may have resulted in 'imbalance' between vegetative and reproductive growth. The limit in photosynthetic leaf area relative to crop load would have resulted in an inability of those vines to mature the fruit to the same level as vines with a lower crop load. The effect of node number on sugar accumulation appeared to be independent of the irrigation method applied, even where PRD received 50% the irrigation level of the control.

In 2001/2002, where PRD and control received the same amount of irrigation, there was a different response of the yield to pruning weight ratio than in the other seasons of the study. In that season, a cool spring with heavy rains throughout spring and early summer (Appendix E) resulted in excessive early spring growth. Thus, for 2001/2002 there was

no limitation in vegetative growth relative to reproductive growth, and increasing crop load actually resulted in higher pruning weights (Table 3.7). The yield to pruning weight ratio in the 120 node vines was therefore more comparable to the 30 and 60 node vines in 2001/2002. This might account for the apparent lack of differences in the rate of sugar accumulation in 2001/2002, as there was no limitation in the photosynthetic capacity of the vines in terms of leaf area, relative to crop load; thus enabling a larger vine to mature a larger crop load. However, the observed decline in the rate of sugar accumulation in the 120 node control vines may show that photosynthetic carbon became limiting as the season progressed, despite having sufficient leaf area to mature a larger crop. This indicates that where PRD and control vines received the same amount of irrigation, the control vines experienced water stress and photosynthetic limitation, whereas PRD vines were able to maintain photosynthetic rates throughout the season. This is very likely due to increased water-use efficiency of the PRD irrigation strategy, which maintained lowered stomatal conductance levels despite receiving the same level of irrigation water (Figure 3.4) (Dry and Loveys, 1999; Dry *et al.* 2000a; Stoll *et al.* 2000).

The combination of PRD and node number on juice TA and pH was compared at similar TSS levels, in order to remove the effect of node number on the rate of ripening (Table 4.4). There was no significant effect of PRD or node number on pH and TA of berry juice in either 2000/2001 or 2001/2002. However, in 2003 there was an observed increase in juice pH and a decrease in TA with PRD that was independent of node number. The result for 2002/2003 is similar to that obtained with PRD on Cabernet Sauvignon (Table 4.1), and shows that under some conditions PRD irrigation can result in an increased loss of organic acids from the fruit, most likely due to increased respiration of malate as a response to the irrigation treatment rather than changes in berry microclimate. There were large differences in pH and TA observed between seasons (Table 4.4). The 2000/2001 season was a hot, dry season whereas the 2001/2002 and 2002/2003 seasons were milder (Appendix E). In 2000/2001, higher temperatures may account for the increase in pH and drop in TA observed across all the treatments, at the same TSS level (Kliewer, 1971).

Table 4.4: Effect of PRD on pH and TA on Shiraz fruit at 23.5 – 24 °Brix pruned to differing node number from the vintages 2001 – 2003. Irrigation level: 2000/2001 and 2002/2003: PRD = 50% Control; 2001/2002: PRD = Control (ANOVA; n=30; T= irrigation treatment; P= pruning; T x P = interaction effect; ns = not significant).

		30 node	60 node	120 node	P (T)	P (P)	P (TxP)
2001							
pH	Control	3.67	3.73	3.67	ns	ns	ns
	PRD	3.69	3.71	3.64			
TA (g/L)	Control	4.22	4.09	3.89	ns	ns	ns
	PRD	4.39	4.01	4.25			
2002							
pH	Control	3.29	3.27	3.30	ns	ns	ns
	PRD	3.24	3.23	3.30			
TA (g/L)	Control	8.31	7.67	7.82	ns	ns	ns
	PRD	8.67	8.36	8.00			
2003							
pH	Control	3.38	3.44	3.41	<0.05	ns	<0.05
	PRD	3.46	3.40	3.48			
TA (g/L)	Control	7.74	7.32	7.14	<0.01	ns	ns
	PRD	6.82	6.76	6.22			

4.4 Conclusions

The pH and TA of grape juice are important criteria for juice and wine quality and largely determine the pH of wine after fermentation (Boulton, 1980). Low acid levels in harvested grapes can accompany either high or low sugar levels but produce unbalanced and flat wines (Ruffner, 1982a). The decrease in TA and the increase in pH observed with the PRD treatment on Cabernet Sauvignon in both vintages of the study, and in Shiraz in one vintage of the study would significantly affect the resulting TA and pH of the wine at the time of crushing. This would potentially decrease the quality of the fruit, and would additionally require addition of tartaric acid prior to fermentation, increasing the costs of winemaking. Furthermore, anecdotal evidence indicates that a threshold level of malic acid is required in wine post-fermentation, in order for malolactic fermentation to be initiated (P. Grbin, pers. comm..). Levels of malic acid below this threshold would result in 'stuck' malolactic ferments, and potentially increase the time of fermentation

and possibly require multiple additions of malolactic cultures. Again, this could lead to an increase in the costs of ferments. Thus, the current data do not indicate that PRD necessarily leads to an increase in fruit quality with respect to fruit acidity, although in some instances PRD caused no change in fruit pH or TA. Decrease in fruit acidity with PRD very likely demonstrates increased metabolism within the respiratory pathway, which does not necessarily lead to the reduction by oxygen but which could also indicate an increase in flux to pathways of secondary metabolism, which require respiratory pathway intermediates (Taiz and Zeiger, 1998). It is therefore possible that a reduction in grape berry acidity could equate with increased synthesis of secondary metabolites which are important compounds for grape and wine quality (Singleton, 1988).

However, it should be noted that high acid levels not only affect the palatability of grapes used as table varieties, but when they occur in certain varieties of winegrapes, they may be associated with low sugar concentrations, resulting in poor wine quality (Ruffner, 1982a). The increased rate of ripening in PRD fruit in some instances, in addition to potential decreases in TA, would prevent relatively 'unripe' fruit at the time of harvest. Furthermore, the current study has shown that working with crop loads that retain an adequate balance of vegetative and reproductive growth will prevent potential delays in fruit ripening and the limitation of final sugar levels attained. This will also allow for adequate levels of sugar and acidity to be attained by a given harvest date. Relevant outcomes of this work emphasize changes in malic acid as the primary factor governing final pH and TA as a result of the irrigation treatment. Thus, further research into the factors governing its metabolism will shed light on the potential to manipulate final pH of grapes and wine through viticultural practices. Chemical signals mediated by the PRD treatment have been shown to be the most likely reason for the decreases in malic acid content observed, rather than alterations in bunch microclimate caused by environmental factors such as bunch exposure and temperature. Further work into the effects of plant signals on the enzymes and products of the pathways of sugar and organic acid metabolism will be a relevant research objective for future research.

CHAPTER 5: THE EFFECT OF PRD ON GRAPE BERRY AND SEED DEVELOPMENT

5.1 Introduction

5.1.1 The pattern of grape berry development

The development of the grape berry has been described as a 'double sigmoid' growth curve, which is characterised by two stages in which berry size increases rapidly, with an intermediate or 'lag' stage in which there is little or no growth (Figure 5.1) (Coombe and Hale, 1973; Coombe, 1992; Coombe and McCarthy, 2000). The initial growth phase is characterised primarily by rapid cell division and expansion in both the cells of the seed and fruit pericarp (Coombe, 1960). It is during this stage that the final cell number of the seed and pericarp is determined (Coombe, 1976). In turn, this effectively limits the final volume that the berry can attain during the second growth stage where fruit size increases due to cell expansion only, rather than due to cell division (Coombe, 1976). Thus, any factor affecting cell number in the initial growth stage, will indirectly affect the final berry size attained at harvest.

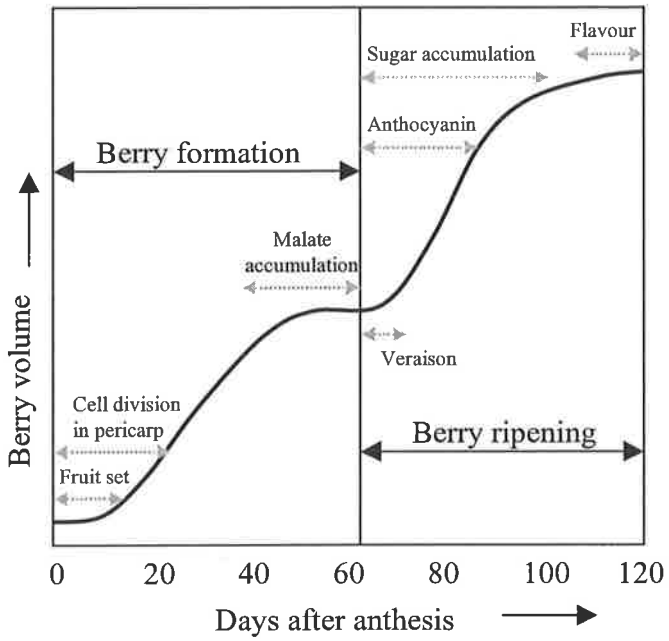


Figure 5.1: Typical double sigmoid growth pattern of a grape berry showing physiological processes associated with successive developmental stages (adapted from Coombe and McCarthy, 2000).

During the initial growth phase, the rate of cell division and expansion in the seed and pericarp reaches a maximum, and then declines as the fruit approaches veraison (Coombe, 1960). The rate of growth in the seed and pericarp has been shown to be similar during this period for the varieties Sultanina and Muscat of Alexandria, and generally reach a peak between 20-30 days after anthesis, which declines by approximately 45 days after anthesis (Coombe, 1960). During the second growth phase, the pericarp expands rapidly due to accumulation of photosynthate to reach two to three times its original size (Coombe, 1960). At this stage, although the embryo continues to develop, the seed has reached its maximum weight. Thus, the increase in fruit size observed is primarily due to the increase in pericarp volume (Coombe, 1960). A recent study by Ristic (2004) has classified seed development into three consecutive stages: I. Seed growth, which describes the initial phase of seed expansion to maximum fresh weight occurring up to veraison; II. A short intermediate phase when there is no change in seed fresh weight and the embryo continues to mature; III. Seed drying and maturation which occurs post-veraison, when seed fresh weight declines although the embryo continues to mature.

5.1.2 The effect of water deficit on berry size

The effect of water deficit bringing about a reduction in grape berry size has been well-documented, and is dependent upon the timing and severity of the deficit (McCarthy, 1997; McCarthy, 2000). It has been shown that berries are most sensitive to water deficit in the post-flowering period, with a less severe response when the stress is applied during the pre-veraison period (Smart *et al.* 1974; McCarthy, 1996; McCarthy, 2000). In both cases, the reduction in berry size is irreversible, even when optimal irrigation levels are restored following the deficit (Smart *et al.* 1974; Van Zyl, 1984; McCarthy, 1996; McCarthy, 2000). Following veraison, the application of water stress has been shown to have only a minor effect on berry size, which has been shown to be recoverable following re-application of optimum irrigation level to the vines (Van Zyl, 1984; McCarthy, 1996; McCarthy, 2000). It was initially proposed that the observed decrease in berry weight following an early-season deficit was due to a reduction in cell division and thus cell number (Matthews *et al.* 1987). It is possible that a post-flowering stress could cause a

reduction in cell division as the stress falls within the period where maximum rates of cell division have been reported in the pericarp (Harris, 1968). However, McCarthy (2000) suggested that this cannot explain the inability of the berry to expand when irrigation was restored following the post-flowering deficit, as mesocarp cells can potentially increase 300-fold or more during the phase of cell enlargement (Coombe, 1976). Rather, it was suggested that a pre-veraison stress reduced the ability of grape pericarp cells to expand. This hypothesis was supported by work which correlated the DNA content of grape pericarps to cell number, which indicated that a post-flowering deficit did not cause a reduction in cell division, but that the resulting restriction in berry volume was due to a limitation in pericarp volume alone (Ojeda *et al.* 1999, 2001). The reduction in the ability of the pericarp cells to expand was proposed to be most likely due to an alteration in cell wall synthesis and structure in response to the deficit, the properties of which are not yet known (Ojeda *et al.* 2001). However, in other plant species cell growth in terms of inhibition of cell expansion by water stress is closely followed by an inhibition in cell wall synthesis in rapidly growing tissues (Hsiao, 1973).

5.1.3 The influence of the seed on grape berry development

Although there is extensive literature describing the effect of water deficit of grape pericarp development, there has been little attention given to seed development under water stress. It has been suggested that final berry size is determined by pre- and post-flowering events, namely seed number and seed developmental characteristics (Ebadi *et al.* 1996a). Berry size differences have been shown to be closely associated with differences in the extent of seed development, particularly in the period after flowering (Coombe, 1960; Ebadi *et al.* 1996a,b) and it is conceivable that factors affecting seed development will similarly affect pericarp development.

Early work done on the effect of seed maturity on delayed ripening in cv. Concord grapes (Cawthon and Morris, 1982) indicated that the physiological and biochemical changes associated with veraison were only initiated following maturation of the seed, as determined by seed coat colour. The accumulation of ABA in pericarp tissues that is associated with veraison did not occur until the seeds matured, and where seed maturity

was delayed, veraison was delayed (Cawthon and Morris, 1982). However, as seedless grape varieties also show typical increases in ABA associated with veraison (Inaba *et al.* 1976), it appears that the presence of seeds is not necessary to induce ABA accumulation, but rather has the potential to delay or inhibit its accumulation in the pericarp. The changes occurring in the seed and the seed coat before and during veraison may therefore give an indication of seed maturity, and thus the timing of veraison.

5.1.4 Developmental changes occurring in the seed at veraison

Seed fresh weight increases until veraison, followed by a decline in seed fresh mass (Kennedy *et al.* 2000a). Although seed dry weight continues to increase slightly post-veraison, indicating that the seed continues to grow through the ripening period of fruit development, the timing of veraison is characterised by the onset of seed desiccation in terms of its fresh weight (Kennedy *et al.* 2000a). The underlying cause of this desiccation is unknown, but it has been reported that when a grape seed reaches maturity, it abscises from the placental tissue of the fruit, effectively removing the original nutrient and water supply (Skirvin, 1971). This may initiate the changes causing fresh weight loss in the whole seed. Prior to the onset of veraison, the seed coat accumulates tannins, in the form of both flavan-3-ol monomers and proanthocyanidin polymer (Kennedy *et al.* 2000a; Downey *et al.* 2003; Ristic, 2004).

The onset of veraison-associated changes in the berry has been linked to the accumulation of maximum levels of proanthocyanidin polymer (Kennedy *et al.* 2000a; Downey *et al.* 2003; Ristic, 2004). The increase of the flavan-3-ol monomers in the seed coat at veraison is much sharper that that for proanthocyanidin polymer, and generally peaks between one and three weeks later than the polymeric tannin, followed by a rapid decline (Kennedy *et al.* 2000a; Downey *et al.* 2003; Ristic, 2004). It has been proposed that the peaks in polymeric tannin and flavan-3-ol monomers coincides with the successive phases of seed development (Ristic, 2004); such that polymeric tannin peaks at the end of the initial seed growth phase and flavan3-ol monomers peak at the end of the intermediate phase of seed development, indicating the onset of ripening and pericarp

expansion. At the onset of the third phase of seed development where seed drying is initiated, the seed coat enters a phase of oxidation, where the extractability of the seed tannins declines, and the seed coat colour darkens (Kennedy *et al.* 2000a; Downey *et al.* 2003). The onset of this darkening in seed coat colour is what has been previously described as 'seed maturity' (Cawthon and Morris, 1982). The impact of environmental factors on the development of seed tannins has not yet been extensively studied. One study compared the effect of different irrigation regimes on seed tannin composition, and showed that the content of seed flavan-3-ol monomers per berry was decreased where the level of irrigation applied was reduced (Kennedy *et al.* 2000b). Irrigation treatments have not been shown to affect total proanthocyanidin content or composition, although a slightly higher rate of proanthocyanidin decline post-veraison has been reported under reduced irrigation (Kennedy *et al.* 2000b).

5.1.5 The contribution of seed tannins in vinification

Grape berry tannins are extracted during vinification, and contribute significantly to the chemical properties of the wine, namely in terms of organoleptic properties such as bitterness and astringency, and through their stabilising effect on wine colour (Somers and Evans, 1977; Robichaud and Noble, 1990; Gawel, 1998). These tannins are derived from the skin and the seed, and the two sources differ in their tannin composition and content. Both seeds and skins contain the flavan-3-ol monomers catechin, epicatechin and epicatechin-gallate, which together with epigallocatechin form the units of the more complex polymeric proanthocyanidins, although seeds and skins differ in their composition of flavan-3-ol subunits (Downey *et al.* 2003). At harvest, 75% of the total extractable proanthocyanidin is in the seeds, indicating that the seeds are an important source of tannin during vinification (Downey *et al.* 2003). However, as the degree of polymerisation of seed tannins is significantly lower in seeds than in skins, the contribution of the proanthocyanidins from the two sources will differ in their contribution to the sensory properties of the wine (Prieur *et al.* 1994; Souquet *et al.* 1996; Downey *et al.* 2003). Alterations in the quantity of seed-derived tannins per berry may affect the sensory properties of the resulting wines, depending on the length of vinification and wine style. As irrigation has not been shown to affect the composition of

grape seed tannins, but does affect the content of seed-derived flavan-3-ols and potentially proanthocyanidin polymer at harvest (Kennedy *et al.* 2000b), this has implications for winemaking.

The aim of the current study is to investigate the effect of the PRD irrigation technique on berry size, taking into consideration the contribution of seed size to final berry weight. Within this, possible alterations in seed development as a result of PRD will be determined, in terms of seed fresh weight and tannin composition. Finally, changes in berry size have implications not only for yield at harvest, but may also cause compositional changes in fruit and resulting wines. Changes in the concentration and content of seed-derived tannins in the fruit will be assessed during fruit development and at harvest, and will be discussed in terms of the implications for winemaking.

5.2 Materials and Methods:

5.2.1 Sample collection and processing

Twenty-five berry samples were collected from seven two-vine plots of three replicates of PRD and control irrigated vines of the Cabernet Sauvignon experiment at Langhorne Creek (Section 2.1.2, Chapter 2). Berries were stored at 4°C for transport, and then frozen at -80°C for later analysis. For processing, frozen berries were weighed, and then immediately de-seeded while still partially frozen. Seeds were dried on paper-towel, weighed, counted and then flash-frozen in liquid nitrogen. Frozen seed samples were immediately stored at -20°C. Due to a slight loss of berry juice due to fruit thawing during seed removal, pericarp weight was more accurately determined by subtracting total seed weight from the weight of the original 25-berry sample.

5.2.2 Seed tannin extraction

Seed tannins were extracted from whole seeds using an adaptation of the method of Ristic (2004). Frozen seeds were placed in a 50 mL centrifuge tube and covered with 40 mL 70% (v/v) acetone. The centrifuge tubes were sealed and placed on an orbital shaker in the dark. Seed tannins were extracted from whole seeds over 24 h. Acetone was removed from the seed tannin extracts using a rotary evaporator, with the water bath set at <35 °C. The remaining solution was immediately frozen at -20 °C. Frozen extracts were later thawed at 4 °C and diluted with water to give a final volume of 10 mL. The extract was then made up to 20 mL with methanol to give a final concentration of 50 % (v/v) methanol. The 50 % (v/v) methanol extract was then diluted 1:5 giving a final concentration of 10 % (v/v) methanol. The extracts were then filtered using a 0.45 μm membrane, and a 250 μL aliquot was transferred to an HPLC vial, and stored at 4 °C for less than 24 h prior to HPLC analysis.

5.2.3 HPLC analysis of the seed tannin extract

The method used was adaptation of that of Lambert (2002). The column used was a Merck LiChrospher RP C18 column (5 μm, 250 x 4 mm) preceded by a guard column of the same material. The solvents used were orthophosphoric acid (0.2% v/v) (Buffer A) and 20% Buffer A in acetonitrile (Buffer B). Twenty μL of the seed tannin extract was injected onto the column for pre-veraison and veraison samples, and 10 μL was injected for post-veraison samples. The elution scheme used a flow rate of 1 mL/min at 30 °C, with a linear gradient of Buffer B from 0 to 15% in 15 min, 15% to 16% from 15 to 40 min, 16% to 25% from 40 to 45 min, and isocratic up to 48 min. From 48 to 50 min Buffer B was increased 25% to 60%, and 60% to 100% from 50 to 55 min, then isocratic for 5 min, followed by a 5 min re-equilibration of the column to pre-run conditions. The final run time was 65 min. Standards of catechin, epicatechin and epicatechin-gallate were purchased from Extrasynthese (Germany). The method gave the elution times 19.8 min for catechin, 24.9 min for epicatechin and 46.5 min for epicatechin-gallate. Seed proanthocyanidin eluted as a single peak at 53.0 min. Quantities of flavan-3-ol monomers and proanthocyanidin polymer were calculated from a standard curve for catechin, which was linear within the range of concentrations of the current study (Figure 5.2).

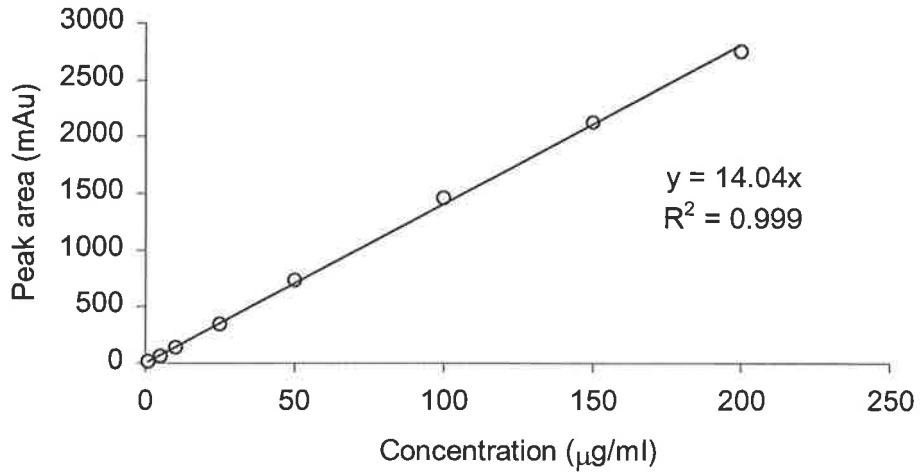


Figure 5.2: Standard curve for catechin

5.3 Results and Discussion

5.3.1 Berry and seed development

The physiological changes in the fruit at the onset of veraison were different for the two vintages studied. In 2001/2002, veraison was initiated on 24/01 where 5% of the berries were coloured (Figure 5.3). However, in both PRD and Control fruit for 2001/2002, the period defined as 'veraison' between which the fruit changed from 5% coloured to 100% coloured was unusually long, and only complete by 7/02 (Figure 5.3). The following year, the onset of veraison was recorded on 28/01 and the fruit rapidly coloured with veraison complete within one week (Figure 5.3). Despite the delay in the completion of veraison in 2002, in both seasons fruit reached maturity to 23.5°Brix within a similar time period, at approximately 54 and 50 days post-veraison for 2001/2002 and 2002/2003 respectively.

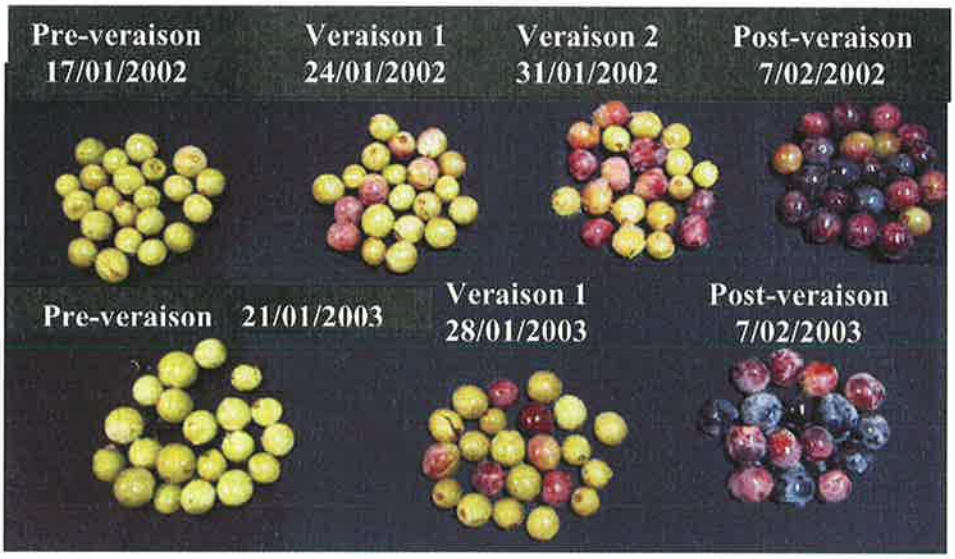


Figure 5.3: Timing of visual changes associated with veraison in Cabernet Sauvignon at Langhorne Creek in the 2001/2002 and 2002/2003 vintages.

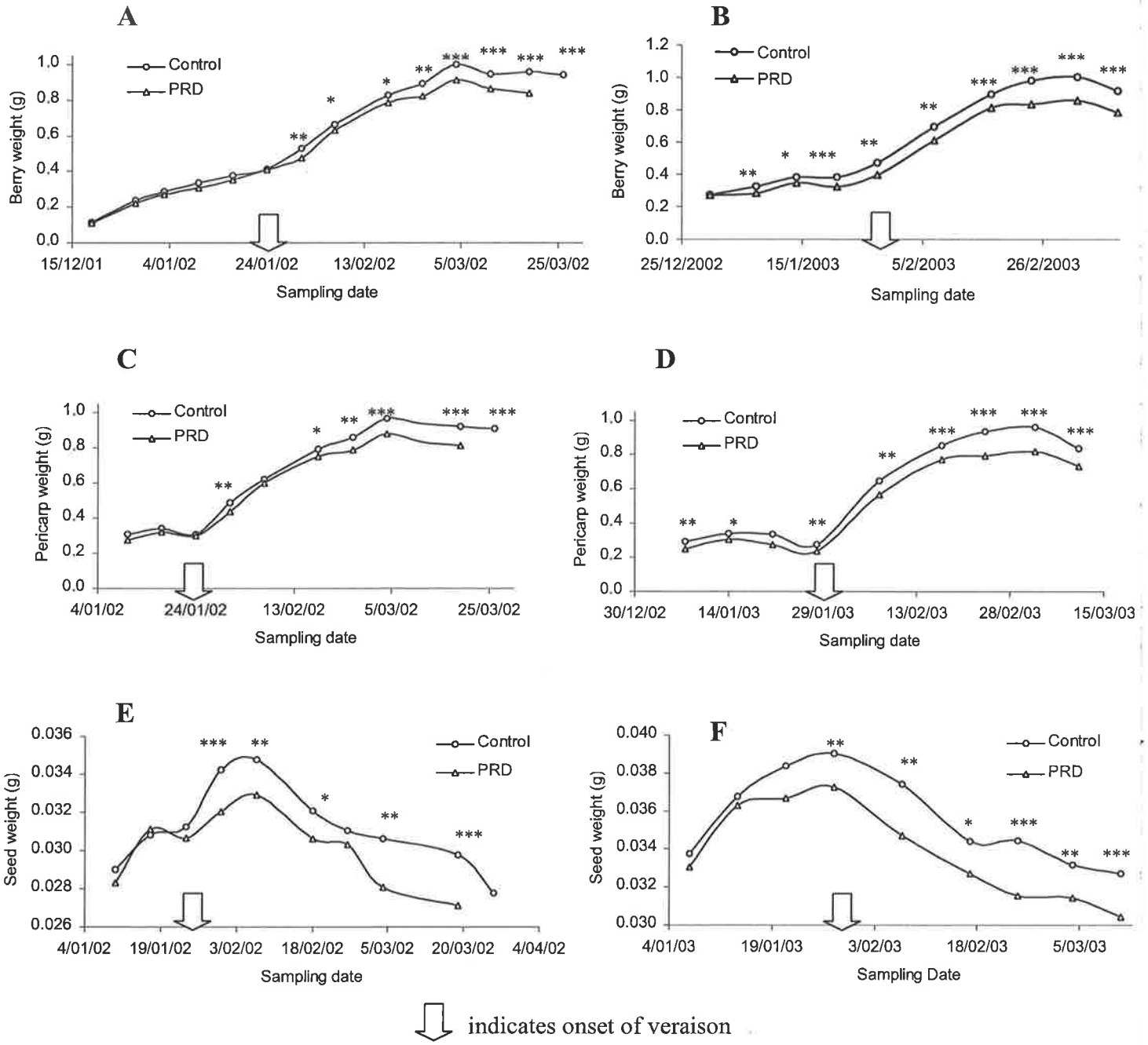


Figure 5.4 The effect of PRD on developmental changes in berry components for Cabernet Sauvignon at Langhorne Creek over two vintages. **A** Berry weight 2001/2002; **B** Berry weight 2002/2003; **C.** Pericarp weight 2001/2002; **D.** Pericarp weight 2002/2003; **E.** Seed weight 2001/2002; **F.** Seed weight 2002/2003 (ANOVA, n=42, asterisk indicates significant difference * = $P<0.05$; ** = $P<0.01$; ***=$P<0.001$; points of significance on 26/03/2002 indicate a difference between Control at 23.5°Brix and PRD from 19/03/2002 at 23.5°Brix).

Table 5.1: Maximum and final differences in the weight of berry components observed with PRD for Cabernet Sauvignon at Langhorne Creek for the 2001/2002 and 2002/2003 vintages.

Sampling date	Difference type	% Control>PRD		
		Seed	Pericarp	Berry
19/03/2002	Maximum	10	14	14
Harvest 2002	Final	1	12	12
24/02/2003	Maximum	9	18	18
Harvest 2003	Final	7	14	17

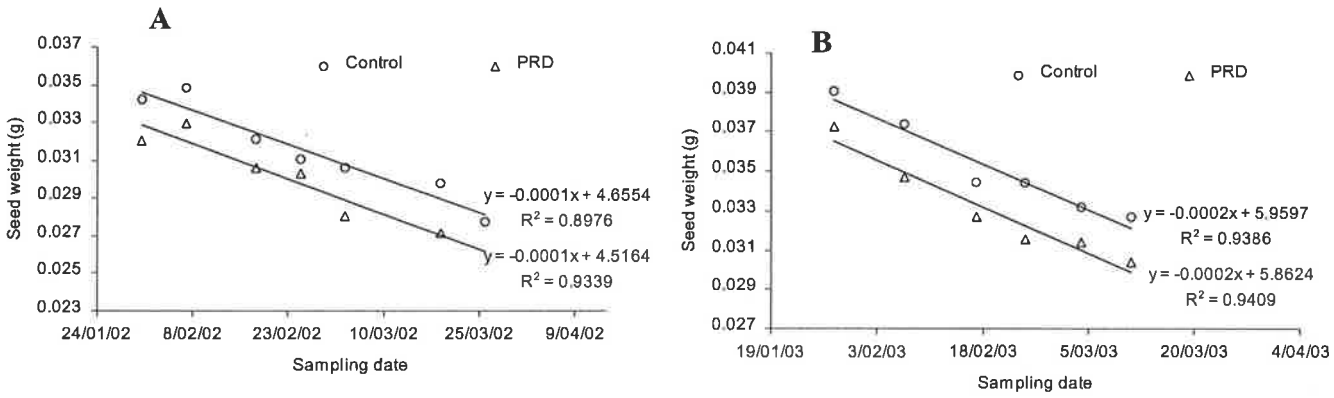


Figure 5.5 Linear regression analysis of the rate of seed fresh weight loss in PRD versus Control-treated Cabernet Sauvignon in **A.** 2001/2002, **B.** 2002/2003.

The pattern of berry growth showed the typical double sigmoid pattern (Figure 5.4 A, B) that has been described by Coombe and Hale (1973). The PRD treatment caused a restriction in the increase of berry volume, as was evident post-veraison. This occurred in both seasons of the study. In 2001/2002 this restriction in berry volume was not evident pre-veraison (Figure 5.4 A), whereas in 2002/2003 PRD-treated fruit had lower values of berry and pericarp weight from three weeks prior to veraison (Figure 5.4 B, D). In previous studies, the PRD treatment has been shown to have minimal effect on berry weight (Dry, 1997; Stoll, 2000) despite a 50% reduction in water applied, which would have been sufficient to cause a water deficit under a standard irrigation regime. The current data show that despite rigorous irrigation scheduling, the PRD regime brought about a typical water deficit response in terms of fruit weight (Ojeda *et al.* 1999, 2001).

Seed fresh weight increased to a maximum and then decreased due to desiccation (Figure 5.4 E, F). In the 2001/2002 vintage, seed fresh weight continued to increase after the onset of veraison, reaching a maximum on 7/02. In 2002/2003, however, seed fresh weight reached a maximum at veraison, following which seed desiccation occurred. A study by Ristic (2004) on cv. Shiraz fruit, showed a strong correlation between the completion of seed growth (as estimated by maximum fresh weight) and the onset of berry colouring. The 2001/2002 data may indicate that, compared with the 7 day 'veraison' period of 2002/2003, the relatively longer 14 day 'veraison' period of that season may have been caused by a slightly longer, or possibly delayed period of seed growth. A comparison of climatic data between the two seasons for the month of January at Langhorne Creek showed that in 2002 there were 3 days where the temperature was 35 ºC or greater, whereas in 2003 there were 9 such days (Appendix D). This could have hastened fresh mass accumulation in the seed and pericarp in the 2002/2003 season (Ebadi *et al.* 1996a). This suggestion brings into consideration the results of Cawthon and Morris (1982), who showed that a delay in seed maturation caused a delay in the increase in ABA in berry tissues associated with the onset of berry ripening and fruit colouring.

In both vintages, the PRD treatment resulted in lower seed fresh weight relative to the control, which was evident from one week pre-veraison (Figure 5.4 E,F). Seed number was not affected by the PRD treatment (results not shown), and the mean number of seeds per berry was 1.16 and 1.25 in 2001/2002 and 2002/2003 respectively. The percentage differences in pericarp, seed and berry weights between the PRD and Control treatments for both vintages are shown in Table 5.2. On the dates when the greatest difference in berry weight was observed in response to PRD, this was reflected in maximal differences in both seed and pericarp weight. However, PRD caused a greater reduction in pericarp weight than seed weight, which is more clearly evident in the final sampling date at harvest (Figure 5.4 C,D). Until now, only the restriction in pericarp weight has been considered as the primary factor contributing to a water-deficit induced decrease in berry mass (Ojeda *et al.* 2001). However, as seed weight contributes only 3-5% of berry weight at harvest, the influence of the PRD treatment on seed weight will have little influence on final fruit yield. The reduction in seed weight observed with PRD

may reflect changes in seed developmental physiology caused by the water deficit. It must be noted that in both seasons, the lower seed weight due to PRD irrigation only became evident during the period of seed expansion. This indicates that physiological changes in seed development occurring before or close to the onset of seed expansion were affected` by the PRD treatment. According to the work of Ojeda *et al.* (1999, 2001) a water-deficit induced restriction in pericarp volume is caused by a limitation in cell volume rather than by a decrease in cell number in the pericarp. Assuming that seed and pericarp growth would be similarly affected by the prevailing environmental conditions, it is possible that a deficit was experienced by the PRD-treated vines in both seasons of the study. In 2003, PRD vines entered water deficit relative to controls close to the time of seed expansion, as shown be a decrease in stem water potential (Figure 3.3 B). This may later have resulted in a limitation in the ability of seed cells to expand during the period of rapid growth. During the period of seed desiccation, a linear regression analysis of the slope of fresh weight decrease over time was the same for both the PRD and control treatments, even though seed weight was lower in the PRD-treated seeds (Figure 5.5). This indicates that the rate of water loss from the seed post-veraison was not increased by the PRD-treatment, which again suggests a pre-veraison restriction in seed expansion as the primary factor limiting seed weight in the PRD treatment.

5.3.2 Seasonal variation in the developmental profile of seed tannins

In both seasons of the study, total flavan-3-ol monomers (catechin, epicatechin and epicatechin-gallate), as well as polymeric tannin (proanthocyanidin) increased to a maximum up to the veraison period, followed by a decline toward harvest (Figures 5.6 and 5.7). The timing of the peaks of each of these tannin categories, expressed per seed, differed between seasons. In 2002, proanthocyanidin levels were at a maximum on 24/01, coinciding with the onset of veraison as observed by 5% of fruit colouring (Figure 5.6). The levels of flavan-3-ol monomers peaked on 7/02, coinciding with the date on which 100% of fruit was coloured, which has been termed 'post-veraison' (Figure 5.6). The results were quite different for the 2002/2003 season. In that vintage, flavan-3-monomers reached high levels at the onset of veraison, although the maximum level per seed occurred when veraison was complete, or 'post-veraison' (Figure 5.7). Proanthocyanidin

polymer reached maximum levels per seed only post-veraison (Figure 5.7). Given that the essential difference between the two vintages was the length of the 'veraison' period, the current data can be interpreted to reflect this difference.

According to the observations of Ristic (2004), the completion of seed expansion and the onset of veraison is signalled by a peak in polymeric proanthocyanidin, whereas the post-veraison events signalling the onset of berry ripening and the increase of pericarp volume are characterised by a peak in seed flavan-3-ol monomers. In 2002, the peaks in the quantity of proanthocyanidin polymer and flavan-3-ol monomers per seed were approximately two weeks apart, which indicates a delay in the completion of veraison. Despite this, the pericarp had begun to expand before the peak in flavan-3-ol monomers had occurred (Figure 5.4 C), although berry colouring was delayed (Figure 5.3). In 2003, veraison was complete within one week (Figure 5.3). The changes in seed tannins indicate that the maximum levels of flavan-3-ols and proanthocyanidin occurred on the same date, post-veraison. These data show that where the time taken for the fruit to complete the physiological changes associated with veraison was reduced relative to the previous season, that the peaks in flavan-3-ol monomers and proanthocyanidin polymer were shifted closer together.

The enzyme which mediates the conversion of leucocyanidin, the precursor of both the flavan-3-ols and proanthocyanidins, is not yet known (Davies and Schwinn, 2003). Presumably the flavan-3-ols and proanthocyanidins would act as competitive sinks for this precursor at different stages of seed and berry development, which may indicate alterations in the activity of one or more enzymes mediating the final stages of this pathway (Kennedy *et al.* 2000). The data of the current study have shown that where pre- and post-veraison stages in fruit development are clearly defined, the separation of the stages of proanthocyanidin and flavan-3-ol synthesis in the seed can serve as biochemical markers for the onset and termination of veraison. However, seasonal differences which can potentially affect fruit development (Appendix D) may have caused an acceleration of the biochemical changes occurring in the seed at veraison, which resulted in a similar acceleration in the completion of veraison and the onset of berry ripening.

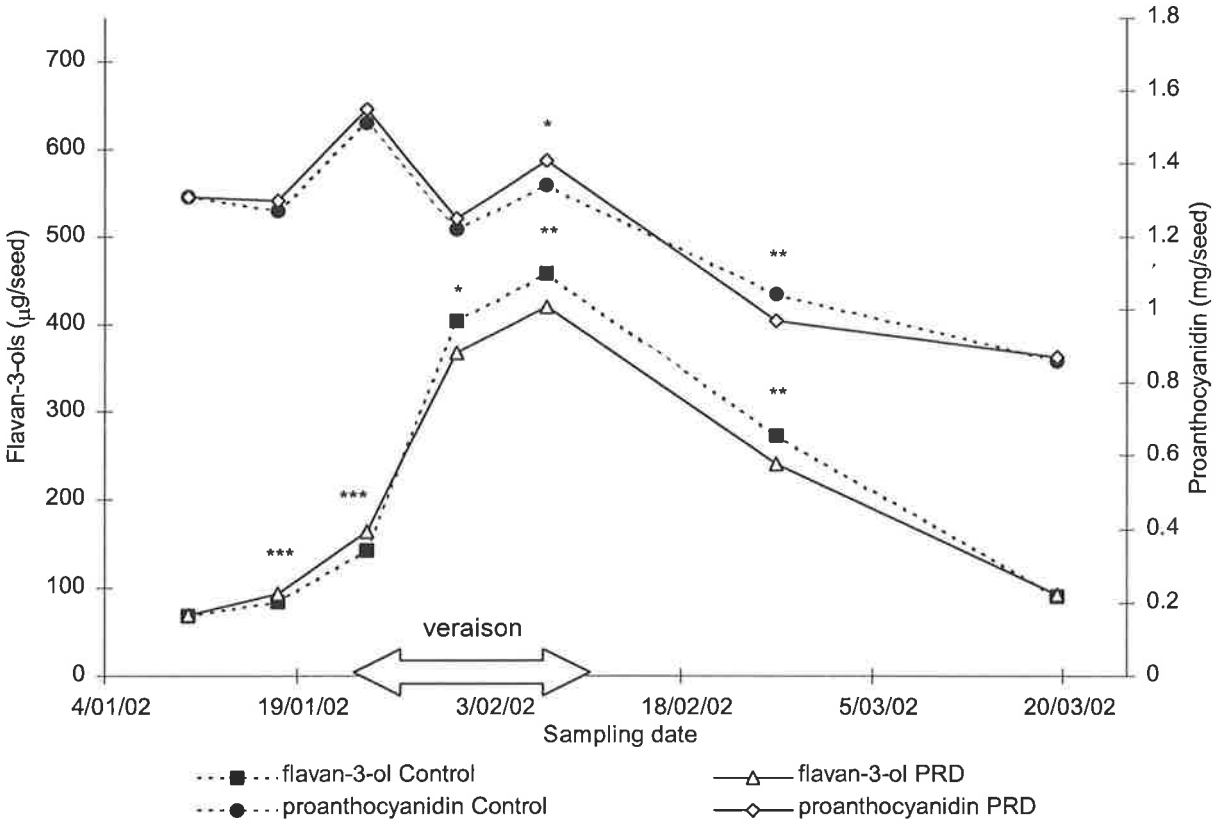


Figure 5.6: Developmental profile of seed proanthocyanidin (mg/seed) and flavan-3-ol monomers (µg/seed) in PRD- and Control-treated Cabernet Sauvignon fruit from Langhorne Creek (2001/2002 vintage) (ANOVA; n=42; * = P<0.05; ** = P<0.01; *** = P<0.001).

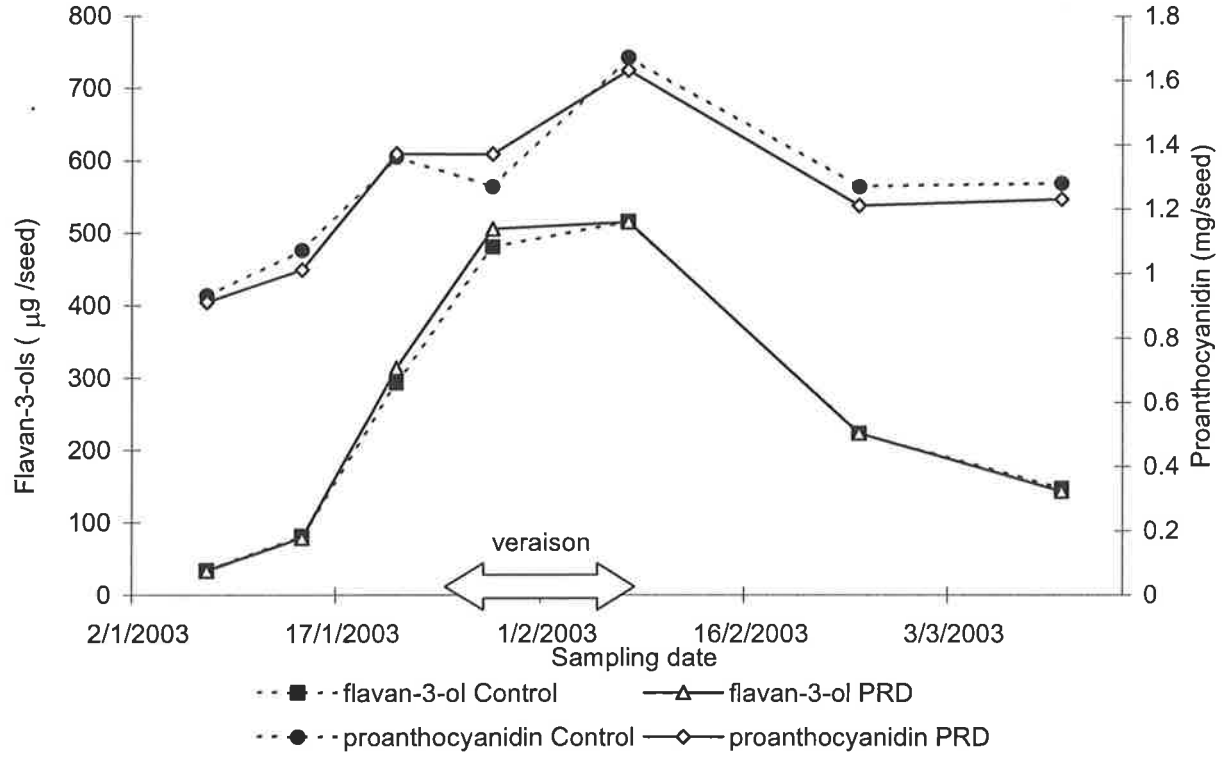


Figure 5.7: Developmental profile of seed proanthocyanidin (mg/seed) and flavan-3-ol monomers (µg/seed) in PRD- and Control-treated Cabernet Sauvignon fruit from Langhorne Creek (2002/2003 vintage) (ANOVA; n=42; no significant differences).

5.3.3 The effect of PRD on grape seed tannin composition

In both vintages of the study, the PRD irrigation treatment did not significantly alter the timing of the peaks of total proanthocyanidin or flavan-3-ol monomers relative to the control treatment (Figures 5.6, 5.7). Pre-veraison, and at the onset of veraison in 2002, total flavan-3-ols per seed accumulated to significantly higher levels in the PRD treatment (Figure 5.6). When each flavan-3-ol monomer was studied individually, the pre-veraison and veraison fruit of the PRD treatment had higher epicatechin and epicatechin-gallate levels when expressed per seed, per unit seed weight, per berry or per unit berry weight (Table 5.2). Catechin was only significantly higher in the PRD fruit at the onset of veraison in 2002, for all units of expression (Table 5.2). Interestingly, these initially higher levels of flavan-3-ol monomers were lower per seed in PRD-treated fruit post-veraison, that is, at the completion of berry colouring (Figure 5.6; Table 5.2).

This difference was not significant when expressed per unit seed weight (Table 5.2), so that the assumption can be made that the decrease in seed weight observed under PRD irrigation at this date is responsible for this decrease, which would have decreased the total surface area of the seed coat. In 2002/2003, there were no significant differences in flavan-3-ol content per seed due to the PRD treatment (Figure 5.7; Table 5.3). However, when seed size differences were taken into consideration by expressing flavan-3-ol concentration per gram of seed weight, the veraison and post-veraison periods were characterised by higher levels of the flavan-3-ols in PRD-treated fruit (Table 5.3). By harvest, differences in flavan-3-ol monomers per seed or per gram seed weight were small or negligible for both vintages of the study, most likely due to oxidation of those present in the seed coat at veraison. PRD did not have a clear significant effect on the content of proanthocyanidins per seed in either 2001/2002 or 2002/2003 (Figures 5.6 and 5.7; Tables 5.2 and 5.3). However, as for the flavan-3-ols, when seed size differences were taken into consideration by expressing proanthocyanidin concentration per unit seed weight the veraison and post-veraison concentration was increased by PRD in both seasons of the study (Tables 5.2 and 5.3). Thus, PRD was shown to have little effect on seed tannins which was carried through across both vintages of the study, except when expressed as a concentration per unit seed weight.

Table 5.2: The effect of PRD on developmental changes in seed flavan-3-ols and polymeric proanthocyanidin in Cabernet Sauvignon for the 2001/2002 vintage (ANOVA; n=42; * = P<0.05; ** = P<0.01' ***=P<0.001; ns = not significant; nd = not determined).

Unit of concentration												
Catechin	µg/seed			µg/mg seed wt			µg/berry			µg/g berry wt		
	Control	PRD	P	Control	PRD	P	Control	PRD		Control	PRD	P
Pre-veraison	28.95	30.93	ns	0.94	0.99	ns	32.46	34.96	*	86.88	100.2	***
Veraison	14.61	15.41	**	0.47	0.50	*	15.56	16.78	***	46.01	51.00	**
Post-veraison	154.07	141.64	ns	4.49	4.42	ns	189.7	169.8	ns	358.19	361.1	ns
Harvest	13.70	14.10	ns	0.51	0.52	ns	17.43	16.52	ns	18.73	20.29	*
Epicatechin	µg/seed			µg/mg seed wt			µg/berry			µg/g berry wt		
	Control	PRD	P	Control	PRD	P	Control	PRD	P	Control	PRD	P
Pre-veraison	14.57	17.43	***	0.47	0.56	***	16.47	19.77	**	44.09	56.94	***
Veraison	56.68	65.33	**	1.81	2.13	***	60.22	71.08	***	176.61	216.1	***
Post-veraison	258.86	235.55	*	7.38	7.20	ns	330.2	284.8	***	501.28	454.1	*
Harvest	48.10	47.38	ns	1.70	1.75	ns	58.45	55.81	ns	62.77	68.42	ns
Epicatechin-gallate	µg/seed			µg/mg seed wt			µg/berry			µg/g berry wt		
	Control	PRD	P	Control	PRD	P	Control	PRD	P	Control	PRD	P
Pre-veraison	40.20	47.09	***	1.31	1.51	***	45.15	53.31	***	120.94	153.4	***
Veraison	71.31	83.31	***	2.28	2.72	***	75.95	90.74	***	223.01	276.6	***
Post-veraison	177.29	160.94	**	5.08	4.92	ns	227.71	195.7	***	345.78	308.9	*
Harvest	27.74	30.82	*	0.98	1.14	**	33.55	35.87	ns	36.40	44.30	***
Proanthocyanidin	mg/seed			µg/mg seed wt			mg/berry			mg/g berry wt		
	Control	PRD	P	Control	PRD	P	Control	PRD	P	Control	PRD	P
Pre-veraison	1.27	1.30	ns	41.15	41.72	ns	1.42	1.48	ns	3.81	4.29	*
Veraison	1.51	1.55	ns	48.50	50.49	*	1.61	1.68	*	4.75	5.15	*
Post-veraison	1.34	1.41	*	39.17	43.38	**	1.74	1.73	ns	2.65	2.77	ns
Harvest	0.86	0.87	ns	30.61	32.10	ns	1.05	1.02	ns	1.14	1.25	**

Table 5.3: The effect of PRD on developmental changes in seed flavan-3-ols and polymeric proanthocyanidin in Cabernet Sauvignon for the 2002/2003 vintage (ANOVA; n=42; * = P<0.05; ** = P<0.01' ***=P<0.001; ns = not significant).

Unit of concentration												
Catechin	µg/seed			µg/mg seed wt			µg/berry			µg/g berry wt		
	Control	PRD	P	Control	PRD	P	Control	PRD		Control	PRD	P
Pre-veraison	126.40	138.10	ns	3.30	3.79	ns	160.4	173.1	ns	423.0	547.0	**
Veraison	237.22	255.62	ns	4.78	5.84	**	312.83	329.6	ns	978.5	1227	**
Post-veraison	261.37	264.09	ns	5.82	6.73	**	337.80	348.1	ns	487.90	598.4	**
Harvest	69.87	68.99	ns	2.25	2.38	ns	86.75	88.55	ns	99.47	116.7	*
Epicatechin	µg/seed			µg/mg seed wt			µg/berry			µg/g berry wt		
	Control	PRD	P	Control	PRD	P	Control	PRD	P	Control	PRD	P
Pre-veraison	60.5	64.4	ns	1.58	1.76	ns	76.5	80.8	ns	202.1	255.6	***
Veraison	100.09	102.46	ns	2.02	2.33	**	132.41	132.8	ns	414.75	491.8	*
Post-veraison	134.66	132.60	ns	2.99	3.39	*	173.95	174.7	ns	251.59	298.6	*
Harvest	63.85	61.21	ns	2.05	2.10	ns	79.86	78.60	ns	91.81	103.6	ns
Epicatechin-gallate	µg/seed			µg/mg seed wt			µg/berry			µg/g berry wt		
	Control	PRD	P	Control	PRD	P	Control	PRD	P	Control	PRD	P
Pre-veraison	106.6	111.1	ns	2.79	3.04	ns	134.9	139.2	ns	356.0	437.0	***
Veraison	143.04	147.46	ns	2.87	3.35	**	189.21	190.9	ns	589.36	704.6	**
Post-veraison	121.67	118.63	ns	2.68	3.01	*	157.83	156.5	ns	227.97	268.1	*
Harvest	13.53	12.83	ns	0.44	0.44	ns	17.00	16.52	ns	19.78	21.74	ns
Proanthocyanidin	mg/seed			µg/mg seed wt			mg/berry			mg/g berry wt		
	Control	PRD	P	Control	PRD	P	Control	PRD	P	Control	PRD	P
Pre-veraison	1.36	1.37	ns	35.67	37.67	*	1.72	1.71	ns	4.55	5.53	***
Veraison	1.27	1.37	ns	25.12	31.11	***	1.68	1.78	ns	5.27	6.67	***
Post-veraison	1.67	1.63	ns	37.42	41.67	*	2.16	2.16	ns	3.12	3.68	*
Harvest	1.28	1.23	ns	40.82	41.89	ns	1.59	1.57	ns	1.83	2.06	**

5.3.4 Hypothesis for seed tannin production under PRD irrigation

According to the difference in the results obtained for seed tannin content per seed, and concentration per gram of seed weight, an hypothesis is proposed below (Figure 5.8). With the slight reduction in seed size observed at veraison in PRD-treated fruit, the surface area occupied by the seed coat would have been similarly reduced. As seed flavan-3-ols and proanthocyanidins are located almost exclusively in the seed coat (Pratt, 1971; Thorngate and Singleton, 1994) the area over which seed tannin was produced at this stage would have been decreased in smaller seeds. Thus, any increases in the concentration of seed proanthocyanidin or flavan-3-ols per g seed weight would reflect an increased synthesis within the seed coat (Figure 5.8).

This increase in seed tannin would not necessarily have been reflected on a per seed mass basis, as larger seeds in the control treatment would have a larger seed coat area, and thus a larger area for tannin production. Therefore, where seed flavan-3-ols and/or proanthocyanidin concentration was increased by PRD on a per gram seed weight basis, this was assumed to reflect an increase in synthesis in the seed coat itself. At the end of veraison (post-veraison), the oxidation of the seed tannins has already commenced (Kennedy *et al.* 2000a). Therefore, although the levels of proanthocyanidin and/or flavan-3-ols were at a maximum in the seed coat, the concentration of these compounds does not reflect synthesis alone. Direct comparison of their concentration between irrigation treatments is not possible at this stage. However, the increase in seed tannin concentration initiated by the PRD treatment at the onset of veraison very likely reflects an increase in synthesis at this stage, as oxidation of seed tannins would not yet have commenced.

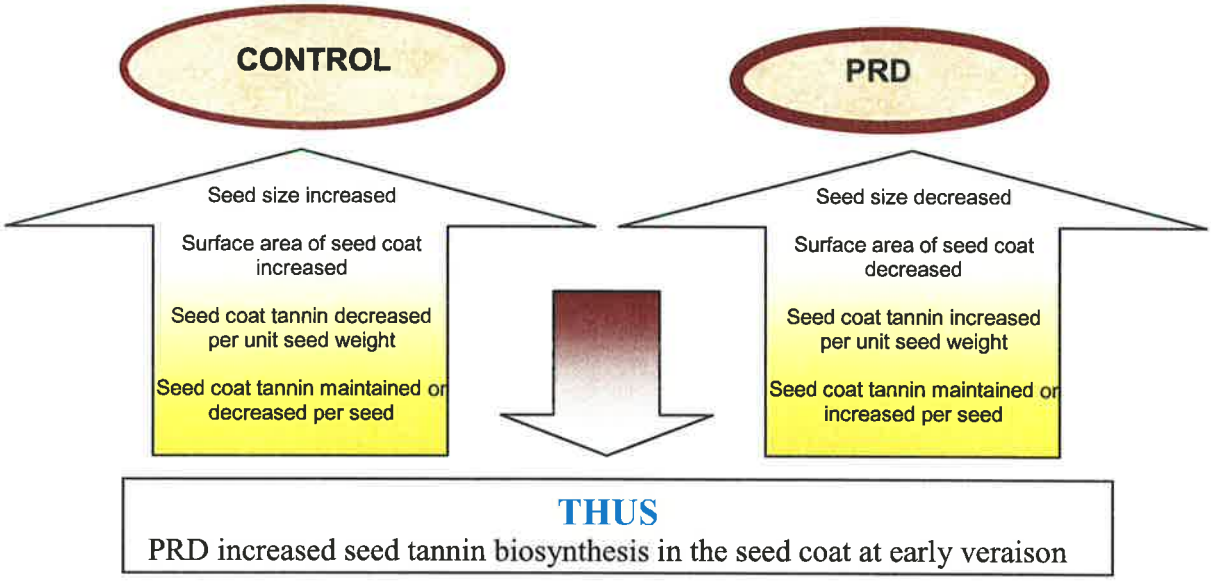


Figure 5.8: Hypothesis for seed tannin production under PRD irrigation

In order to discuss the underlying biological implications of this increase in seed tannin synthesis in the seed coat, it must be looked at in the light of seed coat function. Apart from being a conduit and source of nutrients in early seed development (King *et al.* 1997; Sheen *et al.* 1999; Weber *et al.* 1995; Wobus and Weber, 1999), the seed coat has a dual function in the mature seed by a) conferring resistance to pests and pathogens and b) promoting seed dormancy and preventing precocious germination under adverse environmental conditions. It is the latter of these two functions that needs consideration in the current study, as the implication of a deficit-irrigation strategy is that reproductive organs will receive metabolic signals from the parent plant, which may regulate development of the seed to ensure survival under a perceived environmental stress. For example, the seed coat may have a high degree of impermeability to water or oxygen, thus inhibiting germination even under favourable environmental conditions (Villiers, 1987). Furthermore, phenolic compounds in seed coat cells have shown to increase the impermeability of seed coats to water, reducing their germinability (Marbach and Mayer, 1974). In grape seeds, the bulk of phenolic compounds occur in the seed coat, in the form of seed proanthocyanidins and flavan-3-ol monomers (Fuleki and Ricardo da Silva, 1997; Peng *et al.* 2001). As these compounds undergo a complex process of oxidation during the later stages of seed development, decreasing their solubility and reactivity to oxygen,

(Kennedy *et al.* 2000a; Downey *et al.* 2003; Ristic, 2004) this leads to a concomitant decrease in seed coat permeability to oxygen (Werker, 1980/81; Werker, 1997) and water (Marbach and Meyer, 1974; Werker *et al.* 1979). Thus, seeds with a higher level of oxidised tannin may have a higher degree of seed coat impermeability and thus resistance to germination under adverse environmental conditions.

To date, no research has been done in grape seeds with respect to the effect of seed phenolic concentration on seed germinability. In grapevines, ABA accumulation during seed maturation has been associated with seed dormancy, and its diffusion out of grape seeds is necessary to ensure their germination (Broquedis, 1984; le Page-Degrivy, 1990). In *Vitis labruscana* cv. Campbell Early grape seeds, decreased germination potential of the seeds was correlated with increased ABA levels in the flesh and seeds (Liu *et al.* 1999). Furthermore, precociously germinating somatic embryos of *Vitis vinifera* have been found to have lowered ABA levels relative to those found in zygotic embryos early in embryo development, and this is thought to be responsible for the phenomenon (Faure *et al.* 1998). Interestingly, the localisation of ABA in grape seed cells has been investigated using immunogold electron microscopy, and has shown that ABA is localised in electron-dense substances, thought to be phenolic compounds (Huang *et al.* 1999). The potential exists that ABA, which is directly responsible for regulating the germination potential of grape seeds, is associated with seed phenolic compounds, in all likelihood the seed tannins. Thus, there is a strong likelihood that grape seed tannins are responsible for determining the permeability of the seed coat and henceforth the germinability of the seed, in association with other metabolites such as ABA.

In PRD-treated vines, the developing fruit will receive hormonal and biochemical signals that indicate the plant is experiencing a water deficit, even though the plant itself is not under hydraulic stress (Dry *et al.* 2000). PRD has been shown to cause an increase in xylem sap ABA levels in response to partial drying of the rootzone, although leaf water potential was unaffected by the treatment (Stoll *et al.* 2000). In fruit, alterations in xylem sap ABA have been correlated with changes in ABA concentration of fruit tissues, in both immature and developing grape berries (Antolin *et al.* 2003). As signals in the

xylem sap are transmitted to fruit, presumably developing seeds will respond to these signals indicating a water deficit, as is evident from the seed weight decrease demonstrated in the current study. A peak in ABA has been observed in both grape seeds and flesh shortly before veraison (Xu *et al.* 1995) and it is noteworthy that the changes in seed tannins associated with the PRD treatment occur around the time of veraison, before seed desiccation and tannin oxidation commences. It is unlikely that differences in seed tannin concentration would be observed in mature seeds, as oxidation is advanced and little tannin can be extracted. Thus, any initial differences in total tannin prior to oxidation would not be seen. The fact that PRD caused an increase in seed flavan-3-ols and proanthocyanidin per gram seed weight at veraison could reflect an increase in seed coat impermeability following oxidation of that tannin in mature seeds. This hypothesis requires further testing, including analysis of ABA levels in developing seeds under PRD and also an assessment of seed germinability of PRD-treated seeds, which is outside the scope of the current study.

5.3.5 Seed tannin concentration in fruit and wine

Irrigation deficit has been shown to cause a reduction in seed flavan-3-ol content per berry at harvest, although there was little effect on the content of seed proanthocyanidin per berry (Kennedy *et al.* 2000b). In that study, the content of seed polyphenols per seed, per gram seed weight or per gram berry weight was not considered. Thus, the potential of reduced berry weight under deficit to alter the final ratio of seed tannins to extracted juice in the wine product was not considered. In the current study, there was little effect of PRD on the final concentration of seed flavan-3-ols and proanthocyanidin per berry at harvest in both vintages (Tables 5.2 and 5.3). However, due to a significant decrease in berry weight under PRD irrigation, the treatment significantly increased the concentration of both seed flavan-3-ols and proanthocyanidin per gram of berry weight. As PRD did not affect the content per seed in most cases in the current study (Tables 5.2 and 5.3) at harvest, this increase was most likely due to decreased pericarp volume with PRD in both seasons, which altered the relative concentration per gram berry weight between PRD- and control-treated fruit. This result shows that a smaller pericarp volume in fruit from

water deficit-treated vines has the potential to alter the ratio between seed tannin and the available volume of juice which can be extracted from the fruit. Although, extraction efficiency of seed tannins during fermentation would be variable, the potential exists that where the concentration of seed tannins is increased by a deficit treatment such as PRD, this could result in a higher concentration per unit volume of wine. This would affect the astringency (Robichaud and Noble, 1990, Gawel, 1998) of the resultant wines. In the current study, the contribution of seed tannin to total wine phenolics has not been assessed, as there are a number of phenolic compounds which occur in wines such as anthocyanins, seed tannins, skin tannins as well as other phenolics e.g. gallic acid, caffeic acid and caftaric acid (Ribereau-Gayon and Glories, 1986; Singleton, 1988; Lambert, 2002). However, total polymer or 'tannin' which is a combination of seed and skin tannin together with bound anthocyanin, was increased in wines made from PRD-treated fruit, which resulted in an overall improvement in wine colour (Table 6.7). A recently-developed method has allowed for the distinction between seed and skin tannins in wine based on the difference in the cleavage products of proanthocyanidin in the presence of phloroglucinol (Peyrot des Gachons and Kennedy, 2003). This showed that by the end of fermentation the contribution of skin and seed proanthocyanidin to the total in red wine is equal (Peyrot des Gachons and Kennedy, 2003), which indicates that seed tannins will contribute significantly to the final phenolic properties of the wine. Whether increasing the proportion of seed tannins in wine phenolics would confer a benefit or disadvantage to the winemaker is not yet clear.

The contribution of seed tannin as a copigment to wine anthocyanins is the source of debate (Boulton, 2001). Scheffeldt and Hrazdina (1978) showed that a grape seed extract did not significantly alter the colour response of 200 μM malvidin 3,5 diglucoside at pH 3.2. However, a recent study by Lambert (2002) showed that the addition of a grape seed extract to 500 μM malvidin-3-monoglucoside enhanced the absorbance of a malvidin-3-glucoside solution, albeit a small response relative to that observed in the presence of flavanols. In that study, the contribution of monomers, dimers, oligomers or proanthocyanidins from the seed extract to the colour response could not be distinguished. The individual components of grape seed extract have shown variable

responses in the presence of oenin solutions, with catechin and epicatechin decreasing (Malien-Aubert *et al.* 2001, 2002) or improving (Darias-Martin *et al.* 2001; Lambert, 2002) colour intensity and stability. In the presence of procyanidin dimers or trimers, the red colour of an oenin solution has been reported to be more stable under thermal conditions (Malien-Aubert *et al.* 2002; Lambert 2002). Thus, the effect of seed tannin on wine colour is still not clearly understood and requires further investigation. PRD has the potential to increase the contribution of seed tannins to the final wine volume, and may therefore affect both wine astringency and colour stability.

5.4 Conclusions

PRD caused a significant decrease in berry weight, which was reflected in decreases in the weight of both the pericarp and seed. The degree to which pericarp weight was restricted under the PRD treatment was greater than that for the seed. Decreased seed weight due to PRD resulted in an increase in both seed proanthocyanidin and flavan-3-ols per gram seed weight at certain stages of fruit development. This may reflect increased synthesis in the seed coat in response to signals from the plant under PRD treatment, e.g. ABA in the xylem sap. As a result of the decreased pericarp weight, the contribution of seed tannins to final berry weight, i.e. seed tannin per gram berry weight, was significantly increased with PRD. This could potentially influence the concentration of seed tannins in vinification, which would affect wine astringency and potentially colour stability through copigmentation and polymerisation of anthocyanin pigments and seed tannins. Finally, a delay in the completion of veraison was observed in the 2002 season which was also associated with a delay in seed maturity. This resulted in clear separation of the timing of increase in a) proanthocyanidin at the onset of veraison, and b) flavan-3-ols at the end of veraison (post-veraison) in seeds. In the following season, veraison was complete within one week, and in that year there was no clear separation of these two events, which occurred at the end of veraison (post-veraison) rather than at the onset of veraison.

CHAPTER 6: THE CONCENTRATION OF ANTHOCYANIN AND PHENOLICS IN GRAPES AND WINE – A QUESTION OF BERRY SIZE?

6.1 Introduction

6.1.1 Berry size as an indicator of fruit and wine colour

Within the wine industry, there is a commonly held perception that small berry size equates with better wine quality, as is measured by wine colour (Matthews and Anderson, 1988; Dry *et al.* 1999; McCarthy, 2000; Ojeda *et al.* 2001). This is proposed to be due to a change in the surface area to volume ratio where fruit size is reduced, leading to an increased proportion of skin solutes (e.g. anthocyanin) extracted into a smaller volume (berry sap) in wines made from smaller berries.

Data justifying this concept has primarily been drawn from pooled samples of cv. Shiraz fruit taken from a range of sources and treatments, and have indicated a significant negative correlation with berry size and anthocyanin concentration on a per gram fruit mass basis (Botting *et al.* 1996; Gray *et al.* 1997). However, anthocyanin concentration was also shown to correlate negatively with yield, bunch number and shoot length; and was positively correlated with canopy density and bunch exposure (Botting *et al.* 1996). These additional factors indicate that berry size may not operate in isolation to bring about changes in anthocyanin concentration. Furthermore, these correlations have not yet been described for fruit sourced at the same degree of fruit ripeness, which has a significant impact on fruit size, particularly in cv. Shiraz where berry shrivel occurs at higher °Brix levels (McCarthy, 1999; McCarthy and Coombe, 1999). A study by Singleton (1972) removed or added free run juice to wine musts from a range of red grape varieties prior to fermentation, in order to simulate a 10% increase or decrease in berry weight without a change in berry composition. In that study, removal or addition of free run juice led to a comparable concentration or dilution effect on flavonoid composition and wine colour, but did not result in significantly detectable sensory differences between wines. Furthermore, it was apparent that removal of juice caused a limitation in the extractability of substances from berry skins. Although it provides an

interesting reference point for the potential effect of berry size on wine composition, the study by Singleton (1972) ignored the possible effect of increased skin weight or anthocyanin content in larger fruit, and made the assumption berry composition would remain the same for a given increase or decrease in berry size. To more accurately describe the effect of berry size on grape and wine composition it will be necessary to compare the composition of fruit and wines from a range of berry sizes produced under field conditions.

6.1.2 The effect of irrigation on anthocyanin concentration in fruit and wine

A second area where a possible effect of berry size on anthocyanin concentration has been identified is the effect of water stress, resulting either from a regulated deficit, reduced water application or non-irrigated vines. An early study on cv. Shiraz first shed light on this phenomenon (Freeman, 1983). That study looked at resultant fruit size and wine colour of four pruning levels from 20 to 160 nodes, superimposed on non-irrigated and irrigated treatments. Although the different pruning levels produced different yield and berry size categories, there was little effect on total wine colour. A far greater effect was observed when total wine colour was compared between irrigated and non-irrigated treatments, across all pruning levels. This increase was proposed to be associated with reduced berry size in the non-irrigated treatments.

Later work has included results from experiments investigating controlled deficit irrigation (Matthews and Anderson, 1988; McCarthy *et al.* 1996) and partial rootzone drying (PRD) (Dry, 1997; Stoll, 2000; Dry *et al.* 2000a; da la Hera Orts *et al.* 2002; dos Santos *et al.* 2003) and has indicated that increased anthocyanin concentration may be observed in fruit under actual or induced (PRD) conditions of water stress. However, greater insight into this phenomenon caused by water stress has been shown where anthocyanin content of berry skins (g per g skin fresh weight) was increased under water deficit (Matthews and Anderson, 1988; Esteban *et al.* 2001) rather than on a per g berry weight basis. This indicates that conditions of water deficit may actually increase the production of anthocyanin in the skin of the berry itself, rather than merely altering the skin to fruit weight ratio. These findings allow the premise to be questioned: is berry size,

through altering the skin to fruit weight ratio, the primary factor regulating anthocyanin concentration?

6.1.3 Factors contributing to wine colour

The colour of wine post-fermentation and after a period of ageing is due to a complex set of reactions occurring in the wine, and does not depend upon anthocyanin concentration in the fruit alone (Somers and Evans, 1977). Anthocyanins are one of a host of phenolic compounds extractable from grapes and wine, and these include flavan-3-ols, procyanidins, flavonols, hydroxycinnamates and hydroxybenzoates (Singleton, 1988). As a result of their structure, the potential exists for chemical interactions to occur between these phenolic compounds during vinification, resulting in the formation of copigmented and polymeric compounds (Bolton, 2001). In the case of the anthocyanins, the formation of stable interactions, either self-association with other anthocyanins or in complex with phenolic compounds, generally leads to an intensification of the perceived pigment colour (Bolton, 2001). A second benefit of the copigmentation and/or polymerisation of anthocyanin is an increase in the long-term stability of wine colour, as these pigments are resistant both to alterations in pH, and the bleaching effect of SO_2 (Somers, 1971). Thus, in approaching the application of viticultural techniques aiming to enhance anthocyanin concentration in fruit, the effectiveness of a practice can only be measured in the composition of that anthocyanin after vinification. A viticultural practice that can potentially enhance fruit colour does not guarantee the stability of coloured pigment post-vinification and wine ageing. Therefore, a second question to be addressed in the current study, is whether PRD can potentially influence the long-term stability of coloured pigments in wine.

6.2 Materials and methods:

6.2.1 Experimental

Experiment 1: This experiment was carried out over three seasons at a PIRSA (Primary Industries and Resources) field trial at Nuriootpa (Section 2.1.1.1, Chapter 2). It was on 10-year old Shiraz vines, and compared three pruning levels of 30, 60 and 120 nodes superimposed on PRD and control irrigation strategies. In 2001/2000 and 2002/2003, PRD received 0.5 ML/ha of water and the control 1 ML/ha. In 2001/2002, PRD and control both received 1 ML/ha. Fifty-berry samples were collected at weekly intervals from veraison to maturity (approx 24°Brix) and frozen at -20°C for later analysis.

Experiment 2:_This experiment was carried out over two seasons at a commercial field trial by Orlando-Wyndham, at Langhorne Creek, South Australia (Section 2.1.2, Chapter 2). It compared PRD and control irrigation treatments on 5-year old Cabernet Sauvignon vines pruned to 60 nodes. In 2001/2002, the control and PRD treatments received 1.19 and 0.84 ML/ha respectively, representing a 30% reduction in irrigation amount. In 2002/2003, the control and PRD treatments received 1.37 and 0.85 ML/ha respectively, representing a 38% reduction in irrigation water applied. Twenty-five-berry samples were collected at weekly intervals from veraison until maturity (approx. 24°Brix) and frozen at -20°C for later analysis. Wine was made from harvested fruit from the 2001/2002 vintage using small-scale winemaking techniques, and underwent malolactic fermentation. Winemaking was carried out in 18L fermenters, and samples were pooled to give 6 treatment replicates and 2 winemaking replicates (Section 2.4, Chapter 2).

Experiment 3: A pot-experiment comparing PRD and control irrigation strategies was set up under shade cloth on 3-year old Cabernet Sauvignon vines (Section 2.2, Chapter 2). The experiment compared 5 replicates of each treatment. Stomatal conductance of the vines was measured on sun-exposed leaves at 11 am throughout the season. The vines produced approximately two small (50-berry) bunches per vine. The bunches were harvested when the fruit reached 26 °Brix, and a five-berry sample, representing approximately 5% of all fruit on the vine, was collected and frozen at -40°C.

6.2.2 Extraction of anthocyanins

The 25-berry or 50-berry sample from Experiments 1 and 2 were later defrosted over 30 min, and immediately homogenised using an Utra-Turrax T 25 (IKA Labortechnik, Staufen, Germany) ensuring that both seeds and flesh were completely crushed. One gram of the homogenate was weighed out into a 10 mL centrifuge tube and frozen at -20°C. For Experiment 3, berry skins were removed from the fruit while still frozen and immediately placed in liquid nitrogen-cooled mortar. The berry skins were then ground to a fine powder under liquid nitrogen, and a 200 mg sample was weighed out into a 2 mL Eppendorf centrifuge tube, and frozen at -40°C. The samples from all experiments were extracted in 50% ethanol (pH 2 with HCl); 10 mL per gram berry tissue, according to the method of Iland *et al* (2000). Extracts were placed on a rotary shaker in the dark for 1 h. Samples were then centrifuged for 5 min at 12 000 rpm, and the supernatant retained. For berry-homogenate and skin samples, 1 mL of the supernatant was diluted in 10 mL or 20 mL of 1N HCl respectively. After a 3 h period, the absorbance of the diluted extract was determined at 520 and 280 nm.

6.2.3 Analysis of wine colour and phenolics

Wine colour was assayed by the methods outlined in Somers and Evans (1977), Levengood (1996) and Lambert (2001). Wine samples were adjusted to pH 3.6 using either 1 N NaOH or 1 N HCl. The following analyses were performed using a UV-visible spectrophotometer. All measurements were corrected for a 10 mm pathlength and dilution factor:

1. A^{520} and A^{420}: In a 1 mm quartz cuvette, absorbance at 520 nm and 420 nm was measured.
2. A^{acet}: Twenty μL of a 10% acetaldehyde solution was added to 2 mL of wine, mixed by pipetting and left to stand 45 min. Absorbance at 520 nm and 420 nm was then measured using a 1 mm quartz cuvette.
3. A^{SO_2}: Thirty μL of a 20% $Na_2S_2O_5$ solution was added to a further 2 mL of wine and mixed by pipetting. Absorbance was read at 520 nm in a 2 mm quartz cuvette.

4. A^{HCl}: A 100 µL aliquot of wine sample was added to 10 mL of 1 N HCl, mixed and left to stand 3 h in the dark. Absorbance at 520 nm and 280 nm was then measured in a 10 mm quartz cuvette, and corrected for dilution.
5. A^{100}: A 100 µL aliquot of wine sample was added to 10 mL acidic buffer (12% EtOH, 0.96 mol/L HCl) and left to stand for 3 h in the dark. Absorbance at 520 nm was then measured in a 10 mm quartz cuvette, and corrected for dilution.
6. A^{20}: A 100 µL aliquot of wine sample was added to 1.9 mL of buffer (12% EtOH, 3g/L KHT). Absorbance at 520 nm was measured in a 10 mm quartz cuvette, and corrected for dilution.
7. $A^{20/Acet}$: A 100 µL aliquot of wine was added to 1.9 mL of buffer (step 6) and 10 µL of 10% acetaldehyde. The solution was left to stand 45 min. Absorbance at 520 nm was read in a 10 mm quartz cuvette, and corrected for dilution.

The results from steps 1 – 4 were interpreted using the equations outlined in Somers and Evans (1977). The results of steps 3 and 5-7 were interpreted using the equations outlined in Levengood (1996) and Lambert (2001). These measures give an estimate of the proportion of red wine pigments present as free monomeric anthocyanins, in copigmentation complex, or bound in polymeric form.

6.2.4 HPLC analysis of wines

The wine samples were centrifuged at 12000 rpm and transferred to HPLC vials. Twenty µL was injected onto a Merck LiChrosopher 100 RP-18 (5µm) 4x250mm HPLC column and photodiode array detection at 520 and 280 nm. The HPLC system used was a Hewlett Packard HP 1100. The method was an adaptation of that used by Lambert (2001). The solvents were water acidified with 1.5% H_3PO_4 (solvent A) and 20% solvent A in acetonitrile (solvent B). The flow rate was maintained at 1mL/min, and the column temperature was 25°C. The method was a linear gradient of 8 – 27% solvent B in 55 min, then from 27 – 52% solvent B from 55 to 64 min, then isocratic at 52% solvent B for a further 5 min, followed by reconditioning of the column. The final run time was 70 min.

6.2.5 Statistical analysis

Data for Experiments 1 and 2 were analysed using Genstat 6.1 software. Experiment 1 compared 6 treatments by 5 replicates using a split-plot ANOVA, which compared the data at three levels: (T) treatments PRD and control, (P) pruning at 30, 60 and 120 nodes; and (TxP) treatment by pruning which compared the interactive effect of all the variables. The total n of the experiment was 30. Experiment 2 used a one-way ANOVA to compare three field blocks (PRD and Control) of seven two-vine plots, giving a total n=42. For the linear regression analyses of Experiment 1 and 2, the Instat software package was used. Using this software, a runs test was used to test each regression for linearity. For Experiment 3 a Student's t-test was used to compare two treatments (PRD and Control) of 5 replicates, using the Microsoft Excel software package.

6.3 Results and Discussion

6.3.1 Effect of berry size and PRD irrigation on fruit anthocyanin and phenolic composition

6.3.1.1 Experiment 1: The effect of node number and PRD on anthocyanins and phenolics in Shiraz grapevines

The effect of pruning level and PRD on grape berry anthocyanins and phenolics was studied over two seasons, 2000/2001 and 2002/2003. The data collected in an additional season in 2001/2002 were not included due to a difference in the irrigation water applied in that vintage compared with the other seasons of the study, and also due to unusually cool weather conditions (Appendix E), which did not show a response in terms of berry weight (Table 3.7). In 2000/2001 and 2002/2003, the three pruning levels produced three different categories of yield and berry weight, with the 30 node vines having the highest berry weight and lowest yield (Tables 3.5 and 3.6). The effect of node number and PRD on the resulting anthocyanin and phenolic composition was investigated at two stages of maturity: pre-harvest (18-20 °Brix) which coincides with maximum berry weight, and harvest (24 °Brix). In 2000/2001, pruning level had a significant effect on the

concentration of anthocyanin and phenolics when the fruit was compared at 18 °Brix (Tables 6.1, 6.2). However, the content per berry was not significantly affected (Tables 6.1, 6.2). Those data appear to be weighted by a slightly higher concentration of both anthocyanin and phenolics in the 120 node PRD treatment (Tables 6.1 and 6.2), which was not great enough to bring about a significant TxP interaction. By maturity, the pruning effect was not observed on anthocyanin and phenolic concentration, but was evident when these factors were compared as content per berry. In 2003, there was no significant effect of pruning on anthocyanin and phenolic concentration, at 20°Brix or 24°Brix (Tables 6.3 and 6.4). However, as in the harvest samples of 2000/2001, there was a clear effect of pruning on anthocyanin and phenolic content per berry, at both maturity levels. This indicates that reduced berry size as a result of increased node number does not significantly alter the concentration of anthocyanin and phenolics in fruit tissue expressed per gram. This means that there is not likely to be a strong surface area to volume relationship in the expression of fruit and wine colour. Instead, it appears that with increasing berry size, the total content of anthocyanin and phenolics per berry is increased, due to a higher surface area and thus larger area over which anthocyanins are concentrated. Thus, the change in surface area to volume ratio with larger berries did not appear to affect the concentration of anthocyanin per gram, as total anthocyanin content was increased.

Table 6.1: Effect of three levels of node number and PRD on grape berry anthocyanins in Shiraz at Nuriootpa in 2000/2001 (n=30; T= irrigation treatment; P= pruning level; TxP= interaction)

Anthocyanin concentration		30 node	60 node	120 node	P (T)	P (P)	P (TxP)
18°Brix (mg/g)	Control	0.98	0.81	0.99	ns	<0.05	ns
	PRD	0.97	0.95	1.24			
18°Brix (mg/berry)	Control	0.89	0.70	0.81	ns	ns	ns
	PRD	0.84	0.82	0.84			
24°Brix (mg/g)	Control	1.64	1.62	1.61	ns	ns	ns
	PRD	1.68	1.65	1.70			
24°Brix (mg/berry)	Control	1.58	1.42	1.21	ns	<0.001	ns
	PRD	1.61	1.36	1.11			

Table 6.2: Effect of three levels of node number and PRD on grape berry phenolics in Shiraz at Nuriootpa in 2000/2001 (n=30; T= irrigation treatment; P= pruning level; TxP= interaction)

Phenolic concentration		30 node	60 node	120 node	P (T)	P (P)	P (TxP)
18°Brix (A_{280}units/g)	Control	1.21	1.20	1.25	ns	<0.05	ns
	PRD	1.21	1.22	1.51			
18°Brix (A_{280}units/berry)	Control	1.07	1.00	1.03	ns	ns	ns
	PRD	1.02	1.06	1.00			
24°Brix (A_{280}units/g)	Control	1.28	1.43	1.50	ns	ns	ns
	PRD	1.37	1.39	1.55			
24°Brix (A_{280}units/berry)	Control	1.24	1.27	1.14	ns	ns	ns
	PRD	1.30	1.14	1.01			

Table 6.3: Effect of three levels of node number and PRD on grape berry anthocyanins in Shiraz at Nuriootpa in 2002/2003 (n=30; T= irrigation treatment; P= pruning level; TxP= interaction)

Anthocyanin concentration		30 node	60 node	120 node	P (T)	P (P)	P (TxP)
20°Brix (mg/g)	Control	0.99	0.93	1.04	<0.001	ns	ns
	PRD	1.18	1.11	1.25			
20°Brix (mg/berry)	Control	1.23	1.01	1.09	ns	<0.01	ns
	PRD	1.25	1.10	1.09			
24°Brix (mg/g)	Control	1.45	1.43	1.45	<0.01	ns	ns
	PRD	1.68	1.57	1.55			
24°Brix (mg/berry)	Control	1.56	1.46	1.35	ns	<0.001	ns
	PRD	1.75	1.49	1.21			

Table 6.4: Effect of three levels of node number and PRD on grape berry phenolics in Shiraz at Nuriootpa in 2002/2003 (n=30; T= irrigation treatment; P= pruning level; TxP= interaction)

Phenolic concentration		30 node	60 node	120 node	P (T)	P (P)	P (TxP)
20°Brix (A_{280}units/g)	Control	1.09	1.08	1.07	<0.001	ns	ns
	PRD	1.25	1.19	1.25			
20°Brix (A_{280}units/berry)	Control	1.36	1.18	1.13	ns	<0.001	ns
	PRD	1.32	1.19	1.07			
24°Brix (A_{280}units/g)	Control	1.33	1.29	1.36	<0.01	ns	ns
	PRD	1.45	1.41	1.47			
24°Brix (A_{280}units/berry)	Control	1.43	1.32	1.27	ns	<0.01	ns
	PRD	1.51	1.33	1.15			

The effect of PRD on the concentration of anthocyanins and phenolics differed between seasons. In 2000/2001, only a slight, non-significant increase in these components was observed with PRD (Tables 6.1, 6.2). In 2002/2003, the concentration of anthocyanins and phenolics was consistently increased by the PRD treatment, and was independent of node number per vine in the intensity of the response (Tables 6.3, 6.4). Pooled over pruning level, PRD increased the concentration of anthocyanins and phenolics by 11% and 9% respectively for fruit at 24 °Brix. The response was observed at both fruit maturity levels studied. A reduction in berry weight occurred at harvest with PRD in the 30 and 120 node treatments in the order of 14% and 18% respectively (Table 3.6). Although this berry weight reduction was not statistically significant, it led to slight reduction in the content of anthocyanins and phenolics per berry. Thus, there was no significant increase in either anthocyanin or phenolic content per berry in response to the PRD treatment (Tables 6.3, 6.4).

PRD has been shown to cause minimal change in berry weight in other experiments (Dry, 1997; Stoll, 2000). Consequently, previous observations of an anthocyanin concentration increase under PRD irrigation were due solely to an increase in anthocyanin content per berry, as berry weight was unchanged (Dry, 1997; Stoll, 2000). This suggested an underlying biochemical response of the anthocyanin metabolic pathway to the induced stress signals caused by PRD, upregulating the final concentration of the anthocyanin product. In previous studies, correlation analysis of potential factors contributing to altered metabolism in the anthocyanin pathway showed that colour was strongly negatively correlated with vigour indices, canopy density and stomatal conductance; and positively correlated with light penetration into the canopy (Dry, 1997; Stoll, 2000). This showed that changes in vine physiology and bunch microclimate were likely to influence secondary metabolism where berry size remained constant. In the current study, slight alterations in berry weight caused by PRD prevented the comparison of anthocyanin content on a per berry basis. However, the results do indicate that where PRD does cause an increase in fruit anthocyanin and phenolic concentration, this is more dependent upon a biochemical response to the treatment, rather than upon berry size alone. Therefore,

increases in colour seen with small fruit produced from deficit treatments may reflect a secondary response to the stress treatment, whereas a primary response to the stress is a reduction in fruit size.

This hypothesis was explored further through linear regression analysis of anthocyanins and phenolics, expressed as concentration per gram fresh weight or as content per berry, plotted against berry weight (Figures 6.1, 6.2). For both seasons of the study, anthocyanin concentration showed a poor relationship with berry weight as demonstrated by low R^2 values (Figure 6.1A, B) relative to higher R^2 values calculated for the relationship between anthocyanin content per berry and berry weight (Figure 6.1 C, D). For analysis of both anthocyanin concentration and content, the derived P-value indicated a significant fit of the data to the regression curve in each case. For anthocyanin concentration, the data from each of the pruning and PRD treatments were distinguished by colour, and it can be seen that although smaller berries from the 120 node treatments were distributed toward the lower end of the berry weight range of the experiment, there was little change in anthocyanin concentration compared with larger berries from the 60 or 30 node treatments (Figure 6.1 A, B). Linear regression analysis of phenolic concentration and content versus berry weight showed a similar response to that seen for anthocyanins. Higher R^2 values were obtained for analysis of phenolic content per berry than for phenolic concentration in both seasons (Figure 6.2). However, the 2002/2003 season showed a slightly stronger relationship between the berry weight and phenolic concentration as indicated by a higher R^2 value than in the previous season of the study (Figure 6.2 A, B). This showed that under the conditions of the 2002/2003 season, there was an influence of berry weight on phenolic concentration.

These results provide an important basis for the discussion of the influence of berry size on grape secondary metabolite concentration, and the implications for wine quality. Regression and correlation analyses from other studies reporting a strong relationship between berry size and anthocyanin or phenolic concentration were performed on a sample set taken from a range of vineyards, localities, soil types and irrigation strategies, as well as a wide range of °Brix levels at harvest (Botting *et al.* 1996; Gray *et al.* 1997).

As each of these factors could potentially have a significant effect on the final concentration of fruit secondary metabolites, it is important to attempt to reduce the number of factors which may increase variation within the sample set. The current study has compared fruit from a single vineyard and soil type, and at similar sugar levels (°Brix). The results have shown a weak relationship between secondary metabolite concentration and berry size, drawn from a wide range of berry sizes produced by a range of node numbers at winter pruning. From the results of the 2002/2003 season, the PRD irrigation technique clearly had a greater effect in increasing anthocyanin and phenolic concentration than did decreased berry weight. Although smaller fruit would have an increased surface area to volume ratio, this does not appear to significantly influence the concentration of skin-derived phenolic compounds. Rather, with increasing fruit weight there was a proportional increase in anthocyanin content per berry, leading to a uniform response in terms of concentration. Consequently, changes in berry size observed in fruit exposed to water-deficit appear to be uncoupled from biochemical changes in the metabolism of secondary metabolites in berry skins.

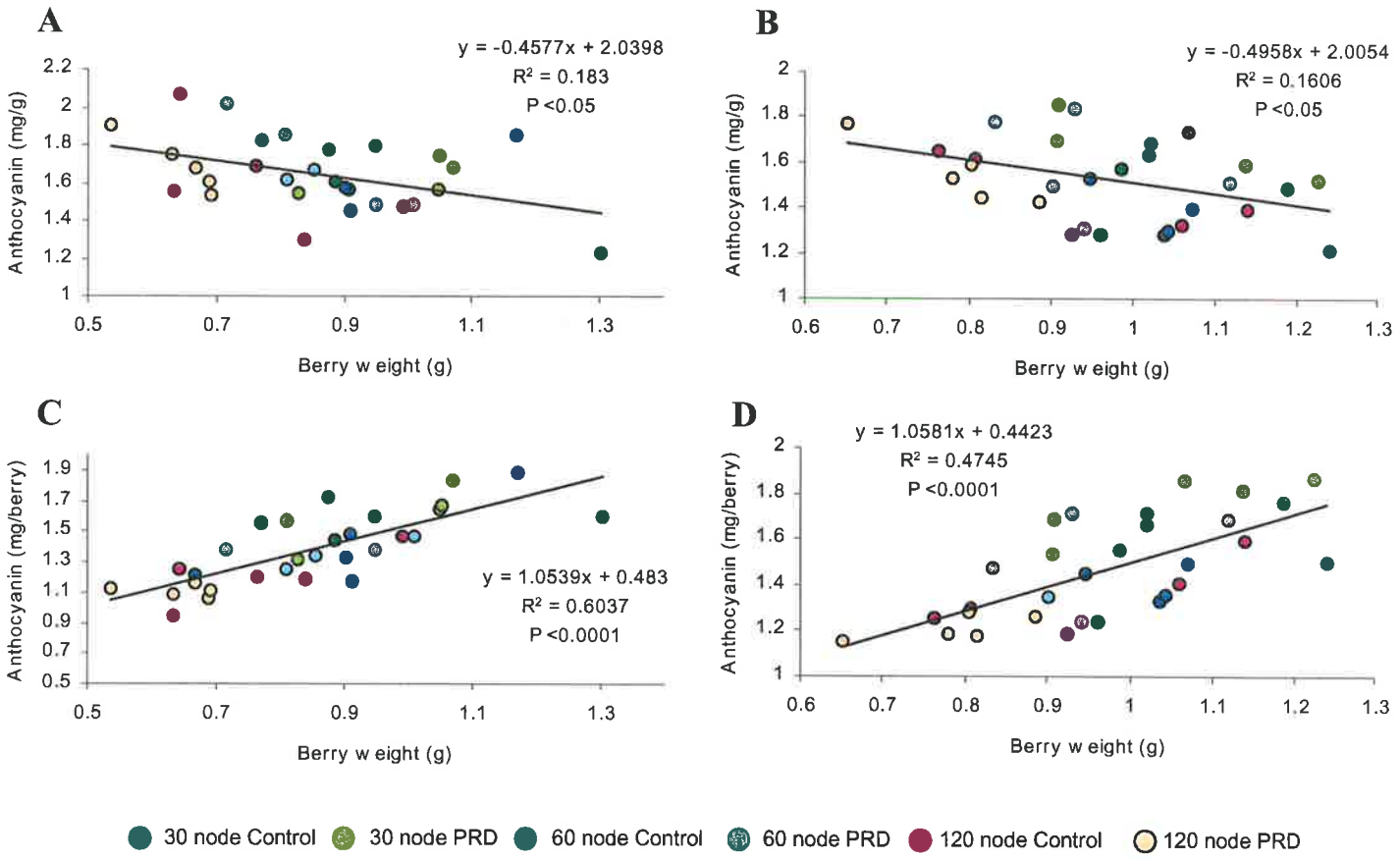


Figure 6.1 Linear regression of anthocyanin versus berry weight for PRD- and Control-treated Shiraz vines pruned to 30, 60 and 120 nodes **A**. 2000/2001 mg/g **B**. 2002/2003 mg/g; **C**. 2000/2001mg/berry; **D**. 2002/2003 mg/berry (n=30)

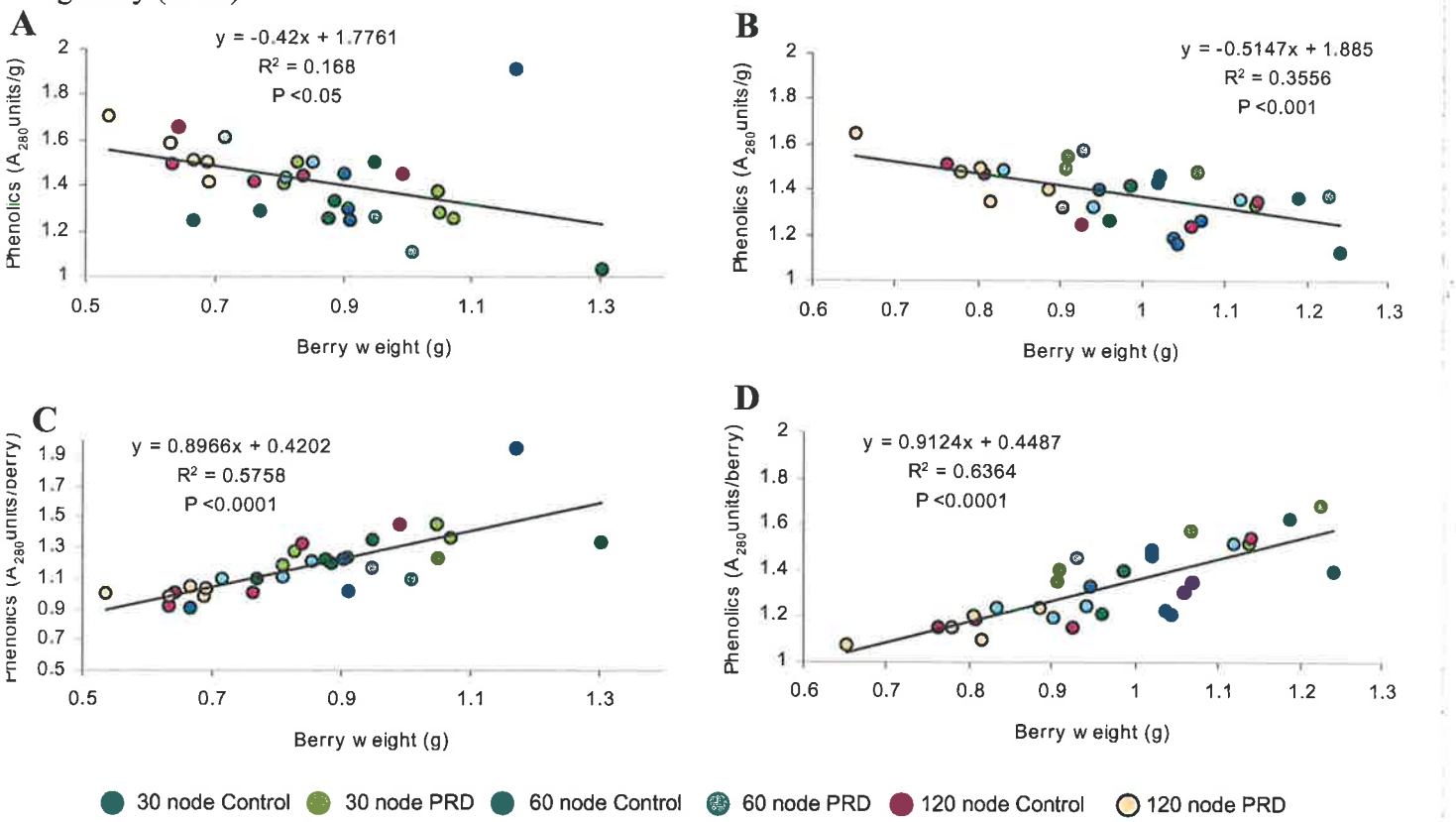


Figure 6.2 Linear regression of total phenolics versus berry weight for PRD- and Control-treated Shiraz vines pruned to 30, 60 and 120 nodes **A**. 2000/2001 mg/g **B**. 2002/2003 mg/g; **C**. 2000/2001mg/berry; **D**. 2002/2003 mg/berry (n=30)

6.3.1.2 Experiment 2: Effect of PRD on anthocyanins and phenolics in Cabernet Sauvignon

In this experiment, there was a consistent reduction in berry weight at harvest in both seasons of the study (Table 3.4). In 2001/2002, PRD berries were 11% smaller than the control and in 2003 this reduction was 12%. This consistent decrease in fruit weight indicates a physiological response of developing berries to water deficit under the PRD irrigation strategy early in the season (Ojeda *et al.* 1999, 2001). Only in 2002/2003, however, was this reduction in berry size associated with a small but significant increase in anthocyanin concentration at harvest (Table 6.5). In both seasons, there was a reduction in total anthocyanin content per berry, which was due to the reduction in berry weight. A linear regression analysis of the 2001/2002 data indicated that there was a strong relationship between anthocyanin content per berry and berry weight, but a weak relationship was found for anthocyanin concentration and berry weight, as indicated by a lower R^2 value (Figure 6.3 A, C). In 2002/2003, analysis of the linear regression results using a runs test indicated that the relationship was non-linear, such that both anthocyanin concentration and content followed a curvilinear pattern when plotted against berry weight. Polynomial curves were fitted to the data set (Figure 6.3 B, D), and showed a similar response to the relationships demonstrated in 2001/2002. Anthocyanin concentration showed a significant, but weak relationship to berry weight, and anthocyanin content per berry was more strongly associated with berry weight, as shown by a higher R^2 value (Figure 6.3 B, D).

With regard to total phenolics, content per berry was slightly, but not significantly reduced in both 2001/2002 and 2002/2003 (Table 6.5). However, this was reflected as an increase in the concentration of total phenolics in both seasons (Table 6.5), although there was no corresponding change in anthocyanin concentration in 2002. A linear regression analysis of phenolic concentration and content showed that there was a stronger relationship of both parameters to berry weight than that seen for anthocyanin, in both seasons (Figure 6.4). Thus, in the case of phenolics, smaller berries were associated with a lower phenolic content per berry, and a higher phenolic concentration per gram berry weight. In the light of the 2001/2002 data where anthocyanin concentration was not

increased with PRD, nor significantly associated with berry weight, it is likely that the increase in phenolic concentration was due to an increase in non-anthocyanin phenolics. This is because in red grapes there is a strong positive relationship between anthocyanins and phenolics, when expressed as both content and concentration (Gray *et al.* 1997). A large proportion of the A_{280} measure of total phenolics comes from anthocyanins which absorb light strongly at both 280 nm and 520 nm (Ribereau-Gayon and Glories, 1986). Thus, where differences occur in the response of anthocyanin and total phenolics to a treatment, it can be assumed that this is due to a change the non-anthocyanin phenolics..

Table 6.5: Anthocyanin and phenolic composition of Cabernet Sauvignon for the 2001/2002 and 2002/2003 vintages (ANOVA, n=42)

Vintage 2001/2002	Anthocyanin		Total Phenolics	
	(mg/berry)	(mg/g)	(A_{280}units/berry)	(A_{280}units/g)
Control	1.69	1.78	1.46	1.54
PRD	1.57	1.81	1.39	1.62
P (ANOVA)	<0.05	ns	ns	<0.05
% PRD>Control	-7	2	-4	5
Vintage 2002/2003	Anthocyanin		Total Phenolics	
	(mg/berry)	(mg/g)	(A_{280}units/berry)	(A_{280}units/g)
Control	1.61	1.79	1.47	1.63
PRD	1.54	1.88	1.43	1.75
P (ANOVA)	ns	<0.05	ns	<0.001
% PRD>Control	-5	5	-3	7

Other components which absorb strongly at 280 nm are the simple phenolics, tannins and flavonols (Ribereau-Gayon and Glories, 1986). It has already been shown that the contribution of seed proanthocyanidin and some flavan-3-ols to the concentration of total phenolics was increased in PRD-treated fruit from this experiment (Tables 5.2 and 5.3). Figure 6.5 shows a linear regression analysis of the 2002/2003 seed proanthocyanidin (tannin) data plotted against berry weight, and shows that at smaller berry weights there is a potential increase in the concentration of seed tannin in fruit. This is due to the berry weight decrease alone, as total content of seed tannin per berry showed no relationship with berry weight. Thus, it is possible that the increase in total phenolics seen in smaller berries, primarily induced by water deficit under PRD irrigation in this experiment, is due to increased contribution of seed tannin to the absorbance of the extract measured at 280 nm

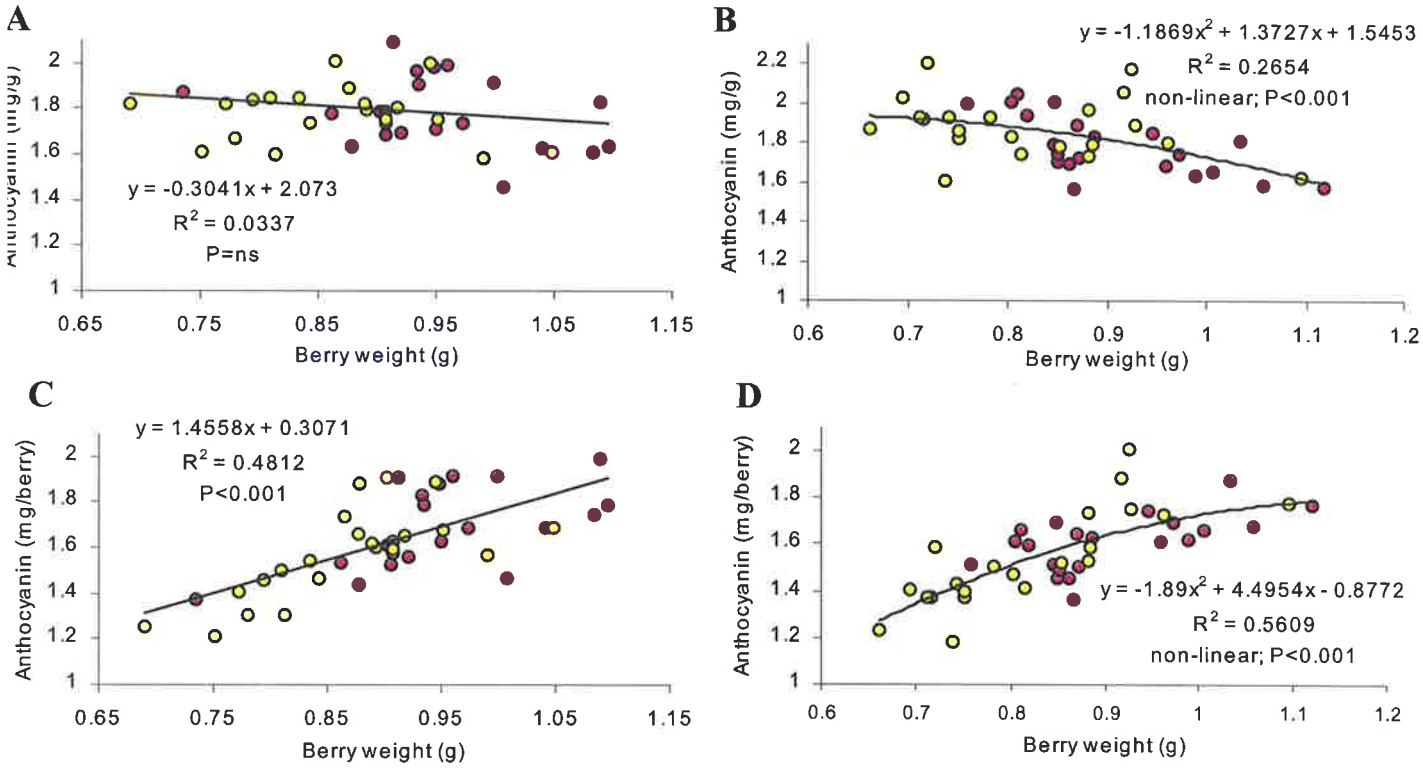


Figure 6.3 Linear regression of anthocyanin versus berry weight for Cabernet Sauvignon **A.** 2001/2002 mg/g **B.** 2002/2003 mg/g; **C.** 2001/2002 mg/berry; **D.** 2002/2003 mg/berry ● Control; ○ PRD; n=42; ns = not significant.

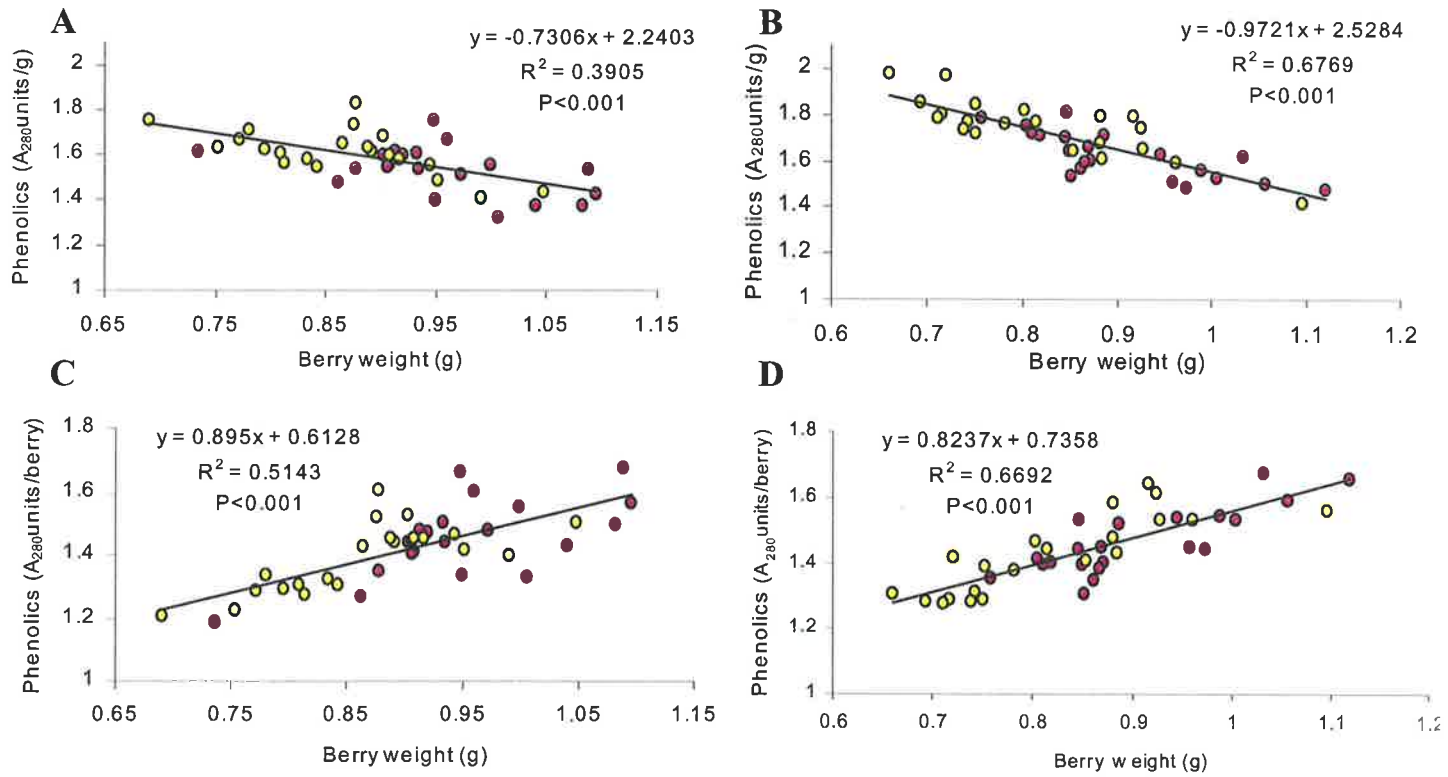


Figure 6.4 Linear regression of total phenolics versus berry weight for Cabernet Sauvignon **A.** 2001/2002 A_{280}units/g **B.** 2002/2003 A_{280}units /g; **C.** 2001/2002 A_{280}units /berry; **D.** 2002/2003 A_{280}units /berry; ● Control; ○ PRD; n = 42.

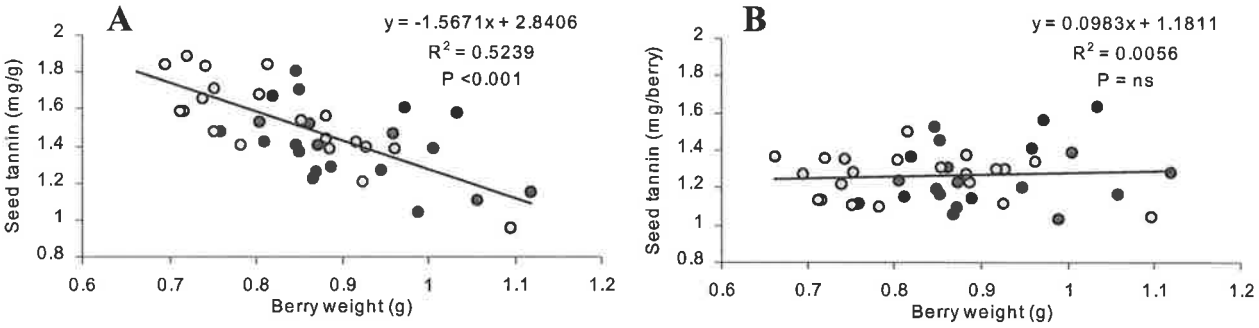


Figure 6.5: Linear regression analysis of the relationship between seed tannin (proanthocyanidin) and berry weight for Cabernet Sauvignon in 2002/2003 **A.** concentration per gram **B.** content per berry; ●Control; ○ PRD; n = 42, ns = not significant

6.3.1.3 Experiment 3: Anthocyanin and phenolic concentration in berry skins of Cabernet Sauvignon

In a PRD experiment on potted Cabernet Sauvignon, the concentration of anthocyanin and phenolics was determined in skins removed from the fruit samples. The PRD treatment caused a small but significant reduction in berry weight and an increase in pH, although °Brix was not significantly affected (Table 6.6). This effect on berry weight and pH is similar to that observed in the field Cabernet Sauvignon (Table 3.4and Table 4.1). The PRD treatment caused an increase in total anthocyanin and phenolic concentration of the berry skins, on a per gram skin weight basis (Table 6.6). This was in the order of 27% for anthocyanins and 25% for phenolics, a more significant response to the PRD treatment than was observed for the field experiment on Cabernet Sauvignon (Table 6.5). This result confirms some of the observations of Experiments 1 and 2, which indicate that a reduction in berry weight may be associated with the PRD response, but is not the underlying factor which results in an increase in anthocyanin concentration. It is more plausible to conclude that there is a biochemical response of the anthocyanin pathway to the PRD treatment, which increases partitioning to the anthocyanin pathway. Previous studies of the PRD treatment have shown a correlation between increased light penetration within the fruiting zone of the grapevine and increased anthocyanin concentration in berry skins (Dry, 1997; Stoll, 2000). It is possible that elevated bunch exposure could increase anthocyanin concentration, as long as fruit temperature was not elevated by the PRD treatment (Mahbrouk and Sinoquet, 1998; Spayd *et al.* 2002). In the

current experiment, vines were grown under reduced light incidence (50% of ambient PAR) and as the potted vines produced very small canopies, all fruit was completely exposed to ambient light conditions. Thus, the changes in anthocyanin and phenolic concentration in berry skins were not due to increased fruit exposure to sunlight, but appear to be the result of a direct biochemical response to physiological signals produced by the PRD treatment itself. Such signals could include elevated levels of ABA in xylem sap (Dry *et al.* 2000b, Stoll *et al.* 2000). Elevated levels of ABA have been found in grape berries at different maturity levels grown under water deficit (Antolin *et al.* 2003) which means that root-derived signals produced by the PRD treatment may potentially be transmitted to the developing grape berry.

Table 6.6: Fruit composition of potted Cabernet Sauvignon trial from the 2003 vintage (Student's T-test, n=10)

	Control	PRD	P	%PRD> Control
Berry weight (g)	0.82	0.71	<0.01	-13
Total Soluble Solids (°Brix)	26.7	26.1	ns	-2
pH	3.24	3.44	<0.001	6
Total anthocyanins (mg/g skin weight)	11.31	14.36	<0.05	27
Total phenolics (A_{280}units/g skin weight)	7.34	9.16	<0.05	25

Altered levels of ABA in developing fruit has the potential to markedly affect anthocyanin metabolism in the berry skins. ABA is known to increase anthocyanin production in grape berries, by upregulating the expression of the enzyme chalcone-flavone isomerase (Hiratsuka *et al.* 2001), and by promoting the formation of anthocyanoplasts (anthocyanin-forming cells) (Kim *et al.* 1997). In the current study, the application of ABA to the roots of potted vines in a simulated PRD experiment did not cause any change in the anthocyanin content or composition of the berry skins (results not shown). Although this does not preclude ABA as being a key plant growth regulator in this response, it is possible that ABA may not operate in isolation to bring about the changes seen with PRD. Water stress has been implicated in the increase of anthocyanin content of cultured grape cells, when cells were subjected to increased osmotic potential of the culture medium (Do and Cormier, 1990; Do and Cornier, 1991). There may be

other signals induced by water stress conditions which enhance the production of anthocyanin. This result is in agreement both with previous studies on PRD and other studies on the effects of water stress on anthocyanin composition of grape berry skins; all of which showed an increase in anthocyanin concentration localised to the skin itself (Dry, 1997; Stoll, 2000; Matthews and Anderson, 1988; Esteban *et al.* 2001). In view of the previous discussion of anthocyanin concentration and berry size, these data show that where decreased berry weight occurs as a response to deficit irrigation or PRD, an increase in anthocyanin concentration in fruit will only occur when there is a similar increase in its concentration in the berry skin. This may explain the results of the 2001/2002 field experiment on Cabernet Sauvignon (Table 6.5) where a significant reduction in fruit size in response to the PRD treatment was not associated with any change in anthocyanin concentration.

6.3.2 Effect of PRD on wine colour and phenolic composition

Wines were made from a single vintage (2002) of Cabernet Sauvignon fruit harvested from Experiment 2. In that vintage, measures of fruit composition indicated an 11% reduction in fruit weight, a 5% increase in the concentration of total phenolics and no change in anthocyanin concentration in response to PRD (Table 6.5). Measures of wine colour and anthocyanin composition were made at bottling, approximately four months from the start of fermentation. Figure 6.6 shows the spectrum of the PRD and control wines at bottling, and shows a higher absorbance for PRD wine not only in the range of maximal red-colour absorption (A_{520}), but also at lower wavelengths. This affected the measure of wine colour density, which reflects the intensity of absorption at both A_{420} and A_{520}, a combined effect of red and brown pigments. PRD wines had a significantly higher colour density both at bottling and after 12 months ageing (Tables 6.7 and 6.8). This effect was retained in the presence of acetaldehyde (modified wine colour density), which restores bleached anthocyanin pigments, thereby increasing the absorbance at A_{520}. Actual and modified wine colour hue was unaffected by PRD at bottling, which is a measure of the tint of the wine (A_{420}/A_{520}) (Table 6.7). However, after 12 months ageing, actual wine colour hue was reduced in PRD wines, primarily due to an increase in the relative absorption of red pigments at A_{520} in PRD during this time (Table 6.8). The

actual and modified degree of red pigment colouration at bottling was increased with PRD, but this effect was not retained after ageing. Total red pigments, which is derived from total red colour absorbance in the presence of a strong acid, were increased with PRD both at bottling and after ageing (Tables 6.7 and 6.8). Similarly, total wine phenolics were increased with PRD, and unlike the other parameters measured, the level did not significantly change after 12 months ageing (Tables 6.7 and 6.8).

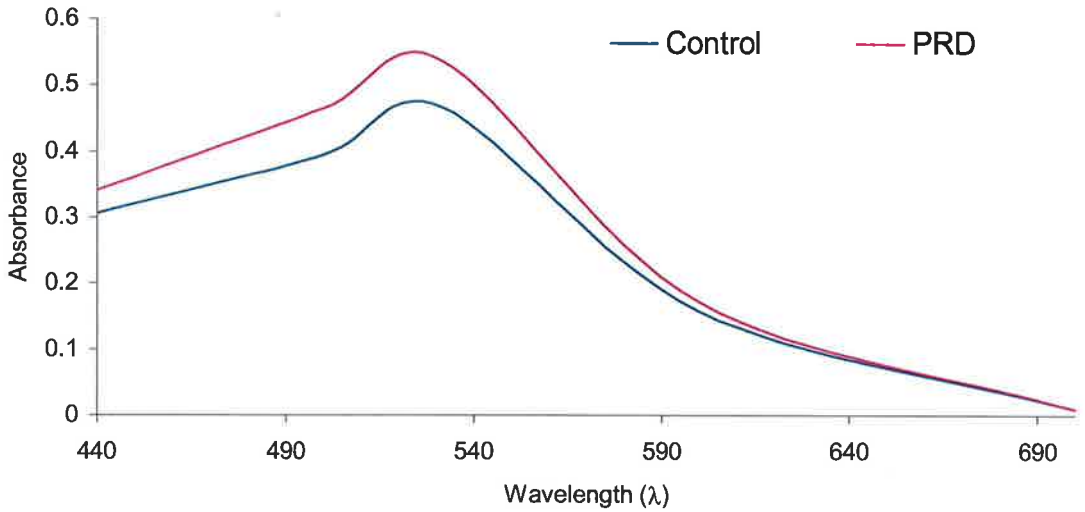


Figure 6.6: Wine spectrum for PRD and control wines at bottling from the 2002 vintage

The forms of red pigments were determined by their response to the effects of SO_2-bleaching and acetaldehyde. This showed that the increases in total colour observed in the PRD wines at bottling was not due to an increase in total free monomeric anthocyanin, but rather by an increase in copigmented and polymeric forms of anthocyanin (Table 6.7). The presence of anthocyanin in these forms may enhance wine colour through increasing the intensity of the absorbance at A_{520} (Bolton, 2001). Analysis of the contribution of the respective forms of red pigments showed a significant decrease in the proportion of anthocyanin contribution to the colour of PRD wines at bottling (Table 6.7). It is clear that PRD-treated fruit which initially showed no change in anthocyanin concentration resulted in wines with improved colour properties. The component that was

altered by the PRD treatment was the contribution of non-anthocyanin phenolics to total phenolics, which was increased by PRD (Table 6.5). What has been brought to question in the current study is the correlation between fruit anthocyanin and wine colour. Previous findings have demonstrated increased colour in wines produced from deficit-irrigated vines (Freeman, 1983; Singleton and Trousdale, 1983) but the original fruit anthocyanin composition was not presented. The current data suggest that the effect of deficit irrigation treatments such as PRD is to alter the phenolic profile of the fruit and wine, which bring about a change in the degree of copigmentation and polymerisation of wine anthocyanins. The increases seen in wine colour with deficit irrigation treatments may therefore not be due to a change in anthocyanin concentration alone, but rather an increase in the intensity of the colour due to the molecular properties of the anthocyanin in copigmented or polymeric form (Bolton, 2001). Thus, as has been shown in the current study, deficit irrigation strategies like PRD may not necessarily improve anthocyanin concentration in the fruit, but still lead to a significant improvement in wine colour. The potential exists to further optimise this response, particularly where anthocyanin concentration is increased in PRD-treated fruit in addition to total phenolics.

After 12 months of ageing, free anthocyanin, copigmented anthocyanin and the resulting wine colour were reduced (Table 6.8). Polymeric anthocyanin was slightly increased, as would be expected during the ageing process. However, the treatment effects were still reflected in the aged wines as in the wines at bottling. Free anthocyanin was higher in the aged PRD wines, although this effect was not significant. This response reflects a higher stability of the anthocyanin in PRD wines, perhaps due to disassociation from the copigmented forms during ageing. Thus, wine quality may be improved in two aspects in PRD wines due to the increase in the polymerisation of pigments: an increase in both wine colour and the long-term stability of coloured pigments.

Table 6.7: Wine pigmentation results at bottling for the 2002 vintage (ANOVA, n=12)

Red wine colour measures	Control	PRD	%	P
Wine Colour Density	7.1	8.1	15	<0.001
Wine Colour Hue	0.69	0.66	-3	ns
Degree of red pigment colouration (%)	21.9	24.3	11	<0.01
Total red pigments (A_{520} HCl)	19.2	20.1	5	<0.05
Modified wine colour density	9.3	10.3	11	<0.001
Modified wine colour hue	0.59	0.58	-1	ns
Modified degree of red pigment colouration (%)	30.6	32.6	6	<0.05
Total phenolics (A_{280} HCl)	34.4	37.3	8	<0.001
Copigmentation results	Control	PRD	%	P
Anthocyanins in copigment complex (mg/L)	30.6	34.1	11	<0.001
Anthocyanins in free monomeric form (mg/L)	247.2	248.7	1	ns
Colour of anthocyanins in polymeric form (A_{520}units)	2.0	2.2	12	<0.001
Total Colour	9.4	10.4	10	<0.001
% Colour due to copigment complex	64.1	65.0	1	<0.01
% Colour due to anthocyanins	14.9	13.6	-10	<0.001
% Colour due to polymer	21.0	21.5	2	ns

Table 6.8: Wine pigmentation results after 12 months ageing for the 2002 vintage (ANOVA, n=12)

Red wine colour measures	Control	PRD	%	P
Wine Colour Density	7.3	8.1	10	<0.01
Wine Colour Hue	0.74	0.71	-4	<0.05
Degree of red pigment colouration (%)	37.9	37.6	-1	ns
Total red pigments (A_{520} HCl)	11.1	12.7	14	<0.05
Modified wine colour density	8.2	9.1	11	<0.001
Modified wine colour hue	0.74	0.70	-5	ns
Modified degree of red pigment colouration (%)	42.5	42.5	0	ns
Total phenolics (A_{280} HCl)	32.3	35.4	10	<0.001
Copigmentation results	Control	PRD	%	P
Anthocyanins in copigment complex (mg/L)	24.5	27.9	14	<0.01
Anthocyanins in free monomeric form (mg/L)	113.2	134.1	18	ns
Colour of anthocyanins in polymeric form (A_{520}units)	2.5	2.7	7	<0.01
Total Colour	8.0	9.0	12	<0.01
% Colour due to copigment complex	60.6	61.5	2	ns
% Colour due to anthocyanins	8.0	8.4	5	ns
% Colour due to polymer	31.5	30.1	-4	ns

The results of the wine spectrophotometric analysis were compared with the results of HPLC analysis to justify some of the conclusions drawn from the study. HPLC analysis of the wines at bottling allowed for polymeric forms of the anthocyanin to elute as a single peak, together with other polymeric molecules, namely tannin. Comparison of A_{280} and A_{520} of free anthocyanins in the wines, and removal of these peaks from the A_{280} spectrum, allowed for an anthocyanin-free measure of wine phenolics to be made (Table 6.9). The data are in agreement with the spectrophotometric analyses, indicating that the total level of free anthocyanin was not affected by PRD. Instead, the concentration of coloured polymeric pigments was significantly increased (Table 6.9). Interestingly, a measure of anthocyanin-free A_{280} showed that free phenolics were also increased in the PRD wines. Total polymer, which partly included an A_{280} contribution of polymeric anthocyanin, was also increased. This led to a higher measure of total phenolics in PRD wines overall. This result may to some extent account for the increases observed in the copigmentation and polymerisation properties of the PRD wines. A higher contribution of non-anthocyanin phenolics and potentially tannin from seeds or berry skins, may have facilitated the binding and stabilisation of anthocyanin in other forms (Ribereau-Gayon and Glories, 1986). Certain phenolics contribute more strongly as potential copigments than others. A recent study showed that the flavonols quercetin and quercetin-3-glucoside are the stongest copigments with malvidin-3-glucoside relative to other wine phenolics (Lambert, 2002). The flavan-3-ols epicatechin and catechin were found to be weaker copigments, as were seed tannins (Lambert, 2002). The concentration of seed tannins, seed-derived catechin and epicatechin-gallate were increased due to PRD in the 2001/2002 season (Table 5.2). These seed phenolics could account in part for the increase in the A_{280} of the PRD wines, but it is possible that quercetin-3-glucoside concentration could be affected by the PRD treatment as well. The content of quercetin glycosides in grape berry skins have been shown to be significantly increased by sun exposure (Price *et al.* 1995; Lee, 1997; Haselgrove *et al.* 2000). As PRD significantly increased canopy-openness, allowing more light to reach the fruiting zone of vines (Figure 3.8), the potential exists that the treatment could affect quercetin glycosides in berry skins. This will be an important component for analysis in future studies of the effect of PRD on fruit and wine composition.

Clearly, the current data show that wine colour is significantly affected by the chemical properties of wine phenolics, through copigmentation and polymerisation. Rather than merely viewing wine colour and thus potential quality in terms of anthocyanin concentration in fruit and wine, more detailed analysis of the contribution of grape and wine phenolics should be a focus of future research.

Table 6.9: Effect of PRD on Cabernet Sauvignon wine phenolics at bottling analysed by HPLC (ANOVA; n = 12; ns = not significant).

	Free monomeric anthocyanin	Pigmented polymer	Total polymer (P)	Anthocyanin-free phenolics		% polymer
				(– P)	(+P)	
	(mg/L)	(A_{520}units/mL)	(A_{280}units/mL)			
Control	232.6	113.2	261.5	328.4	589.8	44.3
PRD	240.3	130.3	297.9	350.5	648.4	46.0
% PRD>Control	3	15	14	7	10	4
P (ANOVA)	ns	<0.001	<0.001	<0.001	<0.001	<0.05

6.4 Conclusions

The results of this study have shed light on some of the current thinking about the effect of berry size on wine quality. The concept that smaller berries produce higher colour in both fruit and wine needs to be reconsidered. Although there is some published data which correlates smaller berries with higher anthocyanin concentration (Botting *et al.* 1996, Gray *et al.* 1997) much of the current thinking backing this concept is speculative. It is based on the assumption that smaller fruit alters the surface area to volume ratio of skin to flesh, effectively increasing the concentration of skin-derived secondary metabolites, namely anthocyanins and phenolics. From the results of the current study, this concept is shown to be incorrect, since smaller fruit resulted in a reduction in anthocyanin per berry, which did not necessarily translate into increased concentration on a per gram fresh weight basis. Rather, in cases where small berries resulted in higher anthocyanin and phenolic concentration, this appeared to be the result of a biochemical response induced by PRD, rather than an increase in surface area to volume ratio alone. This was verified by data indicating that PRD caused an increase in skin anthocyanin concentration, which has been previously shown to be a response to deficit irrigation in

grapevines (Matthews and Anderson, 1988; Esteban *et al.* 2001). Furthermore, the current study has shown that this response is not necessarily an indirect result of the PRD treatment, i.e. due to increased light incidence on developing fruit caused by a restriction in canopy growth. Rather, it appears to be induced by the irrigation treatment itself, and is likely to be mediated by biochemical signals such as increased ABA levels in developing fruit. A clear future direction is to investigate the underlying biochemical response of anthocyanin to stress signals produced by the vine sensing a water deficit. Although ABA is a likely signal to investigate, it is more than likely a combination of factors which brings about the response in berry skins.

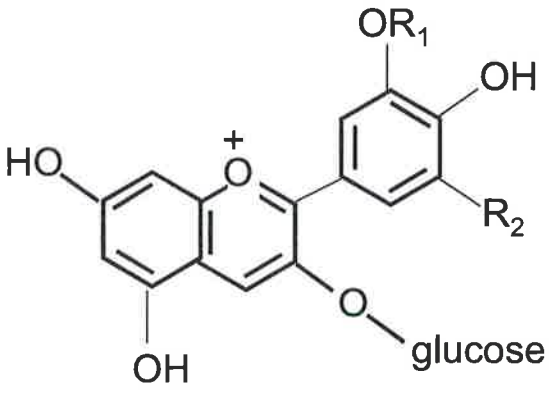
Furthermore, what has been brought to question in the current study is the correlation between fruit anthocyanin and wine colour. The effect of deficit irrigation treatments such as PRD may alter the phenolic profile of the fruit and wine, which bring about a change in the degree of copigmentation and polymerisation of wine anthocyanins. The increases seen in wine colour with deficit irrigation treatments may therefore not be due to a change in anthocyanin concentration alone, but rather an increase in the intensity of the colour due to the molecular properties of the anthocyanin in copigmented or polymeric form (Bolton, 2001). Thus, as has been shown in the current study, deficit irrigation strategies such as PRD may not necessarily improve anthocyanin concentration in the fruit, but nevertheless cause a significant improvement in wine colour and stability. An important future direction for research will be to further characterise the anthocyanin and phenolic profile of PRD-treated fruit and the resultant wines, with an aim to identify the factors most likely to enhance copigmentation and polymerisation of wine anthocyanins.

CHAPTER 7: THE EFFECT OF PRD ON ANTHOCYANIN COMPOSITION AND DEVELOPMENTAL PROFILE

7.1 Introduction

7.1.1 Changes in anthocyanin composition during ripening

The contribution of pigmented molecules to the colour of grape skins of red grape varieties is primarily due to a group of flavonoid compounds: the anthocyanins. The anthocyanins can be subdivided into a number of types which are distinguished by variations in the number of hydroxyl groups in the molecule; the degree of methylation of these hydroxyl groups; and the position, type and number of the sugars attached to the molecule (Mazza and Miniati, 1993). The backbone of the anthocyanin molecule is the anthocyanidin group, which is unglycosylated (Figure 7.1). There are five anthocyanidin types in *Vitis vinifera* red varieties, namely: malvidin, delphinidin, petunidin, peonidin and cyanidin. Each type gives a different colour, depending on the R-groups attached (Figure 7.1). Of these types, malvidin is the most abundant, and is purple-red at wine pH (3.7), giving the typical colour of red wine. The position of the glucose on the aglycone backbone gives a further 3 derivative groups: 3-monoglucoside, 3-acetylglucoside and 3-p-coumarylglucoside anthocyanins (Mazza and Miniati, 1993). Thus, in *Vitis vinifera* species there are a total of fifteen anthocyanin classes that are commonly observed in the fruit and wine.



Anthocyanidin	R_1	R_2	Colour
Malvidin	CH_3	OCH_3	purple-red
Delphinidin	H	OH	pink
Petunidin	CH_3	OH	purple
Peonidin	CH_3	H	purple-blue
Cyanidin	H	H	red

Figure 7.1 The primary structure and forms of the 15 anthocyanins found in *Vitis vinifera*

During the post-veraison increase in the total colour of berry skins, the levels of each anthocyanin type follows a unique developmental profile. An early study by Roggero *et al.* (1986) shed some light on the changes of 3-glucoside anthocyanins in Shiraz berries of three different clones. All the anthocyanin classes were all shown to peak in their content between 19 and 27 days post-veraison in all the clones studied. Following this peak, the levels of the non-malvidin anthocyanin types typically began to decline towards fruit maturity at 47 days post-veraison. The changes in malvidin-3-glucoside differed from the other anthocyanin classes, in that following 19 days post-veraison the levels either became constant or continued to increase, declining only 41 days post-veraison, shortly before the fruit was mature. Thus, the ratios of malvidin-glucosides to non-malvidin glucosides varied significantly depending on the stage of fruit development. Typically, the contribution of non-malvidin anthocyanins to the total pigments declined with fruit development, whereas the proportion of malvidin-glucosides increased as the fruit matured.

A later study on the Tempranillo grape variety also noted that the accumulation of the anthocyanins did not follow a steady pattern, and most types were characterised by a decrease as fruit matured (González *et al.* 1990). However, differences were noted in the accumulation pattern between the glucoside groups of anthocyanins. The acylated anthocyanins were distinguished from the other glucosides by a characteristic pattern of initial accumulation, followed by a decrease in concentration until a minimum was reached, after which a final phase of rapid accumulation was initiated up to fruit maturity (González *et al.* 1990). Therefore, in the case of the acylated anthocyanins, there did not appear to be the typical decrease in concentration associated with physiological maturity. However, since the studies discussed were only conducted over one season, there is little known about the seasonal variation in the developmental profile of these anthocyanin types, nor the effects of environmental factors on their changes during ripening.

7.1.2 The effects of sunlight and temperature on anthocyanin concentration

The level of solar radiation is known to influence anthocyanin levels in red grapes. Early work by Kliewer (1970, 1972) showed that anthocyanin content was greater in berries ripened under high light conditions for Emperor and Pinot Noir varieties. A further study by Smart *et al.* (1985) showed that in grape musts obtained from sun exposed and shaded canopies of Shiraz, colour density, total and ionised anthocyanins were negatively correlated with shading. However, higher levels of anthocyanins in sun exposed fruit has not been shown to be consistent throughout development (Haselgrove *et al.* 2000). In Shiraz, a significantly higher total anthocyanin content per berry was observed in sun-exposed fruit samples harvested 15 and 35 days after veraison. However, at the final harvest date, 46 days after veraison, there was no significant difference in total berry anthocyanins between treatments (Haselgrove *et al.* 2000).

A study by Mahbrouk and Sinoquet (1998) shed some light on the interaction of incident light level on the bunch and anthocyanin content in berries, which draws some of the previous observations together. In that study, bunches receiving light from a level of 2% incident radiation to 18% were compared for anthocyanin and phenolic concentration. It was found that for anthocyanin content, a maximum was reached corresponding to a light incidence of 10%, with a decline on either side of that value so that minimums occurred at 2% and 18% light incidence. This indicates that extreme levels of bunch exposure will be limiting factors for anthocyanin or phenolic content.

Some more recent studies have indicated that the limiting factor under high light incidence is likely to be elevated temperature under these conditions (Bergqvist *et al.* 2001; Spayd *et al.* 2002). A concurrent study of Grenache and Cabernet Sauvignon varieties by Bergqvist *et al.* (2001) compared anthocyanin content of north-facing and south-facing fruit. The difference between these treatments, was that the south-facing fruit received an extended period of late-afternoon sun, leading to fruit development under elevated temperature. Thus, it was an ideal situation to separate the effects of sun-exposure and temperature within a single vine. Interestingly, it was found that as light

intensity increased on the north-facing fruit, there was an increase in anthocyanin concentration. However, in the south-facing fruit, a maximum point was reached, beyond which an increase in light intensity led to a reduction in anthocyanin content. This was apparent for both grape varieties studied. Thus, it appears that where temperature is not a limiting factor, high light intensities are correlated with enhanced anthocyanin production.

This finding was further investigated by Spayd *et al.* (2002), with Merlot. Control treatments of sun-exposed and shaded bunches were set up, to be compared with sun-exposed bunches cooled to the temperature of the shaded control; and shaded fruit heated to the temperature of the sun-exposed control. Again, the results indicated that although a higher level of sun-exposure was associated with increased anthocyanin content, elevated temperature was the limiting factor. Cooled, exposed fruit had higher levels of anthocyanins than exposed fruit under ambient conditions. The converse was observed for shaded fruit, where elevated temperature decreased anthocyanin content. From these results, it is clear that both light incidence and temperature must be considered when investigating the influence of environmental factors on anthocyanin composition.

7.1.3 The effect of bunch exposure on anthocyanin composition

Studies on the relative levels of anthocyanin types as a result of sun exposure have yielded interesting results. Gao and Cahoon (1994) conducted bunch shading experiments at levels of 55 and 95% on Reliance (*Vitis* hybrid) vines. In the 95% shading treatment bunches had a decreased anthocyanin content, characterised by decreases in all the individual anthocyanins, when compared to the 55% shaded treatment and the unshaded control. Interestingly, the proportions of the various anthocyanins were affected by shading. The 95% shading treatment decreased the percentage of delphinidin-3-glucoside compared with the 55% shading treatment. Furthermore, when the 95% shaded treatment was compared with either the sun-exposed or 55% shaded treatment, the percentage of cyanidin-3-glucoside was decreased, and the percentages of peonidin-3-glucoside, malvidin-3-glucoside and acylated cyanidin derivative were increased. These results

indicated that the level of sun-exposure that the fruit receives can influence the relative levels of the individual anthocyanins, as well as affecting the total level of colour. Similarly, this effect was demonstrated in the study by Haselgrove *et al* (2000) on Shiraz where artificially shaded bunches had increased relative levels of the coumarate derivative of malvidin compared with sun-exposed samples. In contrast, in the study by Spayd *et al* (2002), where total anthocyanins were increased in a naturally sun-exposed Merlot trial, this was associated with increases in all of the anthocyanin types. No differences were observed in the relative levels of monoglucoside, acylated or coumarate forms of the anthocyanins with sun-exposure. It is apparent from the available literature that there is not a clear relationship between the different forms of anthocyanins present in the fruit and the level of sun-exposure. Given that the developmental profile of the different anthocyanin forms is variable, depending on the anthocyanin in question and the variety or clone studied (Roggero *et al.* 1986), it is necessary to explore the effect of environmental factors in fruit at different stages of physiological maturity.

7.1.4 The effects of PRD irrigation on anthocyanin composition

In recent years, research into the effects of PRD irrigation on grape composition has shown that this irrigation strategy can potentially influence anthocyanin concentration. Initial studies were conducted by Dry (1997), where PRD treated Cabernet Sauvignon vines on Ramsey rootstocks were irrigated to half that of the controls. In two years out of the three year study anthocyanin levels were increased with PRD, both when expressed as a concentration per gram berry mass, or as content per berry. In 1995 and 1996, the increase in anthocyanin concentration was 45% and 13% respectively. In 1996, anthocyanin concentration was determined at three stages of fruit development. The initial differences in concentration between PRD and control treatments was small, but this difference was enhanced when the fruit was mature. This appeared to be a result of an accelerated decline in anthocyanin concentration in control fruit, whereas under PRD treatment it was maintained. This experiment was continued by Stoll (2000) for a further two years. However, in that study, anthocyanin concentration was only found to be significantly increased with PRD in the second of the two seasons of the study (1998), in the order of 12%. A further level of analysis was conducted on fruit from these seasons,

to determine the relative proportions of the different anthocyanin types. No significant differences were found in the relative levels of mono-, acetyl- or coumarate forms of anthocyanins with PRD in either season of the study. In the 1998 season, where total levels of anthocyanins were increased with PRD, this was found to be primarily due to increases in delphinidin-monoglucoside, petunidin-monoglucoside, delphinidin-acetylglucoside and peonidin acetylglucoside. Levels of malvidin-glucoside and malvidin-acetylglucoside were actually decreased significantly with PRD, in the order of 5% and 7% respectively. Given that malvidin-glucosides are the primary form of anthocyanin pigments in this variety, this represents a significant loss, although there was a net gain in total anthocyanin. It was proposed that these changes in the relative levels of the anthocyanin types with PRD might be due to increases in light within the fruiting zone of vines, as PRD reduced both vine vigour and canopy density (Stoll, 2000). However, these results do not correspond to previous finding on the effects of sun-exposure on anthocyanin composition, where total levels of malvidin-glucosides were always enhanced under increased light intensity (Gao and Cahoon, 1994; Haselgrove *et al.* 2000, Spayd *et al.* 2002). It is therefore necessary to explore the effects of PRD on fruit composition further. The current study investigated the anthocyanin composition of fruit at different developmental stages, and also under different light conditions.

7.2 Materials and methods

7.2.1 Experimental

Experiment 1: This experiment was carried out over two seasons at a commercial field trial by Orlando-Wyndham, at Langhorne Creek, South Australia (Section 2.1.2, Chapter 2). It compared PRD and control irrigation treatments on 5-year old Cabernet Sauvignon vines pruned to 60 nodes. In 2002, the control and PRD treatments received 1.19 and 0.84 ML/ha respectively. In 2003, the control and PRD treatments received 1.37 and 0.85 ML/ha respectively. Twenty-five-berry samples were collected at weekly intervals from veraison until maturity (approx. 24°Brix) and frozen at -20°C for later analysis. Wine was made from harvested fruit from the 2002 vintage using small-scale winemaking techniques (Section 2.4, Chapter 2), and underwent malolactic fermentation. Winemaking was done in 18L fermenters, and samples were pooled to give 6 treatment replicates and 2 winemaking replicates.

Experiment 2: A shading treatment was super-imposed over the field trial described in Experiment 1 for a single season, in 2003 (Section 2.1.3, Chapter 2). Shading boxes were placed over randomly-selected bunches in each plot from fruit set to harvest. Temperature was monitored within the boxes over the season, and was unchanged by the shading treatment. The boxes and shaded bunches were removed when the fruit reached 20.5 – 21.0°Brix, and whole bunches were frozen at -20°C for later analysis. A representative 25-berry sample was later collected from the frozen bunches.

Experiment 3: A pot-experiment comparing PRD and control irrigation strategies was set up under shade cloth on 3-year old Cabernet Sauvignon vines (Section 2.2, Chapter 2). The experiment compared 5 replicates of each treatment. Stomatal conductance of the vines was measured on sun-exposed leaves at 11 am throughout the season. The vines produced approximately 2-3 bunches per vine. The bunches were harvested when the fruit reached 26°Brix, and a representative 5-berry sample was collected and frozen at -40°C.

7.2.2 Extraction of anthocyanins

The 25-berry sample from Experiments 1 and 2 were later defrosted over 30 min, and immediately homogenised using an Utra-Turrax T 25 (IKA Labortechnik, Staufen, Germany) ensuring that both seeds and flesh were completely crushed. One gram of the homogenate was weighed out into a 10 mL centrifuge tube and frozen at -20°C. For Experiment 3, berry skins were removed from fruit while still frozen and immediately placed in liquid nitrogen-cooled mortar. The berry skins were then ground to a fine powder under liquid nitrogen, and a 200 mg sample was weighed out into a 2 mL Eppendorf centrifuge tube, and frozen at -40°C.

The samples from all experiments were extracted in 50% ethanol (pH 2 with HCl); 10 mL per gram berry tissue, according to the method of Iland *et al.* (2000). Extracts were placed on a rotary shaker in the dark for 1h. Samples were then centrifuged for 5 min at 12 000 rpm, and the supernatant retained for analysis.

7.2.3 HPLC analysis of anthocyanins

The extracts were transferred to HPLC vials and 100µL was injected onto a Merck LiChrosopher 100 RP-18 (5µm) 4x250mm HPLC column and photodiode array detection at 520 nm. The HPLC system used was a Hewlett Packard HP 1100. The method was an adaptation of that used by Stoll (2000). A ramped gradient of water : MeOH : 1.5% perchloric acid was used, according to Table 7.1. The flow rate was maintained at 1mL/min, and the column temperature was 25°C. The final run time was 115 min. The anthocyanins were identified by their order of elution relative to a standard of malvidin-3-glucoside (Extrasynthase, Germany), according to the pattern described by Wulf and Nagel (1978). All anthocyanins were quantified according to a malvidin-3-monoglucoside standard curve which had a linear response within the range of concentrations injected onto the column (0.1-1 mg/mL), giving an R^2 value of 0.9927 (Figure 7.2).

Table 7.1: Proportion of solvents used in HPLC separation of anthocyanins

Time (min)	%Solvent A (water)	% Solvent B (100% methanol)	% Solvent C (1.5% perchloric acid)
0	60	25	15
10	55	30	15
40	50	35	15
45	25	45	30
105	15	55	30
107	0	100	0
112	0	100	0
114	60	25	15

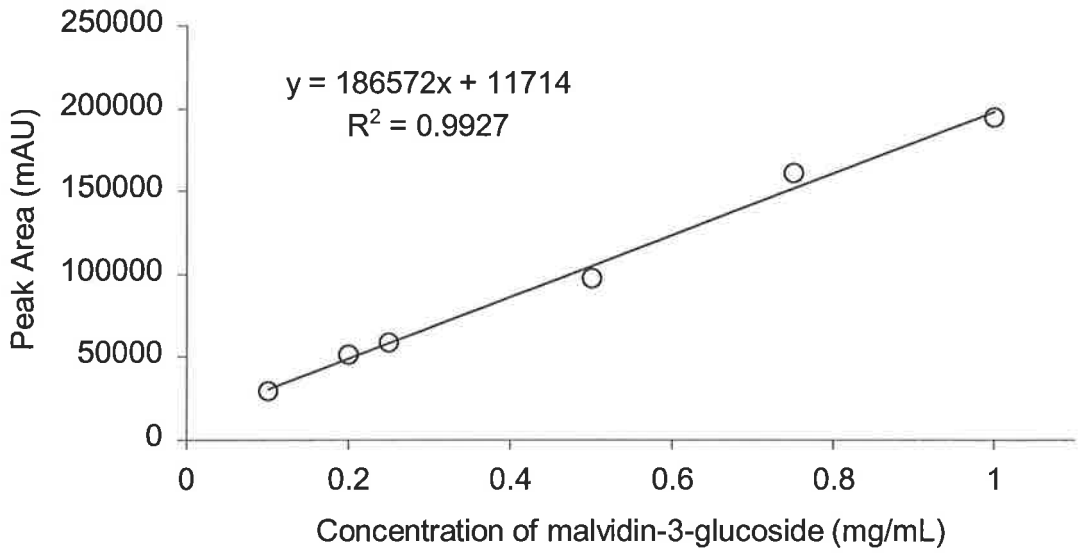


Figure 7.2: Standard curve for malvidin-3-glucoside

7.3 Results and Discussion

7.3.1 Developmental changes in the anthocyanin profile of Cabernet Sauvignon fruit and wine with PRD (Experiment 1)

Over two seasons, 2001/2002 and 2002/2003, a shift in the anthocyanin profile of fruit at harvest (23-23.5°Brix) was observed. This was characterised by an increase in the less abundant anthocyanidin types relative to malvidin-glucosides in PRD-treated berries relative to the control treatment. This was irrespective of the degree of methylation of the anthocyanins, and the trend was evident for the monoglucosides, acetylglucosides and the 3-p-coumaryl glucosides (Figure 7.3). For both the 2001/2002 and 2002/2003 vintages, the most significantly affected anthocyanidin type was cyanidin followed by delphinidin and petunidin (Figure 7.3). Malvidin-glucosides were slightly decreased by the PRD treatment, although this effect was not statistically significant. This result is in agreement with that of Stoll (2000), who demonstrated a shift toward some of the less abundant anthocyanidin types, with a significant reduction in malvidin pigments in that study.

The developmental profile of each anthocyanidin was investigated, from approximately 15°Brix, at which point the fruit had completed veraison, until maturity (Figures 7.4 and 7.5). The final sampling date reflects a time point approximating the time of harvest, as fruit from the different treatments was collected when it reached 23-23.5°Brix. In the 2001/2002 vintage, cyanidin demonstrated a different developmental pattern to the other anthocyanin types (Figure 7.4). Whereas the other anthocyanidins increased throughout development, cyanidin had higher levels early in development, decreasing as the fruit matured. The PRD treatment increased the concentration of cyanidin-glucosides from early in development, and this effect was maintained in the fruit at harvest. In the 2002/2003 vintage, however, the developmental pattern for this pigment was different from the previous season, and increased as the fruit matured, showing a decline only as the fruit approached maturity (Figure 7.5). The PRD treatment did not produce a significant effect on this pigment from early in development as in 2001/2002. Instead, the large differences in this pigment seen at harvest were primarily due to an accelerated decline of cyanidin in the control fruit at maturity, whereas in the PRD fruit, cyanidin levels were maintained (Figure 7.5).

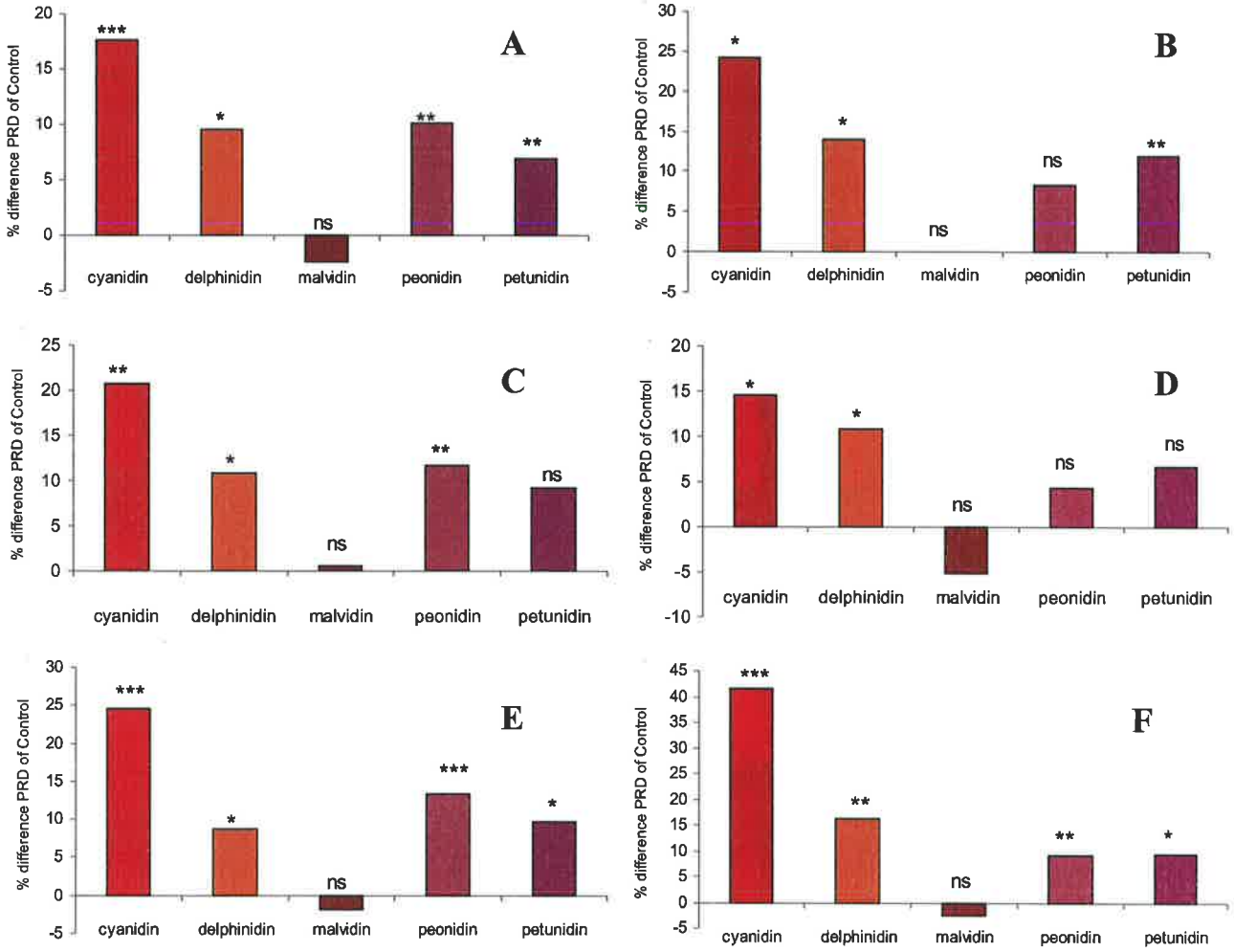


Figure 7.3: Changes in the levels of anthocyanin types in PRD fruit relative to control over two seasons. **A**. 2001/2002 3-monoglucoside anthocyanins; **B.** 2002/2003 3-monoglucoside anthocyanins; **C**. 2001/2002 3-acetyl-glucoside anthocyanins; **D**. 2002/2003 3-acetyl-glucoside anthocyanins; **E.** 2001/2002 3-(p-coumaryl)-glucoside anthocyanins; **F**. 2002/2003 3-(p-coumaryl)-glucoside anthocyanins. (ANOVA; n=42, * = $P<0.05$; ** = $P<0.01$; *** = $P<0.001$; ns = not significant)

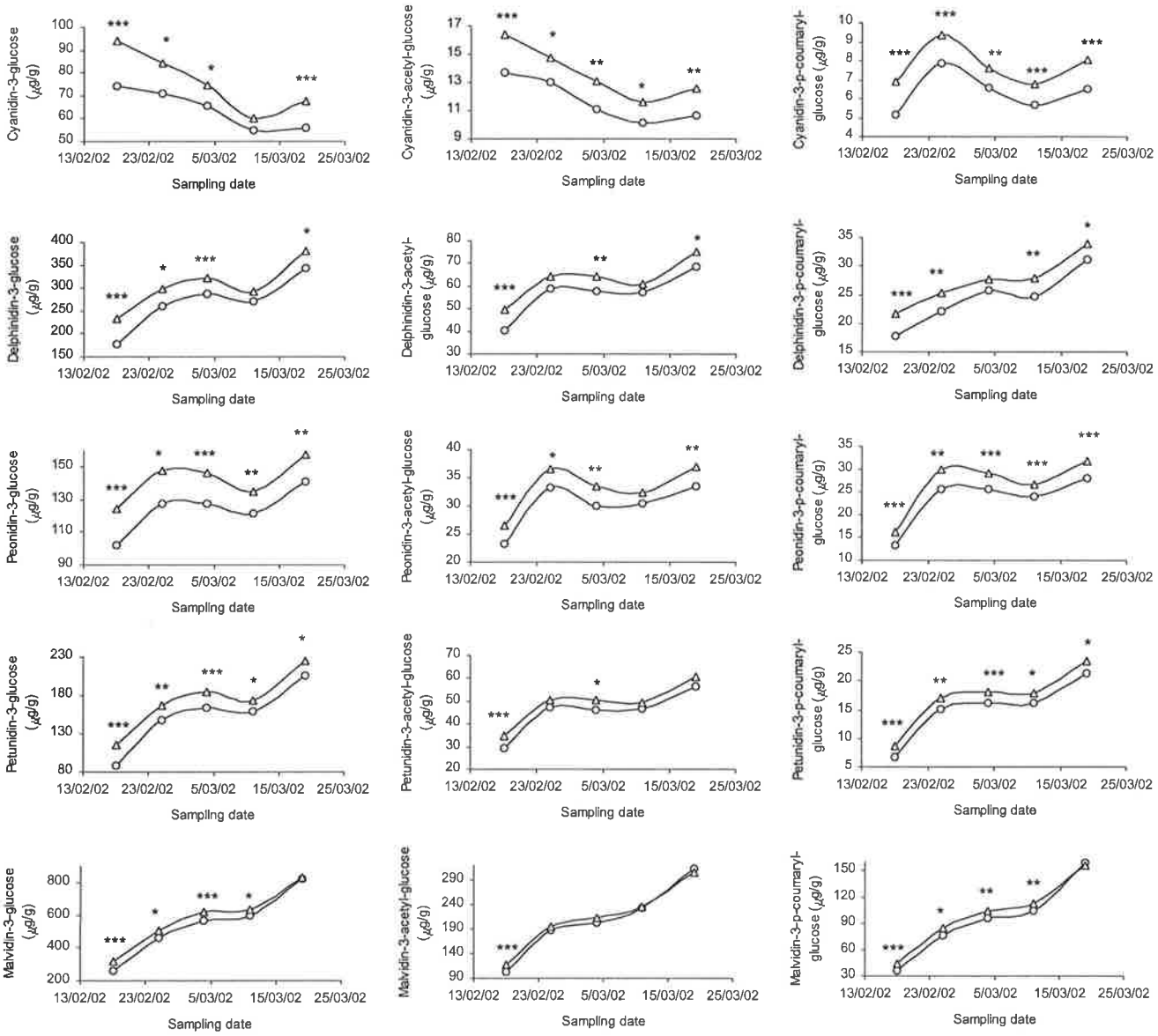


Figure 7.4 Developmental changes of anthocyanidins in Cabernet Sauvignon for the 2001/2002 vintage (Control ○ PRD △ ; ANOVA, n=42, *= P<0.05; **=P<0.01;***=P<0.001).

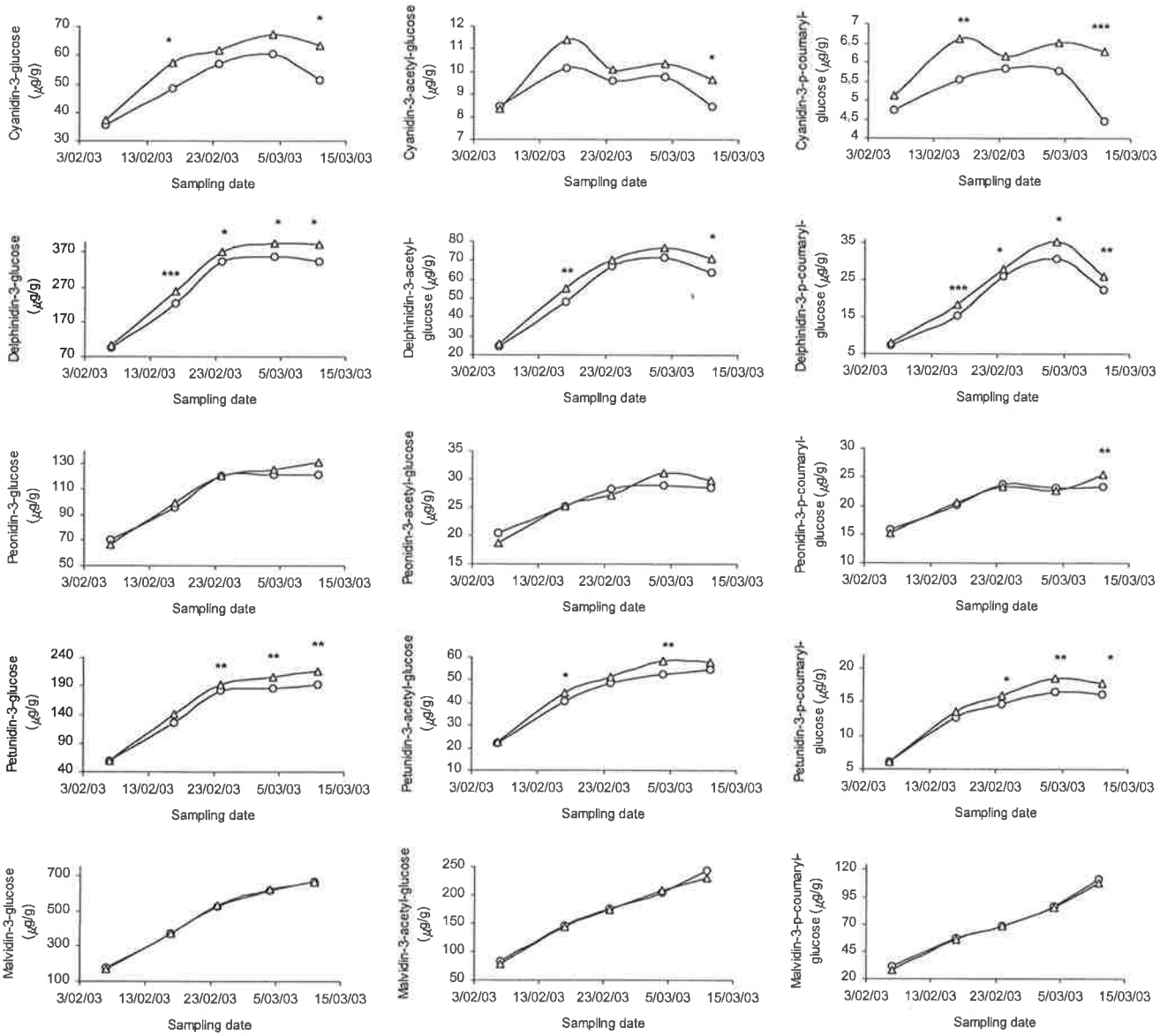


Figure 7.5 Developmental changes of anthocyanidins in Cabernet Sauvignon for the 2002/2003 vintage (Control ○ PRD △ ; ANOVA, n=42, *= P<0.05; **=P<0.01;***=P<0.001).

For the other anthocyanin pigments, the type of curve demonstrated during development differed between anthocyanidin type and between vintages, but followed a pattern of general increase in the concentration of the pigment as the fruit matured (Figures 7.4 and 7.5). Thus, with the exception of delphinidin-acetylglucoside and delphinidin-3p-coumaryl glucoside which declined in concentration at maturity in 2002/2003, the concentration of anthocyanin pigments in the fruit was highest at harvest. In the 2001/2002 vintage, a slight acceleration in the rate of ripening was observed with PRD (Figure 4.1,) which may account for some of the significant differences observed at each time point (Figure 7.4). However, where the PRD treatment caused significant changes in a pigment, it was generally reflected throughout the developmental profile. In the case of the malvidin-glucosides, higher levels were observed early in development in 2001/2002, but by harvest this difference was negligible. In 2002/2003, however, the profile of the malvidin-glucosides did not differ significantly at any stage of development.

These data indicate that the response of the anthocyanin pathway toward the production of different anthocyanin types may be regulated by environmental factors, even when the flux through the whole pathway is not increased. It must be noted that in the 2001/2002 vintage, total anthocyanin concentration was not altered by PRD at harvest, but was increased significantly in 2002/2003 by 5%. Thus, even when total input of assimilated carbon to the anthocyanin pathway was unchanged by the PRD treatment, the alterations in the partitioning of that carbon to the different anthocyanin groups was consistent over both seasons of the study. This raises an important question about the regulation of the anthocyanin pathway under conditions of environmental stress.

Some insight into the regulation of anthocyanin metabolism under stress conditions can be gained from recent work done on the antioxidant properties of anthocyanin in leaves (Manetas *et al.* 2002; Manetas *et al.* 2003; Neill and Gould, 2003). In photosynthetic tissue, light is both an energy resource and potentially destructive by causing photo-oxidative injury and the production of free radicals in the plant tissue (Neill and Gould, 2003). In plants which develop anthocyanins in their leaves under conditions of stress e.g. high light, low temperature or drought, it has been proposed that anthocyanins serve as

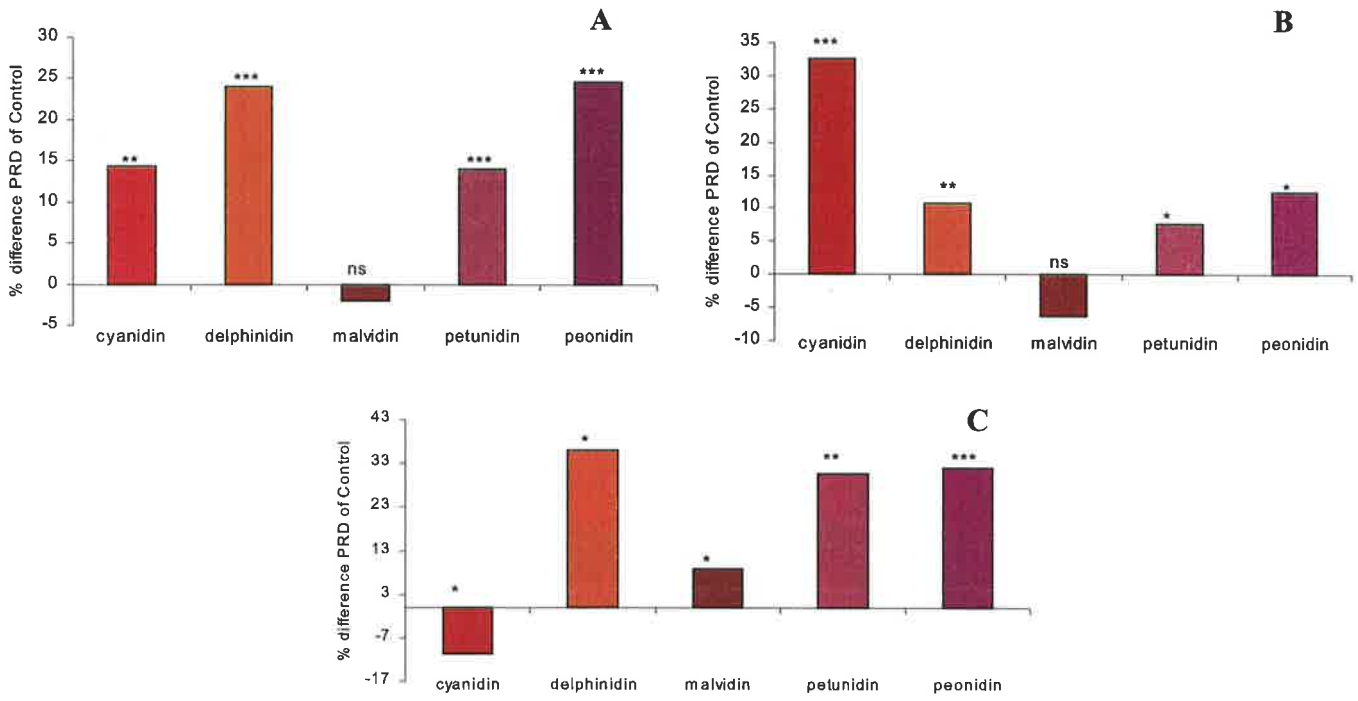
photoprotectants, due both to their ability to strongly absorb light and as antioxidants (Rice-Evans *et al.* 1997; Wang *et al.* 1997; Steyn *et al.* 2002). A study on leaves of *Rosa* sp. and *Riccinus communis* L. showed that in photosynthetic tissue the development of anthocyanins was correlated with lowered carotenoid concentration, and thus a low photodissipative potential via the xanthophyll cycle (Manetas *et al.* 2002). The presence of anthocyanins was proposed to increase photoprotection, allowing for the dissipation of excess light energy.

It must be noted that in grape berries, the fruit is green and photosynthetic pre-veraison, and the senescence characteristics surrounding the changes in the fruit at veraison are accompanied by an increase in anthocyanin, with a concomitant loss of chlorophyll and carotenoid concentration (Razungles *et al.* 1988; Razungles *et al.* 1996; Düring and Davtyan, 2002). The potential of anthocyanin to serve as a means of light energy dissipation in fruit tissue undergoing loss of photosynthetic capacity, and thus requiring an alternative photoprotective mechanism, has not been considered. In the light of the current findings, the changes in the anthocyanin profile may represent a shift in the antioxidant properties of the different anthocyanidin types, toward those with a higher radical scavenging capacity, as has been shown in a study by Pannala *et al* (2001). In that study, the electron-donating ability of a range of flavonoid compounds was studied, including the anthocyanidins malvidin, delphinidin and cyanidin. Of these anthocyanidins, malvidin had the lowest antioxidant capacity, and was given a ranking of 0.63. Cyanidin and delphinidin were higher in radical-scavenging capacity, with rankings of 0.72 and 1.15 respectively. Under the conditions of the current study, PRD vines produced fruit under the action of stress signalling from the roots and leaves; as well as 30% higher levels of fruit exposure to sunlight (Figure 3.8). It is possible that the shift in the anthocyanin profile away from the more chemically sensitive malvidin toward more chemically stable anthocyanins may be occurring as a physiological response to stress conditions. Furthermore, the production of a range of anthocyanin types which absorb light maximally at different wavelengths may also increase the effectiveness of the grape berry skin as a photoprotectant.

The implications of this shift in the anthocyanidin profile in response to the PRD treatment for winemaking needs consideration. The concentration of the different anthocyanidins was investigated in wines made from fruit of the 2001/2002 vintage (Table 7.2, Figure 7.6). Only free anthocyanins were measured in wines at bottling. The shift in the ratios of the anthocyanins in PRD wines was comparable to that seen in the fruit at harvest (Table 7.2). In the fruit, the non-malvidin anthocyanidin types represent approximately 45% of total anthocyanins, whereas in the wine they represent approximately 30%. This decrease may either reflect the degradation of these anthocyanidins during winemaking, but more likely indicates that they are present in another form e.g. bound in a complex (Bolton, 2001).

Table 7.2: Levels of anthocyanin types in fruit and wine from the 2001/2002 vintage. (ANOVA; n=42 for fruit samples; n=12 for wine samples; ns = not significant)

Anthocyanin type	2002 fruit at harvest (μg/g)				2002 wines at bottling (μg/mL)			
	Control	PRD	P	%PRD> Control	Control	PRD	P	%PRD> Control
cyanidin-3-monoglucoside	55.9	67.5	<0.001	21	2.3	2.7	<0.01	14
delphinidin-3-monoglucoside	344.8	382.1	<0.05	11	16.6	20.6	<0.001	24
malvidin-3-monoglucoside	826.0	831.0	ns	1	121.6	119.2	ns	-2
peonidin-3-monoglucoside	140.9	157.4	<0.01	12	6.6	8.2	<0.001	25
petunidin-3-monoglucoside	205.6	224.4	<0.05	9	19.3	21.9	<0.001	14
cyanidin-3-acetyl-glucoside	10.7	12.6	<0.01	18	0.9	1.2	<0.001	33
delphinidin-3-acetyl-glucoside	68.4	74.9	<0.05	10	7.0	7.8	<0.01	11
malvidin-3-acetyl-glucoside	312.8	305.3	ns	-2	41.8	39.2	ns	-6
peonidin-3-acetyl-glucoside	33.5	36.9	<0.01	10	3.5	3.9	<0.05	12
petunidin-3-acetyl-glucoside	56.6	60.5	ns	7	5.7	6.2	<0.05	8
cyanidin-3-p-coumaryl-glucoside	6.5	8.1	<0.001	25	2.0	1.8	<0.05	-11
delphinidin-3-p-coumaryl-glucoside	31.2	33.9	<0.05	9	1.05	1.4	<0.05	36
malvidin-3-p-coumaryl-glucoside	158.5	155.5	ns	-2	6.6	7.2	<0.05	9
peonidin-3-p-coumaryl-glucoside	27.9	31.7	<0.001	14	0.95	1.3	<0.001	32
petunidin-3-p-coumaryl-glucoside	21.4	23.5	<0.05	10	0.81	1.06	<0.01	31
Total anthocyanins	2301.0	2405.0	ns	5	232.6	240.3	ns	3



igure 7.6: Changes in the levels of anthocyanin types in PRD wine relative to control for the 2001/2002 intage. **A.** 3-monoglucoside anthocyanins; **B.** 3-acetyl-glucoside anthocyanins; **C.** 3-(p-coumaryl)-ANOVA; n=42; * = P<0.05; ** = P<0.01; *** = P<0.001; ns = not significant)

The contribution of the non-malvidin anthocyanins to the final wine colour has not been studied in detail, as malvidin-glucosides predominate in the wine and have been given most attention (Bolton, 2001). As the concentration of free anthocyanins in wine decreases with wine ageing (Lambert, 2002), and become primarily bound in polymeric form, the potential for each anthocyanin type to stabilise in the wine will reflect the final colour perceived after ageing. The long-term stability of the non-malvidin anthocyanins, and their potential to bind to other molecules present in wine will influence the final colour of PRD wines, as these forms of the anthocyanins are higher relative to malvidin. Should malvidin be a more stable wine pigment, wines made from the PRD treatment may potentially lose a greater proportion of wine colour during ageing. Thus, further research is required comparing the potential of anthocyanidins such as delphinidin and cyanidin with malvidin in the formation of stable, bonded molecules in wine.

7.3.2 The effect of total shade on the anthocyanin profile of PRD-treated fruit (Experiments 2 and 3)

In the light of the results of Experiment 1, the anthocyanin profile of completely shaded PRD- and control-treated fruit was compared at similar °Brix, in order to assess the importance of light-exposure in the mediation of the changes seen in PRD fruit. The data from Experiment 2 is shown in Table 7.3. Whereas sun-exposed PRD fruit showed a shift away from malvidin-glucosides, with increases in delphinidin-glucosides and petunidin-glucosides, the totally shaded PRD fruit did not. Although this result does not show that increased fruit exposure with PRD is responsible for the changes observed in the anthocyanin profile, it does indicate that the presence of sunlight is necessary for the response in the anthocyanin profile to PRD. The effect of total shade in both the PRD and control treatments was a reduction in total anthocyanin, which was reflected in all the anthocyanidin types (Table 7.3). This response has been shown for Shiraz grapes grown under similar conditions of total shade (Downey *et al.* 2004). The primary change in the anthocyanin profile caused by shading was a decrease in the proportion of the monoglucoside forms of the anthocyanins, and an increase in the proportion of the acylated and p-coumaryl forms of the anthocyanins (Figure 7.7) as has been reported by Haselgrove *et al* (2000). This shift was not evident in the sun-exposed PRD-treatment (Figure 7.7), where all the monoglucoside, acylated and p-coumaryl groups of anthocyanins were increased in equal proportion.

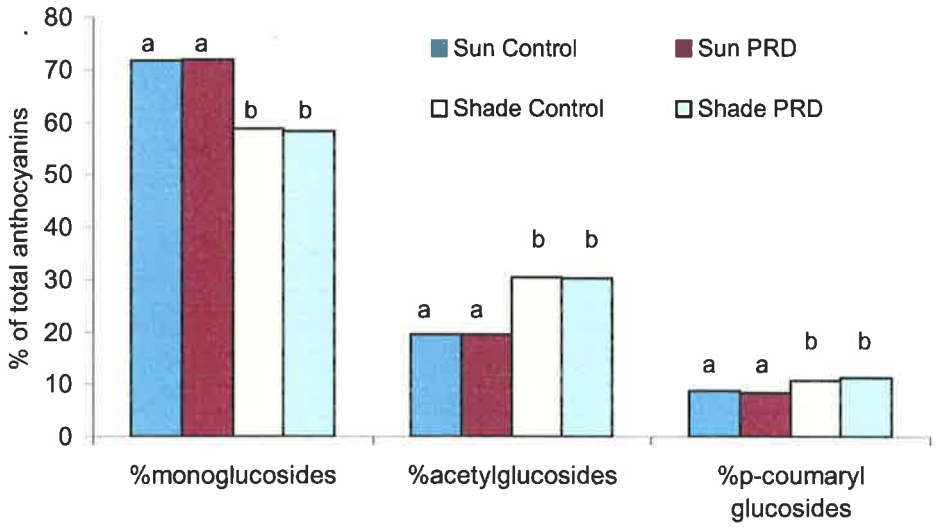


Figure 7.7: Effect of PRD and shading on the percentage of anthocyanins in each glycosylated class from field-grown Cabernet Sauvignon fruit in 2002/2003. (ANOVA; n=42; a,b = significantly different where P<0.01)

Table 7.3: Effect of PRD and total shade on levels of anthocyanin types in field-grown Cabernet Sauvignon at 20°Brix from the 2002/2003 vintage; P=phenolics; A=anthocyanins; MG=3-monoglucoside; AG=3-acetylglucoside; pCG=3-(p-coumaryl)-glucoside. (ANOVA; n=42; ns = not significant)

Treatment	Total P (A_{280} units/g)	Total A (mg/g)	Delphinidin (mg/g)			Petunidin (mg/g)			Malvidin (mg/g)		
			MG	AG	pCG	MG	AG	pCG	MG	AG	pCG
Sun Control	1.5	1550	360	71.2	30.8	190	52.4	16.6	620	204	87.0
Sun PRD	1.6	1630	400	76.8	35.3	210	58.3	18.6	630	208	85.4
Shade Control	1.2	1000	120	38.5	9.7	80	33.4	86.9	390	238	84.5
Shade PRD	1.3	1020	120	35.5	10.0	80	33.3	94.5	400	243	91.2
Sun: PRD relative to Control											
P	<0.001	0.01	<0.05	ns	<0.05	0.01	0.01	<0.01	ns	ns	ns
% difference	8	5	11	8	15	11	11	12	2	2	-2
Shade: PRD relative to Control											
P	<0.05	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
% difference	5	2	0	-8	2	0	0	9	3	2	8
Control: Sun relative to Shade											
P		<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	ns
% difference	20	55	200	85	216	138	57	-81	59	-14	3
PRD: Sun relative to Shade											
P		<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	ns
% difference	24	60	233	116	254	163	75	-80	58	-14	-6

In Experiment 3, the effect of PRD on the anthocyanin profile of potted Cabernet Sauvignon was studied, under limited light conditions, where plants were grown under shade net. The PAR under the shade net was approximately 50% of the ambient light, which ranged from between 1800-2000 PAR measured at midday. Thus, sunlight was a limiting factor in the development of anthocyanins. Stomatal conductance of the leaves was taken as a measure of the effectiveness of the PRD treatment, and PRD reduced stomatal conductance at all stages of the season (Appendix F). As leaf water potential was not measured, no distinction could be made between hydraulic and non-hydraulic responses to the irrigation treatment. As there was little canopy development in the potted vines, all fruit was fully exposed to the sunlight conditions under the shade cloth, and there were no differences in exposure observed between the control and PRD treatments. Thus, any changes observed in the fruit would most likely be due a biochemical response to the PRD treatment on the roots.

In the pot experiment, it was interesting to note that instead of seeing the shift in the anthocyanin profile away from malvidin-glucosides and toward the other anthocyanidin types, there was a significant increase in malvidin-glucosides alone (Table 7.4). Although some changes were observed in the other anthocyanidin types (Table 7.4), these changes were not statistically significant. This result may indicate that where light is limiting, but fruit is not completely shaded, PRD increased the partitioning of carbon toward malvidin-glucoside. This may indicate the upregulation of the anthocyanin pathway into malvidin-glucosides induced by PRD. This increased flux within the anthocyanin pathway in PRD-treated fruit is most likely a direct response to biochemical signals transmitted to developing fruit as a result of a perceived water deficit by the vine (Stoll *et al.* 2000) and does not occur in response to elevated bunch-exposure. However, under the high light intensities experienced by field-grown vines, this increased flux within the anthocyanin pathway appears to be diverted to anthocyanidin forms other than malvidin. This shift may increase the antioxidant and photoprotectant capacity of the berry, which is subject to both water stress and light stress, as has been previously discussed. This hypothesis has not been tested in grape berries, and is only speculative at present.

Table 7.4: Effect of PRD on levels of anthocyanin types in fruit of potted Cabernet Sauvignon from Experiment 3 in 2002/2003. (T-test; n=10; ns = not significant)

Anthocyanin type (mg/g berry skin)	2003 fruit at harvest (μg/g)			
	Control	PRD	P	%PRD> Control
Cyanidin -3-monoglucoside	0.88	0.81	ns	-8
delphinidin-3-monoglucoside	0.12	0.09	ns	-25
Malvidin-3-monoglucoside	4.65	6.04	<0.05	30
Peonidin-3-monoglucoside	0.65	0.65	ns	0
petunidin-3-monoglucoside	0.64	0.61	ns	-5
cyanidin-3-acetyl-glucoside	0.18	0.15	ns	-17
delphinidin-3-acetyl-glucoside	0.02	0.02	ns	0
malvidin-3-acetyl-glucoside	2.56	3.30	<0.05	29
peonidin-3-acetyl-glucoside	0.21	0.19	ns	-10
petunidin-3-acetyl-glucoside	0.20	0.20	ns	0
cyanidin-3-p-coumaryl-glucoside	0.13	0.16	ns	23
delphinidin-3-p-coumaryl-glucoside	0.02	0.02	ns	0
malvidin-3-p-coumaryl-glucoside	1.19	1.81	<0.01	52
peonidin-3-p-coumaryl-glucoside	0.09	0.11	ns	22
petunidin-3-p-coumaryl-glucoside	0.15	0.17	ns	13

Alteration in the expression of the genes has been achieved by recent work on petunia by Winkel-Shirley (2001). The hydroxylation pattern of the anthocyanin B-ring is important in determining the final colour of the pigment (Figure 7.1), and is controlled by two genes: the flavonoid-3′ hydroxylase (F3′H) or flavonoid-3′ 5′ hydroxylase (F3′5′H) genes. Nevertheless, changes in the expression of these genes alone was found to be insufficient to alter flower colour. Rather, maximal expression of the F3′5′H gene has been linked to a specific cytrochrome b_5 in petunia (de Vetten *et al.* 1999). Further investigation showed that when the petunia F3′5′H and cyt b_5 genes were introduced together to carnation, flower colour was altered from red to a deep purple (Brugliera *et al.* 2000). This may indicate that there is a more complex association of genes regulating the anthocyanin pathway than has previously been published (Boss *et al.* 1996). Therefore, the potential point of regulation of the anthocyanin pathway by water stress and light

remains unclear as yet. Furthermore, a recent study on heat stress in rose showed a dramatic shift from the usual 1:1 ratio of pelargonidin:cyanidin anthocyanidins to 2:1 following heat shock (Dela *et al.* 2003). The enzyme controlling this reaction is F3'H, and unexpectedly showed no change in expression due to the heat shock. Clearly, large changes in anthocyanin expression can be caused without a significant alteration in gene expression, suggesting a more complex regulation of the pathway. As yet, there is no explanation for the responses seen in anthocyanin composition to the PRD treatment in the current study, and will require further investigation.

7.4 Conclusions

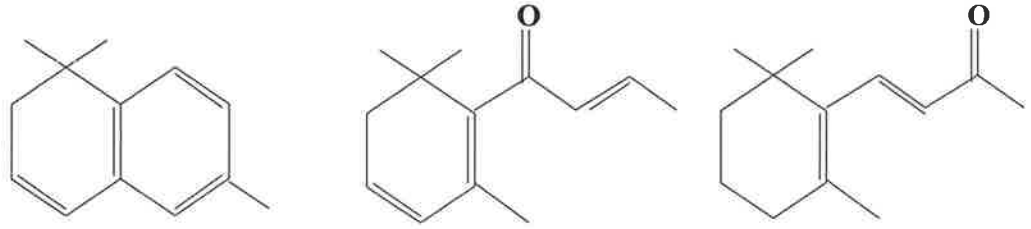
The results of the current study shed some light on the interactions between fruit exposure, PRD irrigation and anthocyanin composition. PRD irrigation affects the regulation of the anthocyanin pathway in the presence of sunlight, possibly to allow greater photoprotection and radical-scavenging capacity under a combination of water stress and high light conditions. This resulted in an alteration of partitioning of carbon away from malvidin-glucosides toward the less abundant anthocyanidin types, namely cyanidin-, delphinidin-, petunidin- and peonidin-glucosides. This trend was observed at an early stage of fruit development, and was not due to degradation or alterations in the rate of accumulation near maturity. Where total levels of anthocyanins were higher in the PRD treatment at harvest, this was primarily due to increases in the non-malvidin anthocyanidins. Under reduced light, PRD appears to cause an increase in the concentration of malvidin-glucosides alone, while the other anthocyanidin types remain unchanged. This indicates that stress signals from the roots alone may mediate the changes in the anthocyanin pathway, and is independent of alterations in fruit exposure in response to the PRD treatment. Instead of directly regulating the anthocyanin pathway, sunlight-exposure appears to be a necessary 'stress signal' to the fruit to alter the partitioning of anthocyanidin types, rather than altering total flux within the pathway. Therefore, a new possibility for research is opened: the sites of regulation of stress signals of the anthocyanin pathway.

CHAPTER 8: QUANTIFICATION OF C_{13}-NORISOPRENOIDS IN GRAPES AND WINE

8.1 Introduction

8.1.1 The C_{13}-norisoprenoids

The oxidative degradation of carotenoids, terpenes with 40 carbon atoms (tetraterpenes) produces derivatives with 9, 10, 11 or 13 carbon atoms (Enzell, 1985). Those derivatives with 13 carbon atoms have been noted for their distinct odour properties, and are termed the C_{13}-norisoprenoids. The C_{13}-norisoprenoids can be divided into two groups, megastigmane and non-megastigmane (Ribereau-Gayon *et al.* 2000). A common example of the megastigmane group is ß-damascenone (Figure 8.1), which has a complex fragrance of flowers, tropical fruit or stewed apple. It can be detected by the human senses at low concentration, with a perception threshold of 2 ng/L in water, and 45 ng/L in dilute alcohol solution (Ribereau-Gayon *et al.* 2000). A further example of a megastigmane C_{13}-norisoprenoid is ß-ionone, which has a characteristic odour of violets. It is not as easily detected as damascenone: 7ng/L in water and 800 ng/L in dilute alcohol solution (Ribereau-Gayon *et al.* 2000). The concentration of ß-damascenone and ß-ionone is extremely variable in wines, with ranges of 5-6460 ng/L and 0-2451 ng/L respectively (Ribereau-Gayon *et al.* 2000). An important non-megastigmane compound is 1,1,6-trimethyl-1,2-dihydronaphthalene (TDN). It has a negative, kerosene-like, "petroleum" odour, and has a perception threshold of approximately 20 µg/L (Ribereau-Gayon *et al.* 2000). It is generally absent in young wines, but increases during wine ageing reaching concentrations of up to 200 µg/L (Ribereau-Gayon *et al.* 2000). Each of the above- mentioned compounds are found in wine of most red and white varieties, and because of their low detection thresholds are considered to be significant in their contribution to wine aroma and flavour (Strauss *et al.* 1987; Sefton, 1998).



1,1,6-trimethyl-1,2 dihydronaphthalene (TDN) ß-damascenone ß-ionone

Figure 8.1: Chemical structure of the aglycones TDN, β-damascenone and β-ionone

8.1.2 The natural formation of C_{13}-norisoprenoids in grapes and wine

The formation of C_{13}-norisoprenoids from the degradation of carotenoids in plant tissues has been extensively studied, but the exact pathway is still unclear. A biogenetic pathway has been proposed, with the breakdown of carotenoids by oxidases as the primary step. The products are proposed to be then modified by oxidases and reductases, with the final step being glycosylation by glycosyltransferases, so that most C_{13}-norisoprenoids are present as bound forms of the free volatile aglycone (see citations of Baumes *et al.* 2002). Although photo-oxidative reactions have also been proposed to play a role in this pathway (Isoe *et al.* 1972), the evidence for a biogenetic pathway in fruit tissues is strong, with recent evidence suggesting close stereochemistry between grape-derived carotenoids and certain C_{13}-norisoprenoids (Baumes *et al.* 2002). Flux though this complex pathway is small, and there are low levels of C_{13}-norisoprenoids in plant tissue. The concentration of free C_{13}-norisoprenoid aglycones is reported to be low in grape tissues, relative to the glycosylated form (Kotseridis *et al.* 1999 a, b). Thus, due to the final steps in the proposed biogenetic pathway, most of the available compound is in the bound form or as oxygenated carotenoid derivatives.

The conditions in wine are very different from those in fruit. It has been reported that high levels of carotenoids are found in grape musts, but that these degrade when the fermentation is allowed to extend to completion (Guedes de Pinho *et al.* 2001). Thus, in fortified wines, where fermentation may be inhibited after 3-4 days by the addition of neutral grape spirit, conditions allow for the persistence of these liposoluble molecules into the wine sample (Guedes de Pinho *et al.* 2001). However, in dry red wines, there is a reported absence of any carotenoids (Guedes de Pinho *et al.* 2001), which most likely indicates that oxidative degradation has occurred. Traditionally, it has been proposed that C_{13}-norisoprenoid compounds are derived chiefly from hydrolytic cleavage of glycosidically-bound precursors in the fruit, at wine pH. However, recent evidence indicates that during alcoholic fermentation, there is a decrease of β-carotene content and an increase of hydroxy- and epoxy-xanthophylls, e.g. neoxanthin, violaxanthin and luteoxanthin (Guedes de Pinho *et al.* 2001). This may occur due to the co-oxidation of carotenoids and lipoxygenases (Ben Aziz *et al.* 1971; Wache *et al.* 2002). It is

conceivable that C_{13}-norisoprenoids may be directly formed from these molecules during wine ageing, rather than due to hydrolysis of glycosides alone.

8.1.3 Quantification of specific C_{13}-norisoprenoids in grapes and wine

A limitation in the quantification of C_{13}-norisoprenoids is the lack of specific, stable standards. Recently, the synthesis of deuterated standards of ß-damascenone and ß-ionone was reported (Kotseridis *et al.* 1998). The use of an internal standard enables the accurate quantitation of trace amounts of these compounds. This provided an opportunity to develop techniques to more accurately quantify these compounds. Methods for the quantification of ß-damascenone and ß-ionone were subsequently reported for grape and wine samples using liquid-liquid extraction, and isolation of volatile precursors on a reverse-phase C_{18} column, followed by analysis by GC-MS (Kotseridis *et al.* 1999 a,b).

A limitation of current techniques is the time involved in sample preparation and processing. In recent years, a technique known as solid-phase microextraction (SPME) has been developed (Arthur and Pawliszyn, 1990), and can be applied to the analysis of headspace volatiles (Steffen and Pawliszyn, 1996). It is an adsorption technique, by which volatile compounds come into equilibrium with a fused silica fibre coated with an adsorbent, e.g. polydimethylsiloxane. The bound compounds can then be thermally desorbed in a gas-chromatograph injection port, and analysed (Figure 8.2). SPME has been applied to determine the headspace volatiles from various plant sources, for example orange juice (Steffen and Pawliszyn, 1996, Jia *et al.* 1998), cantaloupe (Beaulieu and Grimm, 2001), hops (Field *et al.* 1996), truffles (Pelusio *et al.* 1995), apples (Song *et al.* 1997), tomato (Song *et al.* 1998), raspberries, strawberries, blackberries, banana and mango (Ibanez *et al.* 1997; Song *et al.* 1998). It has also been applied to the analysis of wines (de la Calle Garcia, 1996, Hayasaka and Bartelowski, 1999, Francioli *et al.* 1999, Vianna and Ebeler, 2001).

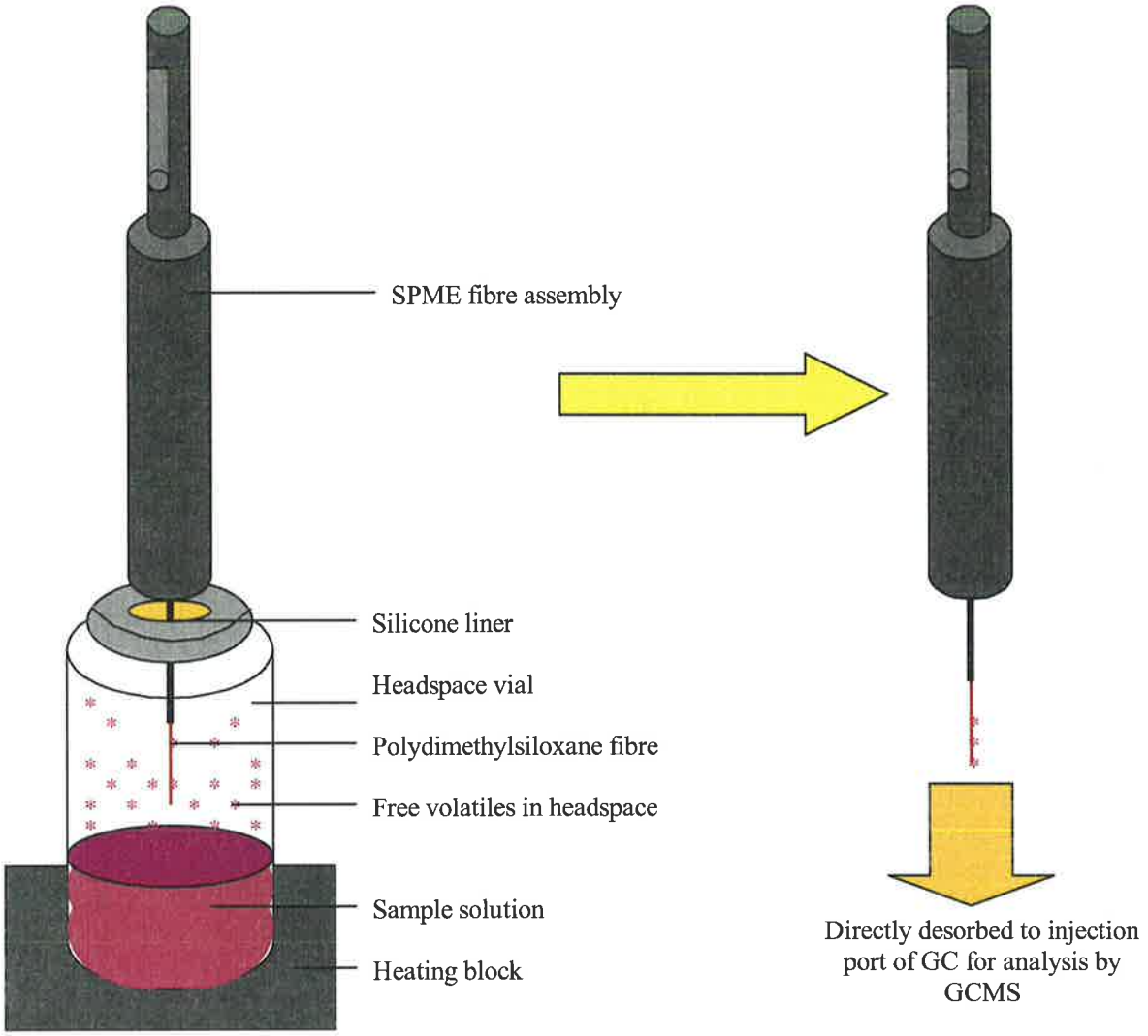


Figure 8.2: The solid-phase-micro-extraction (SPME) technique for the analysis of headspace volatiles by GCMS.

Recently, this technique has been applied to the quantification of C_{13}-norisoprenoid glycoconjugates in Muscadet grapes (Schneider *et al.* 2001). This technique involved the extraction with reversed-phase C_{18} or XAD-2 columns in combination with stable isotope dilution of deuterated ß-damascenone standard. With SPME, the potential remains for the direct sampling of C_{13}-norisoprenoids from the headspace of grape and wine samples. The current study aims to make use of this technique to compare and quantify certain C_{13}-norisoprenoid volatiles in grape berry homogenates and wine samples, in order to relate these back to viticultural practices. Attention has been given to the development of direct sampling techniques using stable isotope dilution and acid hydrolysis without prior

extraction of glycoconjugates from the fruit or wine samples. Under the conditions of acid-hydrolysis, C_{13}-norisoprenoids will be derived both from free forms in the fruit or wine; from glycosylated precursors by hydrolysis; and also partly due to degradation of carotenoids in fruit, or the oxygenated derivatives of carotenoids in wine. Thus, the method will not allow for accurate determination of the volatiles derived from specific sources. Instead, it will quantify the total level of C_{13}-norisoprenoids available from multiple pathways, which may ultimately reflect what may be found in wine samples after fermentation and wine-ageing.

8.2 Materials and Methods

8.2.1 C_{13}-norisoprenoid standards

Standards of β-damascenone, β-ionone and TDN, together with the deuterated analogues [$^2H_4$]β-damascenone, [$^2H_3$]β-ionone (Kotseridis *et al.* 1998) and [$^2H_8$]napthalene were a gift from the Australian Wine Research Institute (AWRI), Adelaide, South Australia.

8.2.2 General headspace-SPME sampling procedure

SPME headspace sampling was performed manually. SPME fibres and fibre assembly were obtained from Supelco. A 2 mL sample of standard or wine, or 2 g of berry tissue were placed in a 10 mL glass vial and sealed with a Supelco PTFE/Silicone septum. The SPME fibre was then inserted through the septum and exposed to the sample headspace at 40°C. Care was taken to ensure that the fibre was exposed at a constant level above the sample surface. After adsorption, the fibre was removed from the sample and desorbed in the GC injection port for 1 min.

8.2.3 Instrumental analysis

SPME - GC/MS analysis was performed using a Hewlett Packard HP gas chromatograph 6890, fitted with a 30 m fused silica SGE BP20 column (0.25mm i.d. and 0.25 μm film thickness), coated with Carbowax 20 M. The split/splitless injection port was heated to 220°C. The split flow was set at 5.0 ml/min and the split ratio was 5.038:1. Ultrahigh-purity helium was used as a carrier gas at a constant flow rate of 1 mL/min, with a column head pressure of 6.98 psi. The temperature program was initially 40°C for 4 min, then ramped to 240°C at a rate of 12°C/min. The temperature was held at 240°C for 5 min, giving a total run time of 25.67 min. The GC instrument was coupled to a Hewlett Packard HP 5972 mass selective detector.

8.2.4 Quantitative analysis

For quantitation of C_{13}-norisoprenoids using selective ion monitoring, 69 *m/z* was used for β-damascenone; 73 *m/z* for [$^2H_4$]β-damascenone (d4-β-damascenone), 177 *m/z* for β-ionone, 180 *m/z* for [$^2H_3$]β-ionone (d3-β-ionone) and 136 *m/z* for TDN. A specific deuterated standard was not available for TDN, and [$^2H_8$]naphthalene (d8-naphthalene) was used as an internal standard. For d8-naphthalene, ion 157 was used for quantitation. The elution times for each compounds were (Figure 8.3): d8-naphthalene = 14.57; TDN = 14.62; d4-β-damascenone = 15.24; β-damascenone = 15.31; d3-β-ionone = 16.28; β-ionone = 16.35.

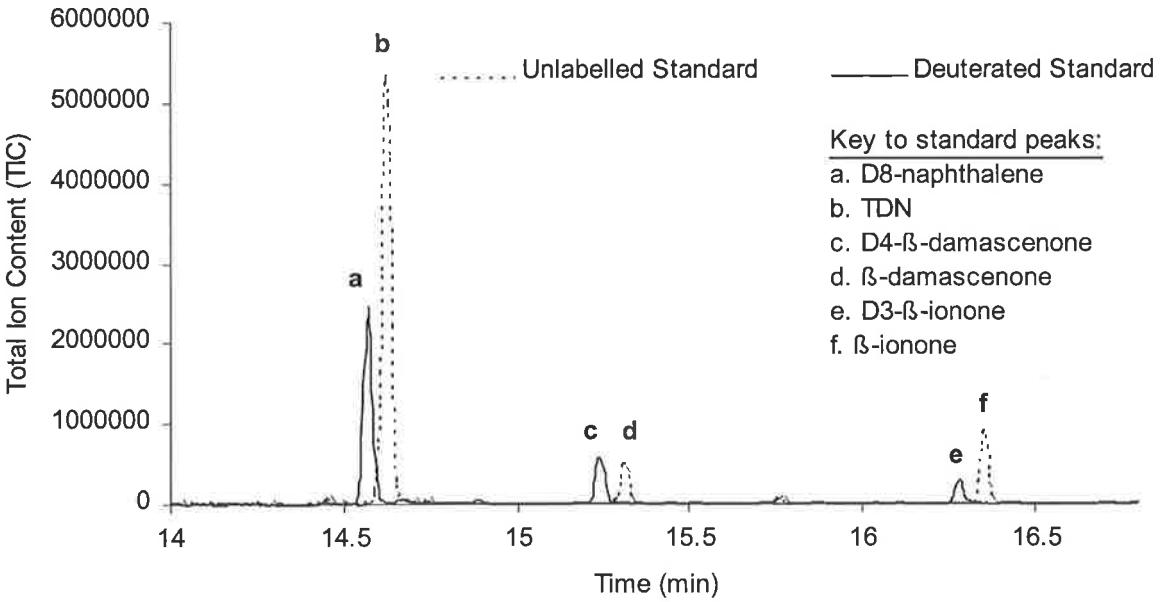


Figure 8.3: Chromatogram of C_{13}-norisoprenoid standards and their deuterated analogues

8.3 Results and Discussion

8.3.1 Comparison of fibres

The extraction efficiency of the SPME method is dependent upon the fibre type used. Three different fibre types were compared for a 25ng/mL solution of each standard. The fibres selected for the experiment had the stationary phases 65 μm polydimethylsiloxane /divinylbenzene (PDMS/DVB); 100 μm non-bonded polydimethylsiloxane (PDMS) and 65 μm carbowax / divinylbenzene (CW/DVB). In the case of the standards used in this study, the PDMS/DVB coating on the fibre had the maximum affinity when the peak areas of the bound compounds were compared (Figure 8.4). Affinity for the standards decreased in the case of non-bonded PDMS, and were lowest when the CW/DVB fibre was used. The PDMS/DVB stationary phase was therefore the fibre type of choice for the development of the method.

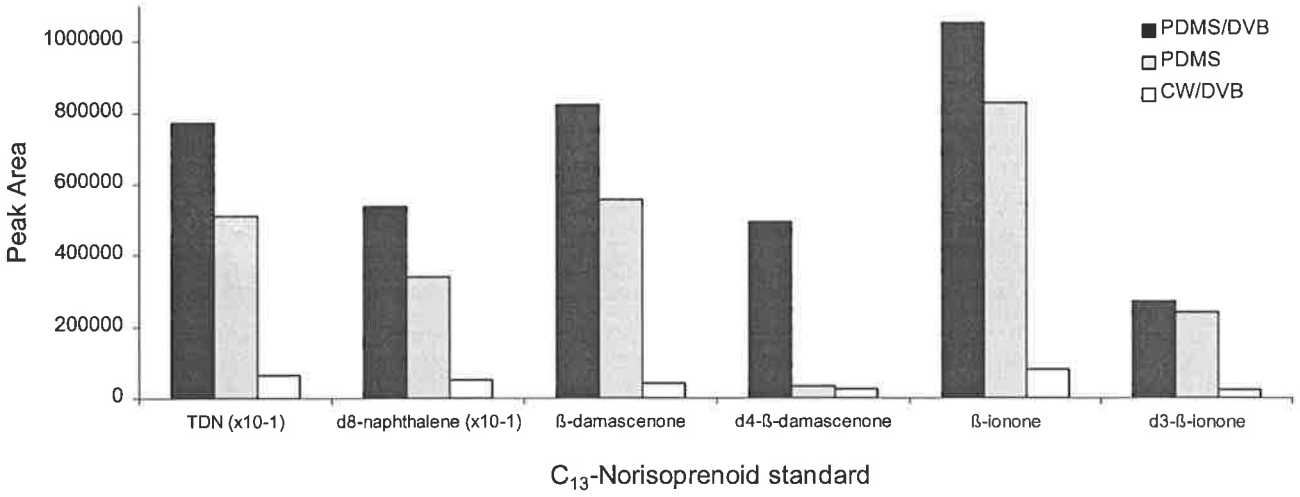


Figure 8.4: Comparison of SPME fibre affinity for C_{13}-norisoprenoids

8.3.2 Adsorption rate of the standards

The adsorption for β-damascenone, β-ionone and TDN to the SPME fibre was assessed at 40°C over a 90 minute period. Unexpectedly, these compounds did not reach equilibriun during this period, and the binding response was essentially linear (Figure 8.5 A-C). This relationship was clear in the case of β-damascenone and β-ionone (Figure 8.5 A, B), but in TDN only became linear after an initial 30 minute binding time (Figure 8.5 C). Given that a linear adsorption rate has been observed, the standard adsorption time selected for the method would be independent, so long as it fell within the linear range. This is possible because the compounds are compared to internal deuterated standards of known concentration. The ratio between the internal standards and the reference compounds remained constant at different adsorption times for β-damascenone and β-ionone, for the ions monitored. In the case of TDN, however, the internal standard d8-naphthalene reached equilibrium within 60 min at 40°C (Figure 8. D). The ratio of TDN to internal standard therefore increased with adsorption time. In order to standardise the method for TDN, a standard adsorption time of 60 min was selected. For simultaneous assay of all three compounds and their internal standards, the selected adsorption time fell within the linear range of the adsorption reaction for β-damascenone and β-ionone.

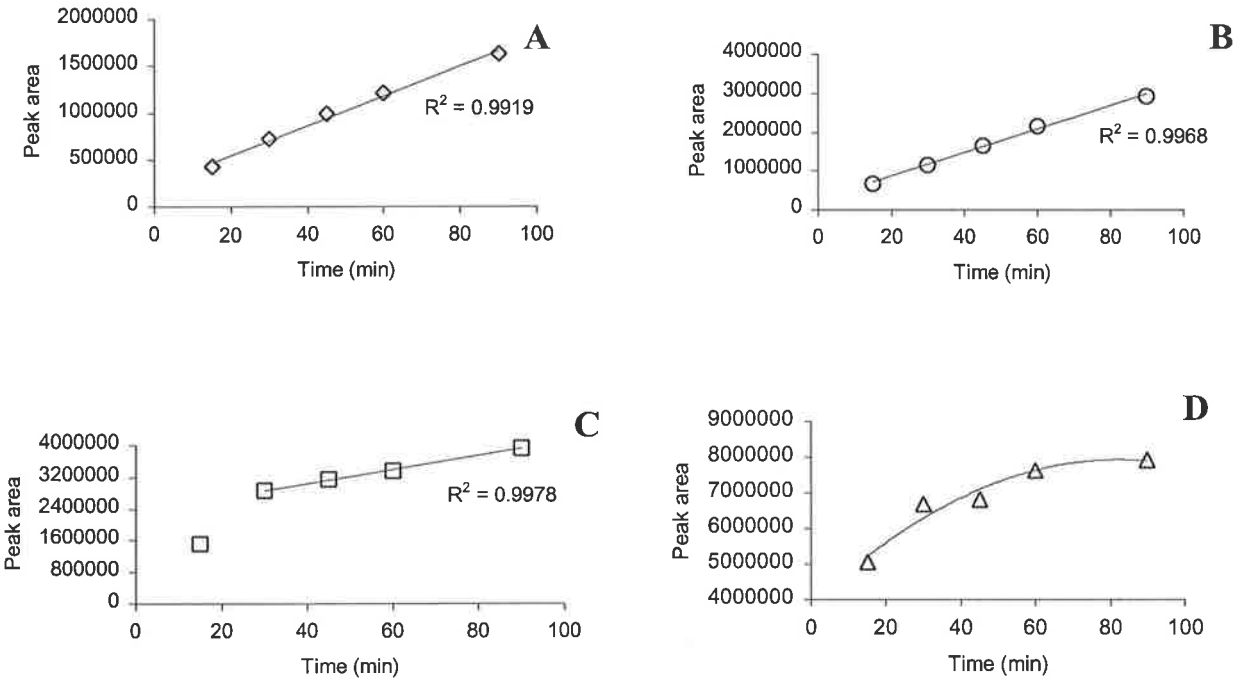


Figure 8.5: Adsorption response of **A.** β-damascenone, **B.** β-ionone, **C.** TDN and **D.** d8-naphthalene

8.3.3 Linear calibration of standards and reproducibility

The linearity of the compounds was assessed within the approximate range of concentrations to be found in grapes and wine. This was assessed when the standards were diluted in nanopure water and in model wine solutions (10% EtOH, 2% tartaric acid, adjusted to pH 3.5 with NaOH). The recovery of β-damascenone and β-ionone and their deuterated analogues was decreased 38% and 45% respectively, when water was compared to dilute alcohol solution. For TDN, the recovery was 23% lower when model wine solution was compared to water, and the reverse occurred for d8-naphthalene, where recovery was increased 18%. The linear relationships for increasing concentration of the standards were therefore different for water and model wine solutions. The analysis of C_{13}-norisoprenoids in grape berry homogenates and wines was based on the linear calibration functions for water, and model wines respectively. In the case of each standard, a linear relationship was observed for the range of dilutions analysed in both water (Figure 8.6 A,B) and model wine (Figure 8.7 A,B). Reproducibility of the recovery of each compound was compared for four sample replicates and the error was found to be less than 5% for all the standards tested.

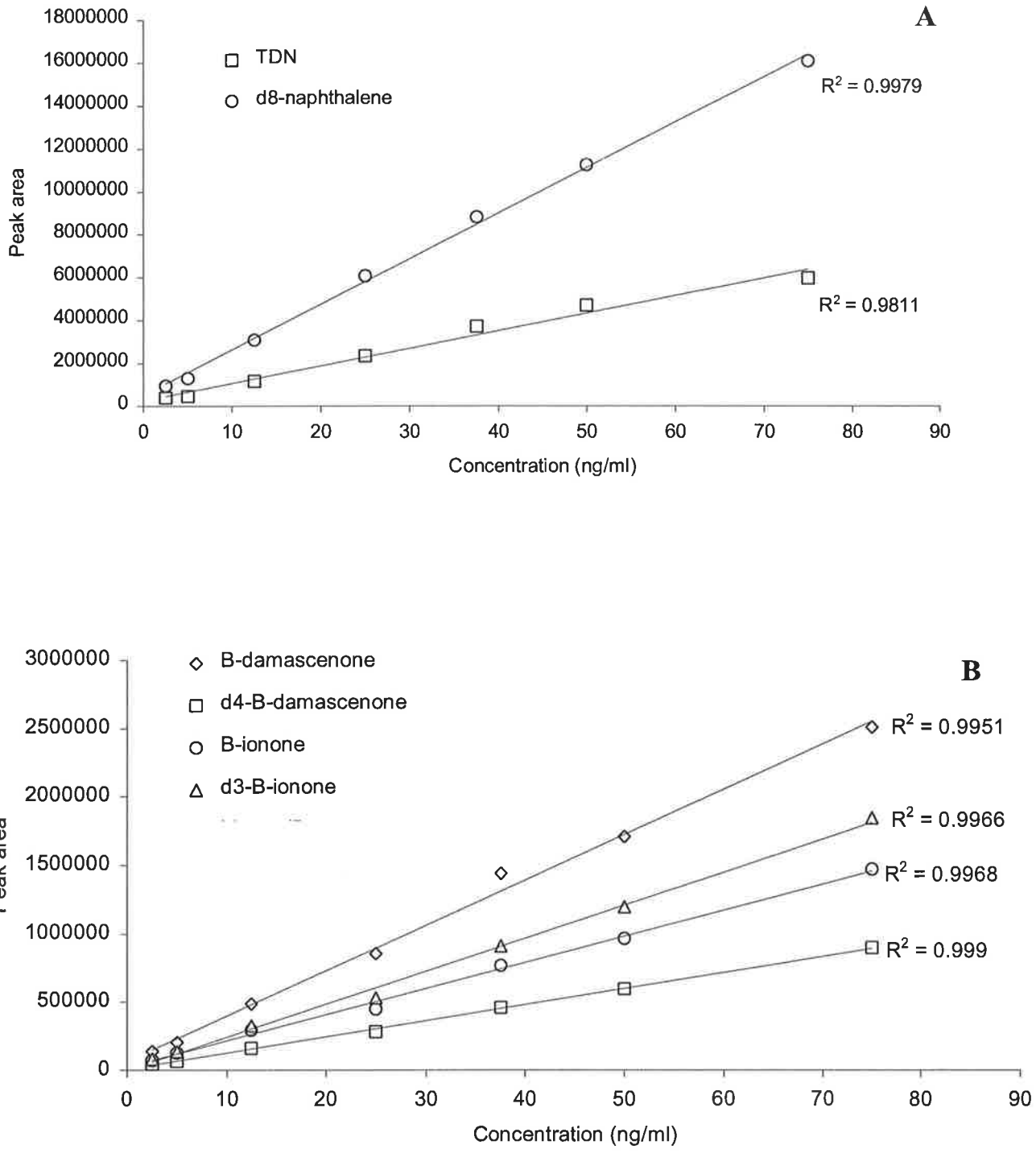


Figure 8.6: Linear calibration functions for **A**. TDN and d8-naphthalene **B**. β-damascenone, d4-β-damascenone, β-ionone and d3-β-ionone in water

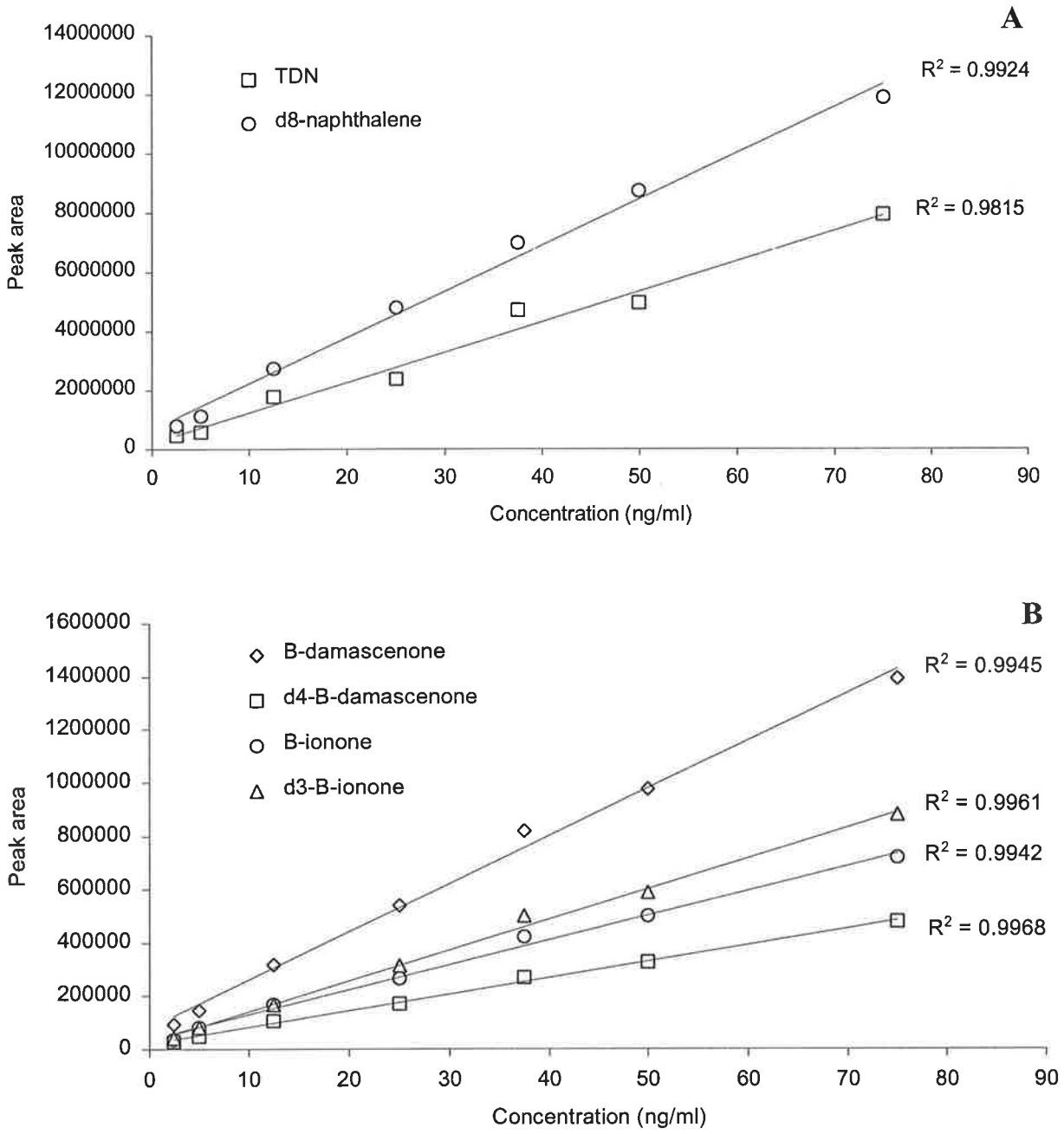


Figure 8.7: Linear calibration functions for **A.** TDN and d8-naphthalene **B.** β-damascenone, d4-β-damascenone, β-ionone and d3-β-ionone in model wine solution (10% EtOH, 2% tartaric acid, pH 3.5)

8.3.4 The effect of salt addition on the recovery of C_{13}-norisoprenoids in model wines

The matrix in which the extraction takes place has an influence on the sensitivity of the extraction using SPME. For a given concentration of a compound, a "salting-in" effect is generally observed, where the amount of compound adsorbed to the SPME stationary phase increases with the addition of salt (NaCl) to the matrix (De La Calle Garcia *et al.* 1996; Hayasaka and Bartowsky, 1999). With increasing salt addition, saturation occurs, followed in some cases by a "salting-out" effect, where recovery of the extracted compound decreases. This effect was observed for 2 mL model wine solutions containing 50 ng of standards of C_{13}-norisoprenoids and their deuterated analogues. Increasing concentrations of NaCl were added until a maximal recovery of standard was observed at 0.25 g of NaCl for TDN and d8-naphthalene (Figure 8.8). For β-damascenone and β-ionone this peak was observed with the addition of 0.5 g of NaCl (Figure 8.6). For the purposes of the current study, which aims for an extraction of all C_{13}-norisoprenoids from wines, 0.25g of salt was the selected amount for addition to wines. This amount would facilitate the extraction of all compounds to be analysed prevent any "salting-out" effect on TDN and d8-naphthalene.

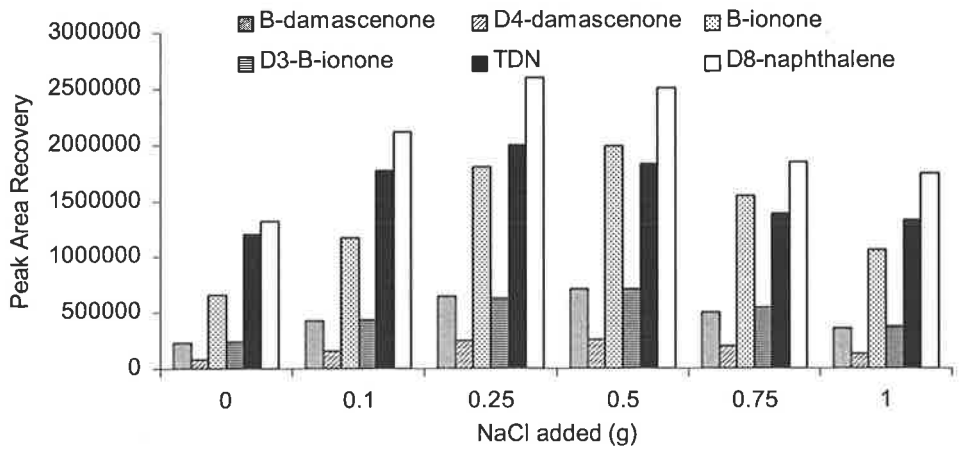


Figure 8.8: The effect of salt addition on the peak area recovery of C_{13}-norisoprenoids

8.3.5 Extraction of C_{13}-norisoprenoids from grape homogenates

C_{13}-norisoprenoids are present as bound glycoconjugates in grape tissues. The extraction of the aglycone for analysis by SPME requires release from the glucose molecule by enzymatic or acid hydrolysis. Previously, acid-hydrolysis of bound β-damascenone has been carried out at pH 2.2 (Kotseridis *et al.* 1999a).

The current study compared the release of β-damascenone and TDN at different pHs. The berry homogenate was diluted with water (250uL/g tissue), and the pH adjusted to 1, 2, and 3 through addition of HCl. The release of β-damascenone and TDN was then monitored after 1 h at 100°C. The release of both compounds was highest at pH 1, but the effect was greater in the case of TDN (Figure 8.9 A). Only a small amount of TDN was released at pH 3, and the increase to pH 1 was 92%. For β-damascenone this was only 32% (Figure 8.9 A). To allow for maximal release of TDN, all experimental hydrolysis was carried out at pH 1.

A further factor affecting the release of glycoconjugates by acid hydrolysis is the temperature at which the reaction is carried out. Previously, it has been shown for β-damascenone that this is optimal at 100°C, but the effect of temperature on the release of this compounds is dependant on grape variety (Kotseridis *et al.* 1999a). For β-damascenone, a clear increase was observed in the amount of the aglycone released from Shiraz homogenates (Figure 8.9 B), from 40°C to 100°C. For TDN, it was found that no release occurred with acid-hydrolysis at 40°C, and only a small amount was released at 70°C. The increase from 70°C to 100°C was 83%. The kinetics of the reaction releasing the aglycone of β-damascenone versus TDN indicate that the thermal and pH stability of TDN precursors may be different. It is assumed under the conditions of the experiment that there is no *de novo* synthesis of C_{13}-norisoprenoids from carotenoids through oxidative degradation, and if so, that it would be minor. This was tested through preparation of a sample with nitrogen passed through the homogenate and headspace to remove oxygen. Compared to the amount released from a standard sample preparation in air (78% nitrogen, 21% oxygen) there was only a small (7%) increase in the relative

levels of TDN formed (Table 8.1). The production of TDN from its lutein precursor (Marais, 1992) was investigated under the extraction conditions, and indicated that a small amount was released from pure lutein by thermal/hydrolytic degradation. However, relative to levels seen in grapes and wine this amount would be insignificant (<3%). Therefore, it is assumed that under the conditions of the experiment, that although a small amount of TDN may be derived from the *de novo* breakdown of lutein, this is at too low a rate to be experimentally significant.

For β-damascenone and β-ionone there was no change in the levels produced without oxidative conditions, indicating hydrolytic release of these compounds into the headspace. Similarly to TDN, the release of small amounts of β-ionone have been reported from thermal/hydrolytic degradation of β-carotene (Marais, 1992). The hydrolysis products of β-carotene were investigated under the conditions of this experiment, and indicated that a small amount of β-ionone was released. However, relative to the levels recovered in fruit and wine, this amount would be negligible. For β-damascenone, no release of this compound was produced from hydrolysis of either β-carotene or lutein, as it is proposed to be derived from neoxanthin (Skouroumounis and Sefton, 2002).

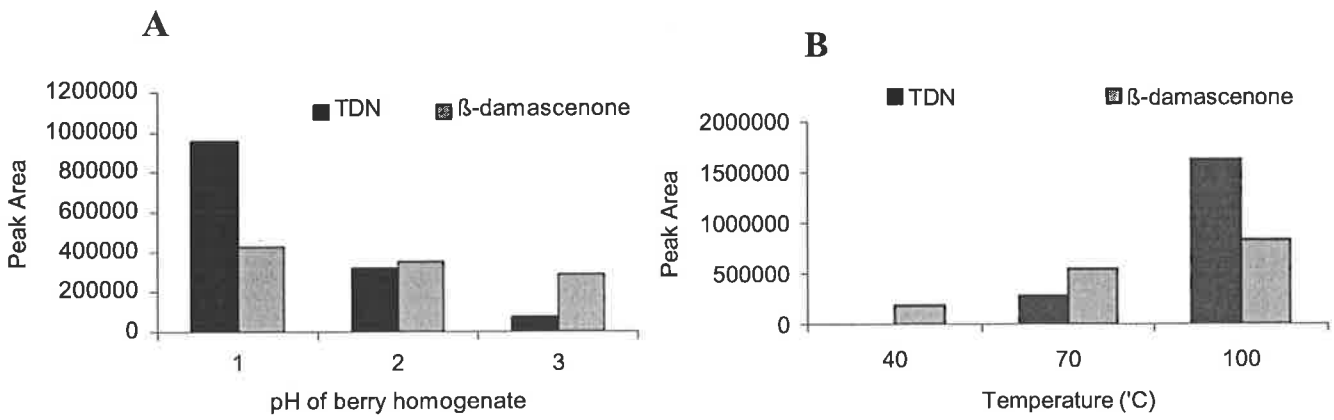


Figure 8.9: Effect of **A.** pH and **B.** temperature on the production of TDN and β-damascenone from Shiraz fruit homogenates

Table 8.1: Comparison of recovered C_{13}-norisoprenoids released under air and nitrogen from a Shiraz fruit homogenate

Headspace conditions	β-damascenone (ng/g)	β-ionone (ng/g)	TDN (ng/g)
Air	51.61	16.85	28.02
Nitrogen	51.75	16.72	26.10
% difference	-0.27	0.74	6.83

8.3.6 Rate of C_{13}-norisoprenoid hydrolysis

The release of C_{13}-norisoprenoids was determined over a 90 minute period at 100°C and pH1. All the compounds under investigation followed a similar trend of release over the period, as is shown for β-damascenone (Figure 8.10). The maximal peak area recovery occurred after 60 min, and remained constant to 90 min. The ion-response ratio of the C_{13}-norisoprenoids to their deuterated analogues increased over the 60 min period, but stabilised after 60 min (Figure 8.10). For the method developed, 60 min was selected as the standard hydrolysis period prior to binding to the SPME fibre. The same conditions were applied for both grape homogenates and wine samples. It must be noted that the peak area recovery of the deuterated standards added was reduced relative to the corresponding amounts seen on the standard curves. This is primarily due to the presence of an abundance of other volatile compounds released during hydrolysis. The binding of multiple compounds reduces the available surface area on the fibre for the binding of reference compounds. For standardisation of the method, it was assumed that the interference in the binding of the C_{13}-norisoprenoid compound would be similar to that of its deuterated analogue. The amount of C_{13}-norisoprenoid recovered was calculated from the ratio with its corresponding standard of known concentration.

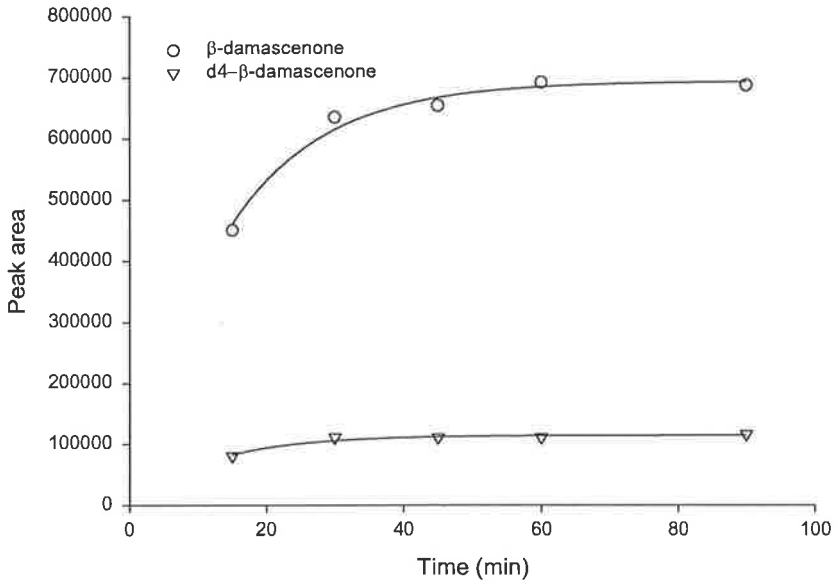


Figure 8.10: The rate of β-damascenone release and spiked d4-β-damascenone recovery from Shiraz berry homogenate over a 90 min period at 100°C and pH 1.

8.4 Conclusions

The development of a direct headspace-sampling method, in combination with stable isotope dilution will greatly reduce the steps previously required in the quantitation of C_{13}-norisoprenoids in grapes and wine. The C_{13}-norisoprenoids are important odour compounds in many grape varieties, and this methodology will open up the opportunity for further research in the environmental regulation of their levels in fruit, and the corresponding recovery as odour compounds in wine. The method developed only allows quantification of hydrolytically-released C_{13}-norisoprenoids in fruit, as the free levels were too low to be detected by SPME. This would allow an estimate of the maximum levels of the compounds which might be released from fruit during vinification. In viticultural research into flavour and aroma of grapes and wines, specific areas have been identified which require further research. Firstly, the effect of environmental factors such as sun-exposure and temperature on the levels of C_{13}-norisoprenoids in fruit and the corresponding wines; and secondly the effect of deficit irrigation practices on their metabolism and final concentration. The methodology described in this chapter will be applied to the determination of C_{13}-norisoprenoids in viticultural field trials.

CHAPTER 9: ENVIRONMENTAL ASPECTS OF C_{13}-NORISOPRENOID METABOLISM IN THE GRAPE BERRY

9.1 Introduction

9.1.1 Developmental regulation of C_{13}-norisoprenoids

The development of C_{13}-norisoprenoid precursors in grape berries occurs post-veraison, with a sharp increase occurring as the berries approach maturity (Strauss *et al.* 1987; Shure and Acree, 1994; Baumes *et al.* 2002). This sharp increase close to maturity coincides with the increase of general grape aroma precursors during this period (Coombe, 2001). At veraison, the changes occurring in the berry signal a senescence-type response, and there is a corresponding decrease in total carotenoid content during the ripening period that closely follows the profile of C_{13}-norisoprenoid increase (Figure 9.1) (Razungles *et al.* 1988, Marais *et al.* 1991, Razungles *et al.* 1996, Baumes *et al.* 2002). In the varieties Carignan, Grenache and Shiraz, this reduction in carotenoid content follows an initial sharp decrease at veraison, levelling off towards maturity (Razungles *et al.* 1988; Razungles *et al.* 1996).

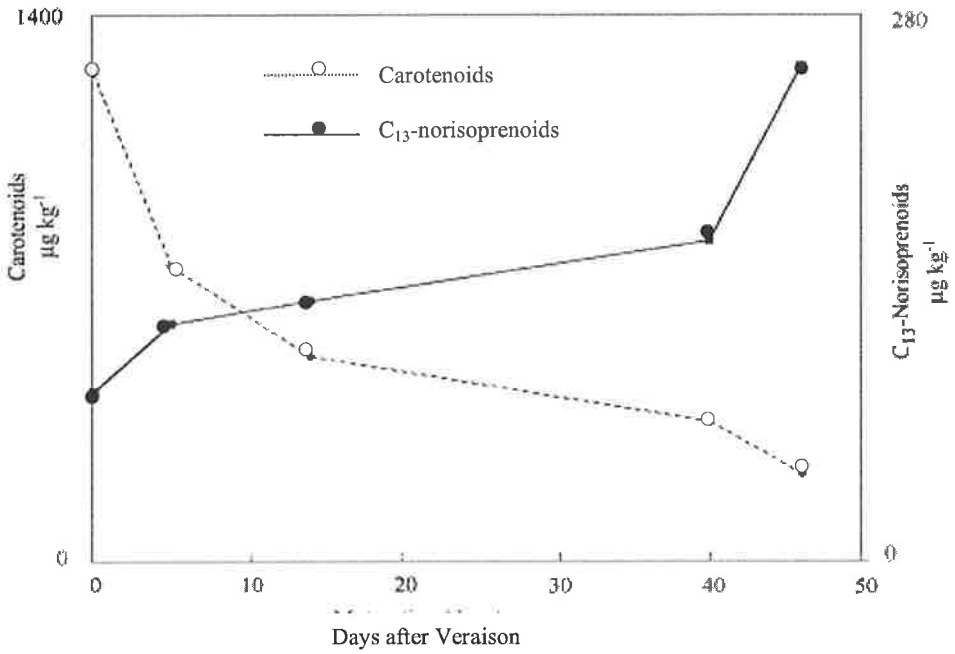


Figure 9.1: Change in the levels of carotenoids and C_{13}-norisoprenoid glycoconjugates during the maturation of Muscat berries (from Baumes *et al.* 2002)

Much of the research into grape C_{13}-norisoprenoids in recent years has attempted to link carotenoid and C_{13}-norisoprenoid metabolic pathways. Of all the C_{13}-norisoprenoids, β-damascenone has received the greatest attention in attempts to derive a biogenetic pathway from carotenoids. Early work linked the formation of β-damascenone from the carotenoid neoxanthin (Isoe *et al.* 1973; Ohloff *et al.* 1973) via the pathway shown in Figure 9.2. More recently, this scheme has been verified by the identification of all the pathway intermediates in grapes (Razungles *et al.* 1988; Razungles *et al.* 1993; Sefton, 1998; Sefton *et al.* 1993; Sefton *et al.* 1994; Sefton *et al.* 1996). However, the precise meachanism and optimum conditions under which the conversion between intermediates occurs is still unresolved. Recently, a number of possibilities for the hydrolytic conversion of intermediates under acidic conditions, with potential glycoconjugation of the precursors to β-damascenone has been proposed (Skouroumounis and Sefton, 2002; Puglisi *et al.* 2001). It is possible that the pathway may be mediated to some extent by hydrolysis of precursors to β-damascenone under acidic conditions in fruit or wine.

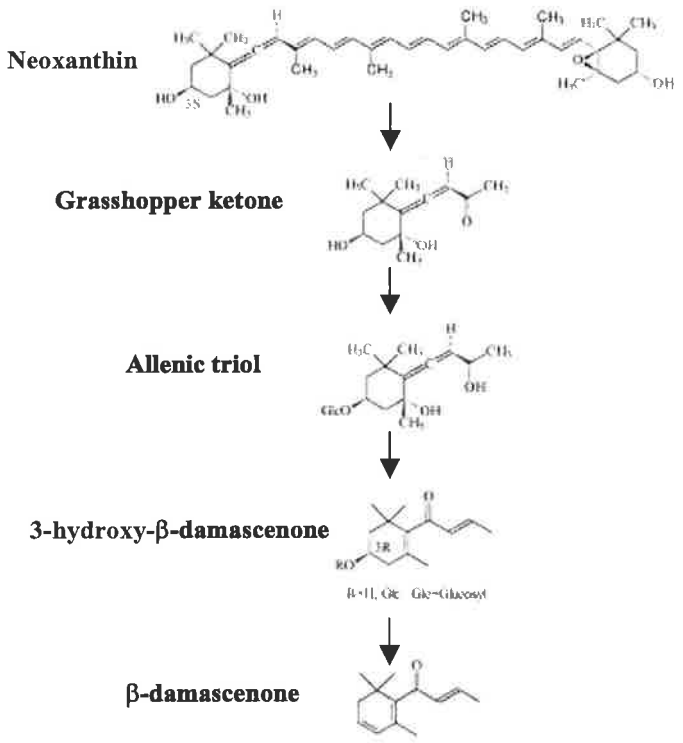


Figure 9.2 Proposed pathway of β-damascenone formation from neoxanthin (from Baumes *et al.* 2002)

However, a biogenic process for β-damascenone formation and its precursors remains more likely within the plant, which requires part of the pathway to be mediated enzymatically (Baumes *et al.* 2002). This is also likely for the other C_{13}-norisoprenoids, for example β-ionone and TDN. A recent study by Wache *et al.* (2002) has shown a potential co-oxidation system, using the enzyme xanthine oxidase in the presence of oxidative or altered thermal conditions. Xanthine oxidase is not a substrate-specific enzyme, and can oxidise a variety of biological compounds, and provided an opportunity to investigate the potential enzyme-mediated degradation of carotenoids and production of C_{13}-norisoprenoid precursors (Wache *et al.* 2002). From the results of the study, the potential co-oxidative formation of β-ionone from β-carotene was demonstrated, as well as co-oxidation of neoanthin to form intermediates in the pathway to β-damascenone formation. Thus, the potential exists for a biogenetic pathway for C_{13}-norisoprenoid flavour/aroma compounds in fruit and wine.

Recent work by Baumes *et al.* (2002) attempted to show a structural relationship between carotenoids and the derived C_{13}-norisoprenoids. The study was based on the premise that grape xanthophylls, which are potential precursors to the C_{13}-norisoprenoids, are cleaved by bond-specific dioxygenases, thus having asymmetric properties. Given this, any derived C_{13}-norisoprenoid aglycones would be expected to show similar properties given that they do not re-enter the primary carotenoid-cleavage pathway. The results indicated symmetry between certain C_{13}-norisoprenoids and carotenoids e.g. the 3R carbon of 3-hydroxy-β-damascenone and the 3S carbon of neoxanthin (Figure 9.2), thereby indicating neoxanthin as a potential precursor to β-damascenone. Furthermore, the study involved ^{13}C-feeding of berries and comparison of the $^{13}C/^{12}C$ ratios of carotenoids, monoterpenes and 3-oxo-α-ionol (a C_{13}-norisoprenoid) in control and labelled fruit. The resulting labelling patterns showed comparable $^{13}C/^{12}C$ ratios in 3-oxo-α-ionol and certain carotenoids at maturity, whereas the monoterpenes did not show a significant amount of labelling at maturity, when carotenoid degradation occurs. The results were proposed to indicate the carotenoid derivation of 3-oxo-α-ionol, and the independent synthesis of monoterpenes and carotenoids in berries, albeit that they have a common precursor: isopentyl diphosphate.

Despite the large amount of evidence linking the carotenoid and C_{13}-norisoprenoid pathways metabolically, there has been some conflicting data. A study by Shure and Acree (1993) on callus cultures of *Vitis labruscana* cv. Concord grapes demonstrated that inhibition of carotenoid synthesis and the xanthophyll cycle with amitrole did not affect the production of precursors to β-damascenone. Furthermore, the addition of β-carotene to the callus medium did not alter the production of β-damascenone precursors, while increased light and sucrose concentration resulted in an increase of the product. In that study, however, the uptake efficiency of both amitrole and β-carotene by the callus tissue was not determined, and the result may indicate inefficient uptake of the substrates. Despite this, it must be acknowledged that further research is still required to provide a clear metabolic link between the two pathways.

9.1.2 Light exposure and the regulation of C_{13}-norisoprenoids and carotenoids

Assuming that there is a close metabolic relationship between carotenoid degradation and C_{13}-norisoprenoid formation, it would be expected that any environmental factor that affects the accumulation or degradation of the source will result in a corresponding change in the product. The hypothesis proposed is that the factors that influence carotenoid metabolism also influence C_{13}-norisoprenoid formation. A key environmental factor in question is light.

Evidence has been put forward that the accelerated degradation of carotenoids after veraison is mediated by sunlight. In Shiraz berries exposed to a variety of light conditions from berry set, increased light was correlated with the degradation of carotenoids at veraison, particularly blue light (Baumes *et al.* 2002; Bureau *et al.* 1998; Razungles *et al.* 1998). In a similar study on Weisser Riesling and Chenin Blanc, lowered carotenoids levels were reported for sun-exposed fruit, relative to the shaded control (Marais *et al.* 1991). Similarly, in a study on the effects of UV exposure on carotenoid composition in Riesling grapes, increased UV exposure was associated with a reduction in carotenoid content of berry skins at maturity (Schultz *et al.* 1998). This was proposed to be due to increased rates of carotenoid degradation in the presence of UV radiation, rather than

decreased synthesis; as carotenoid levels were higher in UV-exposed fruit at veraison, with levels depleted by maturity (Schultz *et al.* 1998). However, a recent study on the influence of UV-B radiation on Cabernet Sauvignon fruit has shown the reverse effect, that where the degradation of ß-carotene from veraison onward was more pronounced when vines were shielded with a UV-B screen (Steel and Keller, 2000). The lutein content of the fruit did not change to the same degree as with ß-carotene, but was decreased when fruit developed under the UV-B screen (Steel and Keller, 2000). These results contradict previous findings on the relationship of sun-exposure and more specifically UV-exposure on carotenoid metabolism in grape berries, and requires further study, particularly in relation to the corresponding concentration of C_{13}-norisoprenoids under different environmental conditions.

A study of Shiraz C_{13}-norisoprenoids indicated that sunlight exposure from set to veraison increased their total concentration at maturity, although this was only in the order of 16-36% for sun exposed fruit when compared with fruit grown with 10% full sun over two seasons (Bureau *et al.* 2000). In the same study a cluster thinning experiment where the leaf area to crop weight ratio was artificially altered in order to test whether C_{13}-norisoprenoid precursors were translocated from leaves to berries showed that the total levels of bound C_{13}-norisoprenoids were unchanged by cluster thinning, demonstrating the independence of this pathway in fruit from that in leaves (Bureau *et al.* 2000).

An earlier study by Marais *et al.* (1992a) on Chenin Blanc and Weisser Riesling grapes and wines also showed the effect of sunlight on increasing the total content of certain C_{13}-norisoprenoids. In this study the selected treatments were naturally shaded and sun-exposed bunches within the canopy. In the acid-hydrolysed grape extracts, TDN was the most significantly affected by sun exposure, in the order of 52% higher in sun-exposed fruit at maturity and 26% higher in the corresponding wines. Unexpectedly, ß-damascenone concentration was shown to be unaffected by sun-exposure in either fruit or wine in the same experiment. This is in contrast to findings by Bureau *et al.* (2000), and requires further investigation into the factors controlling ß-damascenone concentration in

grape berries. It must be noted that in both studies, complete separation of the effects of total canopy shading, and fruit shading were not made. It is still necessary to observe the effects of total bunch shading on carotenoids and C_{13}-norisoprenoids, where canopy sun exposure remains constant.

These studies have not taken into account the effect of temperature on C_{13}-norisoprenoid composition, where temperature is independent of sun-exposure. There have been references to the effects of region on TDN concentration in wine (Marais *et al.* 1992b), where wines from warmer regions (South Africa) had higher TDN concentration that wines produced in cooler climates (Europe). In this instance, sunlight exposure of fruit differed significantly between regions, with the warmer regions having higher levels of average daily sunshine, as well as higher average temperature. It is therefore not possible in this instance to separate the effect of sun-exposure and temperature, and it is necessary to investigate the effect of sun-exposure on fruit and wine composition, where temperature remains constant.

9.1.3 The effect of water stress on carotenoids and its implications for C_{13}-norisoprenoid concentration

In grapevines, water stress can indirectly affect the light environment of developing fruit, through alteration of shoot growth rate, and vine leaf area (Dry and Loveys, 1998). This may therefore lead to an indirect increase in bunch exposure, which may alter levels of carotenoids and C_{13}-norisoprenoids. Furthermore, due to the close relationship of the metabolic pathways for carotenoids (Armstrong and Hearst, 1996) and stress-related plant hormones such as abscisic acid (ABA), it is conceivable that there may also be a direct effect of water stress itself on the metabolism of carotenoids and C_{13}-norisoprenoids, through the action of stress-response signals within the plant.

The effect of water deficit on carotenoids has recently been studied in grapevines by Oliveira *et al.* (2003), on *Vitis vinifera* L. cv. Touriga Nacional. The experiment compared the carotenoid composition of fruit from non-irrigated versus irrigated vines on high water-retention capacity soil and low water retention capacity soils. The deficit

treatment caused a reduction in fruit weight that was independent of soil type. However, carotenoid content was increased 60% by the non-irrigated treatment when the soil had a low water-retention capacity. However, on the high water-retention capacity soil, there was no effect on carotenoid content. Where the effect of water stress caused an increase in carotenoid content, this was found across the spectrum of carotenoids analysed: lutein, β-carotene, neoxanthin, violaxanthin and luteoxanthin.

As yet, this physiological response of carotenoid metabolism to water stress has not been investigated. From the data shown by Oliveira *et al.* (2003), the response of the carotenoids to water stress occurred in fruit from an early stage of development post-veraison, and the effect on carotenoid content was retained as the fruit matured. The fact that the response was only observed on soils of a low water-holding capacity indicates that there may be a physiological response of the roots that necessitates sufficient drying in the soil early in the growing season. The conditions required to mediate such a response is similar to that described for PRD (Dry *et al.* 1996; Stoll *et al.* 2000).

Using PRD, and other deficit irrigation strategies, there may therefore be a potential to mimic the conditions of drying soil on fruit metabolism without exposing the vines to the severe stress which could cause potential damage and yield loss over a number of seasons. It may be possible to increase carotenoid content in fruit under PRD, as well as to potentially increase the precursors to C_{13}-norisoprenoids in grape berries, thus affecting the aroma potential of the resulting wine.

This chapter firstly reports on the effect of altered light environment on the concentration of C_{13}-norisoprenoids in fruit and wine and carotenoids in grape berries. This was achieved through artificial shading of the fruit, and through canopy management. Secondly, the current study aimed to explore the effect of water-deficit (PRD) on carotenoid and C_{13}-norisoprenoid composition in fruit. The purpose of the work was to attempt to elucidate the primary environmental factors affecting the final concentration of C_{13}-norisoprenoids in fruit and the resultant wine, in order to optimise viticultural practices to maximise wine flavour and aroma.

9.2 Materials and Methods:

9.2.1 Experimental:

Experiment 1: This experiment was conducted over one season (2001/2002) at Nuriootpa in the Barossa Valley, South Australia. Five year-old Shiraz vines pruned to 60 nodes were used, and received 1.0 ML/ha of irrigation water over the season from December 2001 to March 2002. Five replicates of two-vine plots were set up in a randomised-block design. Shading boxes were constructed from white polypropylene, and were left white on the surface and painted black on the interior to prevent light penetration and internal reflection. The boxes were designed to allow air movement over the fruit (Section 2.1.3, Chapter 2). Six shading boxes were placed over randomly-selected bunches in each plot from fruit set to harvest. Temperature in the boxes was monitored at 30 minute intervals from veraison to harvest using thermistor probes (Measurement Engineering Australia) positioned within the interior of the shaded bunch. Data were collected using a 16-channel macro 32 Starlog data logger (Measurement Engineering Australia). A measure of photosynthetically active radiation (PAR) within the fruiting zone of the canopy was made at veraison and harvest, as a measure of total light that fruit within the canopy would receive. Shaded bunches were collected at maturity (25 °Brix). Two sets of 50-berry sub-samples were taken from each plot; one set from bunches at ambient conditions within the canopy and another set from the 6 shaded bunches. One 50-berry sample was processed immediately for °Brix, pH and titratable acidity (Section 4.2.1, Chapter 4). The second 50-berry sample was frozen at -20°C for later analysis. A concurrent box-shading experiment was conducted by CSIRO Plant Industry on Shiraz grown at McLaren Vale, South Australia. This was a larger-scale experiment, and allowed for wine to be made from shaded and sun-exposed fruit. Wine sub-samples were obtained from this study at the bottling stage. Under the climatic conditions of McLaren Vale, temperature within the boxes did not differ significantly from ambient conditions.

Experiment 2: This experiment was a GWRDC-funded project conducted at Nuriootpa in 1999/2000 and 2000/2001 by R. Ristic (Section 2.1.1.2, Chapter 2). The field trial was subjected to four canopy-management strategies in order to alter the light environment of developing fruit. A "shade" treatment was set up where the canopy was constrained under shade-nets to create a dense, highly shaded fruiting zone. A "moderate" treatment had a sprawling canopy, and the "sun" treatment had canes trained upward and downward, to create a highly-exposed bunch zone. These three treatments were arranged in a randomised block design of 5 replicates, and were on clone BVRC12. A further treatment was set up in a separate row, on a different clone of Shiraz, PT15N, which represented a typical "Barossa" vine. This "reference" treatment was irrigated at half the rate of the other canopy treatments over the course of the experiment. Over two seasons, 2000 and 2001, a measure of leaf area of randomly selected shoots was made by Ms R. Ristic (Adelaide University) and extrapolated to give an estimate of whole-vine leaf area. A single measure of bunch-exposure (PAR) was also made using a ceptometer positioned horizontally within the fruiting zone, with the sensor facing upward. These data were provided as a reference for discussion in the current study, but a more detailed description of this experiment can be found in Ristic (2004). Fruit was harvested at the same °Brix level, and the average berry weight determined. For winemaking purposes, fruit sampled from the different blocks was pooled, and sub-divided into three replicates. Fifty-berry sub-samples of the fruit from the winemaking were collected, homogenised, and frozen at -20°C for later analysis. Mini-winemaking lots were made from the fruit, and samples were bottled after yeast fermentation and did not go through malolactic fermentation. Samples of fruit and wine samples from this experiment were a kind donation by Ms R. Ristic.

Experiment 3: This experiment was carried out over two seasons at a commercial field trial by Orlando-Wyndham, at Langhorne Creek, South Australia (Section 2.1.2, Chapter 2). It compared PRD and control irrigation treatments on 5-year old Cabernet Sauvignon vines pruned to 60 nodes. In 2001/2002, the control and PRD treatments received 1.19 and 0.84 ML/ha respectively. In 2002/2003, the control and PRD treatments received 1.37 and 0.85 ML/ha respectively. Fruit was collected at 23-24 °Brix and 50-berry lots

were either immediately processed for TSS, pH and TA (Section 4.2.1, Chapter 4); or frozen at -20°C for later analysis of anthocyanins and phenolics (Section 6.2.2, Chapter 6). A measure of photosynthetically active radiation (PAR) within the fruiting zone of PRD and control vines was made at veraison and harvest. Canopy size of the respective treatments was estimated from final shoot number and pruning weight at the end of each season. Wine was made from harvested fruit using small-scale winemaking techniques (Section 2.4, Chapter 2), and went through malolactic fermentation. In 2001/2002, this was done in 18 L fermenters, and samples were pooled to give 6 treatment replicates and 2 winemaking replicates. In 2002/2003, only one ferment was made by Orlando-Wyndham, giving a single treatment and winemaking replicate, which did not go through malolactic fermentation.

9.2.2 Extraction and HPLC analysis of carotenoids

The extraction procedure for carotenoids was adapted from the methods of Lee and Castle (2001) and Steel and Keller (2000). A 1 g sample of grape homogenate frozen at –20 °C were extracted in 10 mL extraction solvent (hexane:acetone:ethanol, 50:25:25, v/v) in the dark for 1h on an orbital shaker. The extracts were then centrifuged at 8000 rpm and the hexane layer removed using a syringe. The hexane extracts were then spiked with a known amount of β-Apo-8′-carotenal (Fluka Biochemica) as an internal standard and dried down in a Savant Speed Vac Plus at low temperature. The dried extracts were resuspended in 1mL diethyl ether, to which was then added 1 mL of 5% methanolic KOH. Saponification was then carried out over a 18 h period in the dark at 4 °C. Following saponification, an additional 1mL of diethyl ether was added to the extract, and then transferred to a 10 mL centrifuge tube. The methanol-KOH fraction was then subsequently removed with 2 successive 3 mL water washes. The water fractions were removed with a syringe, and the final diethyl ether fraction was dried down in a fume hood under a nitrogen stream. The dried samples were redissolved in 250 μL HPLC-grade acetone and centrifuged 10 000 g for 5 min. The samples were transferred to HPLC vials and 100μL was injected onto a LiChrosopher 100 RP-18 (5 μm) 4 x 250 mm HPLC column and photodiode array detection at 450 nm. The HPLC system used was a Hewlett

Packard HP 1100. The method was a ramped gradient of acetone/water: 70% acetone to 100 % acetone from 0 to 25 min. The flow was maintained at 100% acetone from 25 to 35 min and returned to 70% acetone at 36 min. The final run time was 38 min. The flow rate was maintained at 1 mL/min, and the column temperature was 40 °C. The elution times of lutein and β-carotene standards (Sigma-Aldrich Pty. Ltd.) were 13.34 min and 24.52 min respectively. Carotenoids were quantified according to a standard curve of β-Apo-8′-carotenal which had a linear response within the range of concentrations injected onto the column (0 – 10 μg), giving an R^2 value of 0.9994.

9.2.3 Analysis of C_{13}-norisoprenoids

Deuterated internal standards of β-damascenone, β-ionone and [$^2H_8$]naphthalene (d_8-naphthalene) were a gift from the Australian Wine Research Institute (AWRI), Adelaide, South Australia. SPME headspace sampling was performed manually. SPME fibres and fibre assembly were obtained from Supelco. A 2 mL sample of wine, or 2 g of berry tissue were placed in a 10 mL glass vial and adjusted to pH 1 with HCl. Fruit samples were diluted with 500 μL of deionised water to ensure a suspension of the homogenate formed. To wine samples, 0.25 g of NaCl was added. To both grape and wine samples, 10 μL of a 5 μg/mL solution of deuterated standards of β-damascenone and β-ionone, and d_8-naphthalene were added. The samples were sealed with a Supelco PTFE/Silicone septum (Supelco) and heated to 100 °C for 1 h in a heating block. The samples were then transferred to a 40 °C heating block, and a PDMS/DVB SPME fibre was then inserted through the septum and exposed to the sample headspace for 1 h. Care was taken to ensure that the fibre was exposed at a constant level above the sample surface. After adsorption of headspace compounds, the fibre was removed from the sample and manually desorbed to the GC injection port for 1 min. SPME - GC/MS analysis was performed using a Hewlett Packard HP gas chromatograph 5972, fitted with a 30 m fused silica SGE BP20 column (0.25mm i.d. and 0.25 μm film thickness), coated with Carbowax 20 M. The splitless/split injection port was heated to 220°C. The split flow was set at 5.0 ml/min and the split ratio was 5.038:1. Ultrahigh-purity helium was used as a carrier gas at a constant flow rate of 1 ml/min, with a column head pressure of 6.98 psi.

The temperature program was initially 40 °C for 4 min, then ramped to 240 °C at a rate of 12 °C/min. The temperature was held at 240°C for 5min, giving a total run time of 25.67 min. The GC instrument was coupled to a Hewlett Packard HP 6890 mass selective detector. For quantitation of C_{13}-norisoprenoids using selective ion monitoring, 69 *m/z* was used for β-damascenone; 73 *m/z* for [$^2H_4$]β-damascenone (d_4-β-damascenone), 177 *m/z* for β-ionone, 180 *m/z* for [$^2H_3$]β-ionone (d_3-β-ionone) and 136 *m/z* for TDN. A specific deuterated standard was not available for TDN, and d_8-naphthalene was used as an internal standard. For d_8-naphthalene, 157 *m/z* was used for quantitation.

9.2.4 Statistical analysis

Experiment 1 was a simple 4 or 5 replicate comparison and was analysed using a Student's T-test, with Microsoft Excel software. For Experiment 2, the Instat statistical software package was used. A one-way ANOVA was used to assess potential differences within the whole data set, and Tukey's test was for mean separation between individual treatments. For Experiment 3, a one-way ANOVA compared treatment differences using Genstat 6 software.

9.3 Results and discussion

9.3.1 Experiment 1: Effect of total shade on the composition of carotenoids in fruit and C_{13}-norisoprenoids in fruit and wine (Nuriootpa Shiraz)

The effect of the total shade treatment on Shiraz fruit was assessed firstly though analysis of the general measures of fruit quality (Table 9.1) There was no significant change in berry weight, TSS, TA, phenolics or anthocyanins brought about by the shading experiment under these conditions. In shaded fruit, pH was significantly increased, which may represent an alteration in the ion ratios of the fruit under shaded conditions (Table 4.3), since TA was not significantly altered. Under the conditions of this experiment, where temperature was not significantly changed by the shading treatment, it can be concluded that total shade did not affect the primary processes of fruit metabolism. Although total shade has been shown to alter fruit anthocyanin and phenolic composition (Table 7.3), there are data which indicates that shading may not significantly affect the levels of anthocyanins in mature cv. Shiraz fruit (Haselgrove *et al.* 2000).

Table 9.1: Temperature, radiation and fruit composition of shaded and sun-exposed Shiraz treatments in 2001/2002 (T-test, n=5, ns = not significant, nd = not determined)

	Sun	Shade	% Sun>Shade	P
Temperature (°C)	18.6	19.00	-	-
Bunch exposure (PAR % ambient)	19.3	nd	-	-
Berry Weight (g)	1.08	1.04	3.47	ns
Total Soluble Solids (°Brix)	25.6	24.6	3.83	ns
pH	3.4	3.5	-2.77	<0.01
Titratable Acidity (g/L)	7.3	7.0	3.52	ns
Phenolics (A_{280}units/g)	1.3	1.3	-2.75	ns
Anthocyanins (mg/g)	1.9	1.9	-1.95	ns

The effect of total shade on the composition of carotenoids was a significant reduction in the concentration of both lutein and β-carotene relative to the control (Figure 9.3). This was of the order of 70% and 35% for lutein and β-carotene respectively. As there was no significant change in berry weight with the shading treatment, this result was also reflected in an increase in both carotenoids per berry (data not shown). This result contrasts with the finding that carotenoids were higher under shaded conditions, due to a decrease in the rate of degradation of the carotenoid pool (Marais *et al.* 1991; Razungles *et al.* 1998, Baumes *et al.* 2002). However, Steel and Keller (2000) showed that UV-screened Cabernet Sauvignon fruit had decreased levels of β-carotene and lutein at harvest. This indicates that the amount of UV light reaching the fruit may be important in determining the response of carotenoid metabolism to sun-exposure, rather than the total amount of light *per se*.

Despite the uncharacteristic effect observed on carotenoids in shaded grapes the response of the C_{13}-norisoprenoids was similar to that observed by most authors, under similar conditions (Marais *et al.* 1992a; Bureau *et al.* 2000). All C_{13}-norisoprenoids were decreased in the shaded fruit relative to the sun-exposed fruit. This was of the order of 31% for β-damascenone and β-ionone, whereas TDN was 68% lower in shaded fruit. The result for TDN was similar to that of Marais *et al.* (1992b), where TDN was most the significantly affected by sun-exposure of the C_{13}-norisoprenoids in Weisser Riesling

fruit. Whereas previous studies have indicated a negligible or small effect of sun-exposure on the concentration of β-damascenone in fruit, this result indicates that sun-exposure can significantly affect the final levels of this compound. The effect of sun-exposure on quantified levels of β-ionone has not previously been reported, and this result indicates that environmental conditions influence its production similarly to the other C_{13}-norisoprenoids in the current study.

Under the artificial shading conditions of the box experiment, larger decreases in the concentration of carotenoids and C_{13}-norisoprenoids would be expected than under natural shading conditions, where fruit would be exposed to some light. Although the results of this experiment cannot be directly extrapolated to what might be expected to occur under natural conditions, it can be inferred that the production of C_{13}-norisoprenoids may reflect the concentration of the carotenoid source available in the fruit. It is therefore possible that changes in the amount of light reaching the fruit during development may affect the available pools of carotenoids, leading to a similar change in the C_{13}-norisoprenoid products. It appears that higher levels of carotenoids in mature fruit may be associated with increases in C_{13}-norisoprenoid concentration, rather than the reverse (Marais *et al.* 1991; Razungles *et al.* 1998, Baumes *et al.* 2002). The scope of this experiment did not allow for changes in carotenoids and C_{13}-norisoprenoids to be studied over development. Further investigation of the developmental regulation of these compounds in totally shaded and sun-exposed fruit will be necessary to test this hypothesis.

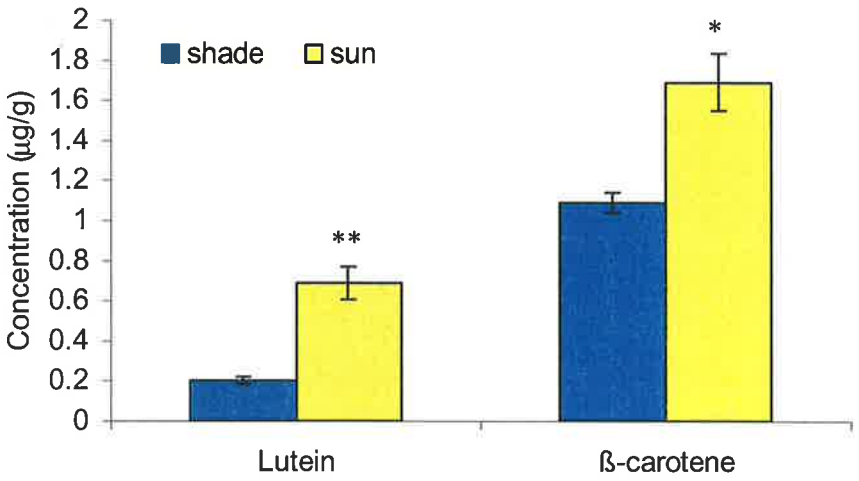


Figure 9.3: Carotenoid composition of sun-exposed and totally shaded berries of Shiraz at 25°Brix grown at Nuriootpa in 2001/2002 (* = P<0.01, ** = P<0.001; n=4; bars are ± SE of the mean).

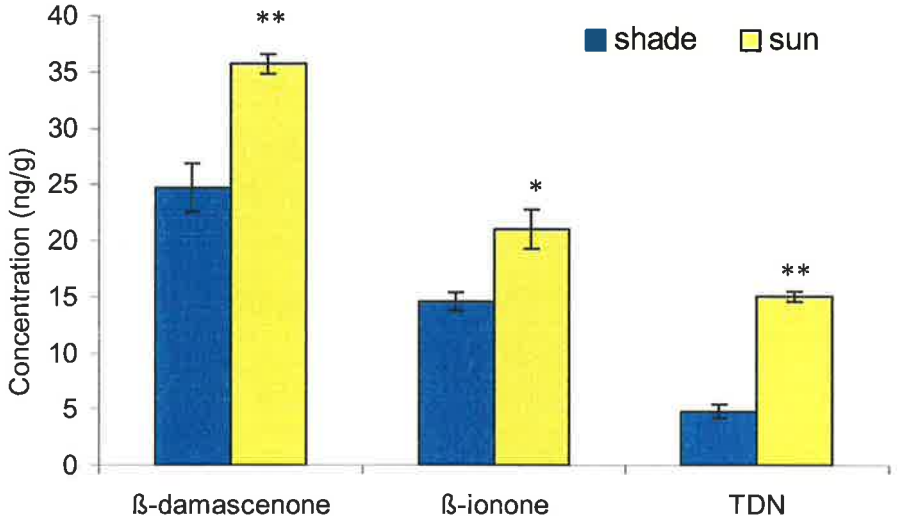


Figure 9.4: Concentration of C_{13}-norisoprenoids in sun-exposed and totally shaded berries of Shiraz at 25°Brix grown at Nuriootpa in 2001/2002 (* = P<0.01; ** P<0.001; *** P<0.0001; n=5; bars are ± SE of the mean).

The concentration of total C_{13}-norisoprenoids (free and bound) was also determined in three replicate wine samples made from totally shaded or exposed Shiraz fruit (Table 9.2). The levels seen in the wine cannot be directly compared with the levels seen in fruit, because the fruit and wine samples were obtained from shading experiments in different localities. However, the effects of the treatment on the total available levels of TDN and β-damascenone in wine are similar to that seen in the fruit. With β-damascenone, however, the difference appears to be far larger, with bound β-damascenone being 92% higher in wine made from sun-exposed fruit than totally shaded fruit. Interestingly, the recovery of β-ionone in wine was not significantly affected by the shading treatment in the McLarenVale Shiraz study, as it was in fruit.

Table 9.2: Total free and hydrolytically-released C_{13}-norisoprenoids of wines made from sun-exposed and shaded Shiraz fruit grown at McLarenVale, 2001/2002 (n=3, ns = not significant).

	C_{13}-norisoprenoid		
	β-damascenone (ng/ml)	β-ionone (ng/ml)	TDN (ng/ml)
Sun-exposed	45.30	22.89	162.23
Shaded	3.66	19.16	39.06
% Sun-exposed>shaded	1138	19	315
P (t-test)	<0.001	ns	<0.001

9.3.2 Experiment 2: Effect of canopy structure on the composition of C_{13}-norisoprenoids in fruit and wine

In this experiment, the three treatments: 'shade', 'moderate' and 'sun' were set up to compare differences in canopy structure and fruit exposure. The 'reference' treatment was set up as in the same vineyard block, on a different clone of Shiraz. Previous analysis of these experiments (Ristic, 2004) have shown that the 'reference' treatment showed consistently higher anthocyanin and phenolic concentrations in both grapes and resultant wines than the other treatments. Furthermore, sensory analysis of wines produced from the 'reference' treatment were ranked significantly higher for quality and for beneficial flavour and aroma attributes (Ristic, 2004). Although on a different Shiraz clone, these differences are not likely to be due to clone alone. Comparison of quality parameters

between clone BVRC12 and PT15N where both clones were pruned to the same level and received the same amount of irrigation indicated a similar response in terms of TSS accumulation and berry weight, although average anthocyanin and phenolic concentration for a single season were lower in PT15N (M. McCarthy, pers comm.) (Appendix G). In the current study, the PT15N clone, or 'reference' treatment produced fruit and wine of a higher quality ranking in terms of colour and aroma, which is most likely due to a non-vigorous canopy resulting from a number of years of reduced irrigation. It received half the irrigation (0.5 ML/ha) of the other treatments over the course of the experiment, and can be compared on this basis with the other treatments.

The effect of the different canopy treatments altered the light environment of developing bunches within the fruiting zone of the vines. The resultant bunch exposure (Table 9.3) differed significantly in the three canopy treatments: 'shade', 'moderate' and 'sun'. This was due to differences in canopy structure and was not the result of changes in the total leaf area of the canopy, although leaf area in the 'shade' treatment differed significantly from the sun treatment in 2000, and from both 'moderate' and 'sun' treatments in 2000/2001 (Table 9.3). The 'reference' treatment had significantly lower leaf area per vine and berry weight than all of the three canopy treatments on both 1999/2000 and 2000/2001 which was most likely a response to the restriction in irrigation water applied relative to the other treatments (Ojeda *et al.* 1999, 2001). However, this reduced leaf area produced a canopy with similar bunch exposure to the moderate treatment in 1999/2000 and was slightly higher than the moderate treatment in 2000/2001. In 'moderate' vines, light penetration to the fruiting zone may have been increased due to shoots drooping under their own weight, due to a larger, sprawling canopy. This would have allowed for comparable measures of bunch exposure between 'moderate' and 'reference' vines, despite the former having a larger canopy size.

Table 9.3: Measures of canopy-openness and fruit weight from canopy management treatments on Shiraz from Ristic (2004) (ANOVA; n=5; ± SE of the mean).

	2000 Vintage			
	Shade	Moderate	Sun	Reference
Total leaf area/vine ($m^{2)}$	12.68^{bc} (±0.82)	12.00^{bc} (±0.66)	13.78^{c} (±0.52)	6.40^{a} (±0.39)
Bunch Exposure (PAR % ambient)	2.37^{a} (±0.36)	31.00^{b} (±1.46)	56.34^{c} (±4.56)	33.00^{b} (±3.07)
Berry weight (g)	1.37^{b} (±0.03)	1.40^{bc} (±0.04)	1.48^{c} (±0.03)	1.10^{a} (±0.02)
	2001 Vintage			
	Shade	Moderate	Sun	Reference
Total leaf area/vine ($m^{2)}$	14.63^{b} (±0.72)	21.00^{c} (±0.71)	22.00^{c} (±1.02)	11.00^{a} (±0.45)
Bunch Exposure (PAR % ambient)	4.45^{a} (±0.44)	27.00^{b} (±2.32)	72.00^{d} (±2.00)	48.00^{c} (±3.83)
Berry weight (g)	1.12^{b} (±0.01)	1.10^{b} (±0.02)	1.10^{b} (±0.03)	0.80^{a} (±0.01)

The effect of bunch exposure on the C_{13}-norisoprenoid composition of fruit and wine was assessed (Figures 9.5 - 9.7). For both seasons of the study, the concentration of β-damascenone per gram fruit homogenate was within the range of the recovered levels per ml in wine made from this fruit in both seasons, which indicates a good extraction of the precursors of this aglycone into the wines (Figure 9.5). The treatment effects on β-damascenone were different in the two seasons studied. In the fruit samples in 1999/2000, a significant effect was demonstrated from the analysis of the whole data set, but the post-test indicated that this was primarily due to a separation of the 'reference' treatment from the other canopy-management treatments in terms of β-damascenone concentration (Figure 9.5 A). Thus, despite large differences in fruit exposure, the strongest effect on β-damascenone was seen in the low vigour 'reference' vines. In the 2001 fruit samples, no significant differences were found after analysis of the whole data set (Figure 9.5 B). However, the wine samples of both seasons showed a statistically significant response (Figures 9.5 C and D). Thus, for fruit of the 1999/2000 vintage and

wines of both vintages there was a clear separation of the 'reference' wine from the other canopy-management treatments in terms of β-damascenone concentration.

In this experiment, TDN was the most significantly affected C_{13}-norisoprenoid by the treatments in both seasons. In fruit from the 1999/2000 vintage, hydrolytically-released TDN concentration increased significantly with increasing light penetration to the fruiting zone (Figure 9.6 A). This was in the order of a 25% increase from the shade to moderate treatment, and a further 45% increase from the moderate to sun treatment. In 2000/2001, TDN levels in fruit showed a similar response to that seen for β-damascenone concentration. There was no significant effect of the canopy-management treatments on its concentration, but the 'reference' treatment had a significantly a higher TDN concentration (Figure 9.6 B). Furthermore, as with β-damascenone, the level of significance of the treatment differences increased from the fruit to the wines (Figures 9.6 C and D). In the wines from both vintages, the concentration of TDN was significantly higher in the 'reference' treatment than the other canopy-management treatments. Although the differences in fruit exposure in the various canopy-management treatments had a small effect on increasing TDN concentration in the wines, this was less than the effect of the 'reference' treatment. The levels of recovered TDN were much higher in wines than in fruit. This may be due to the oxidative breakdown of carotenoids precursors during fermentation (Guedes de Pinho *et al.* 2001), such that the TDN produced by hydrolysis and heat may be derived de novo from carotenoid precursors as well as from glycosides originating from the fruit.

For β-ionone, the canopy treatments did not significantly affect its levels in either the fruit or wine (Figure 9.7). Although the difference was not significant, the reference treatment had a slightly lower β-ionone concentration than all of the canopy treatments for both vintages.

The results for the effects of canopy treatments and resulting bunch exposure on C_{13}-norisoprenoids in fruit and wines shed further light on the question as to how important sun exposure actually is in determining the final recovery of these compounds in wines. It is clear that sun-exposure has little effect on β-ionone in either grapes or wine. This is similar to the result from Experiment 1, where β-ionone was the least affected by sun-exposure in fruit, and not significantly affected in wines. As found by Marais *et al.* (1992b), sun-exposure appears to have the little effect on β-damascenone concentration, but may significantly affect the levels of TDN in fruit, as can be seen from the three canopy treatments. What is of relevance to the experiment which follows, however, are the differences observed between the minimally-irrigated 'reference' treatment, and all of the canopy treatments combined. For the 'reference' treatment, both β-damascenone and TDN were generally significantly higher than in the other canopy-management treatments in both fruit and wine samples. However, when the level of bunch exposure of the 'reference' treatment is compared with canopy management treatments, it falls within the range observed for the 'moderate' treatment. The main differences between the 'reference' treatment and the other canopy management treatments are in the effect of reduced irrigation on canopy size (leaf area) and fruit weight. This indicates that although elevated bunch exposure has an effect in determining the levels of the C_{13}-norisoprenoids β-damascenone and TDN, whole-vine physiology may also be a driving factor influencing their metabolism in fruit, and their recovery in wine. It must be noted that the reference treatment was carried out on a different clone of cv. Shiraz than the other canopy treatments. However, it is likely that the overriding environmental factors affecting canopy and fruit development would outweigh any clonal differences, since the performance of the two clones was similar when pruned and irrigated to the same level (Appendix G).

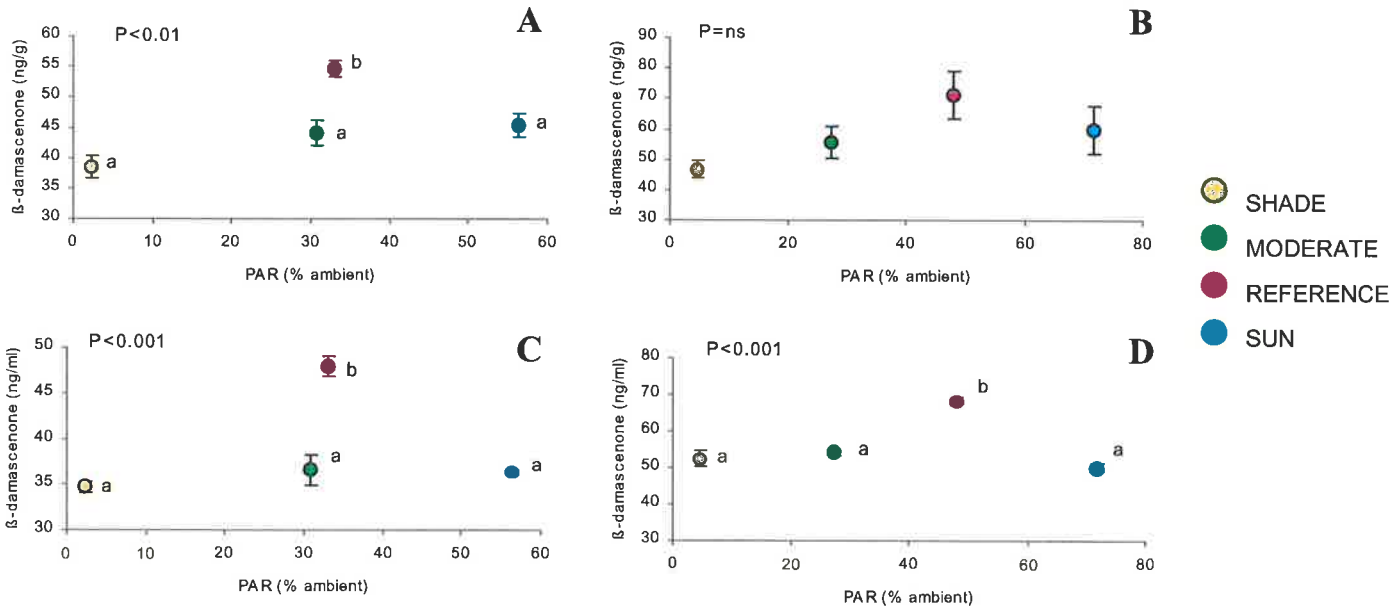


Figure 9.5: The effect of canopy management treatments on bunch exposure and β-damascenone concentration in Shiraz grapes and wine grown at Nuriootpa. **A**. 1999/2000 fruit; **B**. 2000/2001 fruit; **C**. 1999/2000 wine; **D**. 2000/2001 wine. (P=ANOVA; a,b,c=Tukey's post-test; n=12; ns = not significant)

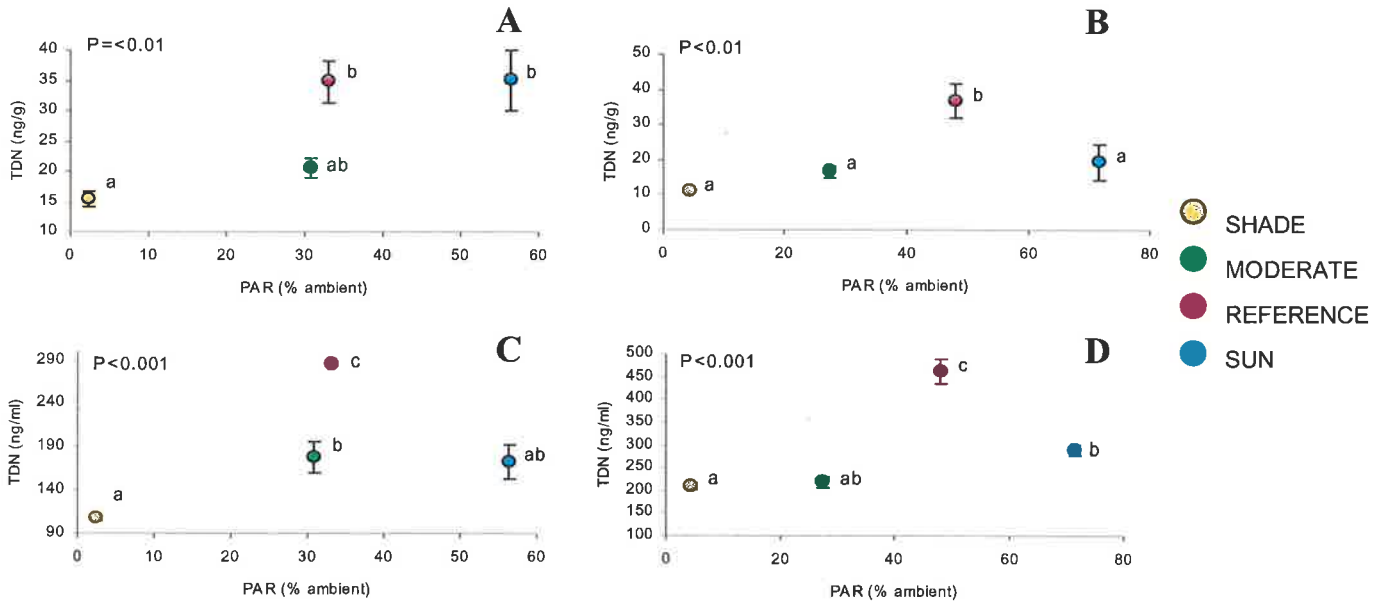


Figure 9.6: The effect of canopy management treatments on bunch exposure and TDN concentration in Shiraz grapes and wine grown at Nuriootpa. **A**. 1999/2000 fruit; **B**. 2000/2001 fruit; **C**. 1999/2000 wine; **D**. 2000/2001 wine. (P=ANOVA; a,b,c=Tukey's post-test; n=12; ns = not significant)

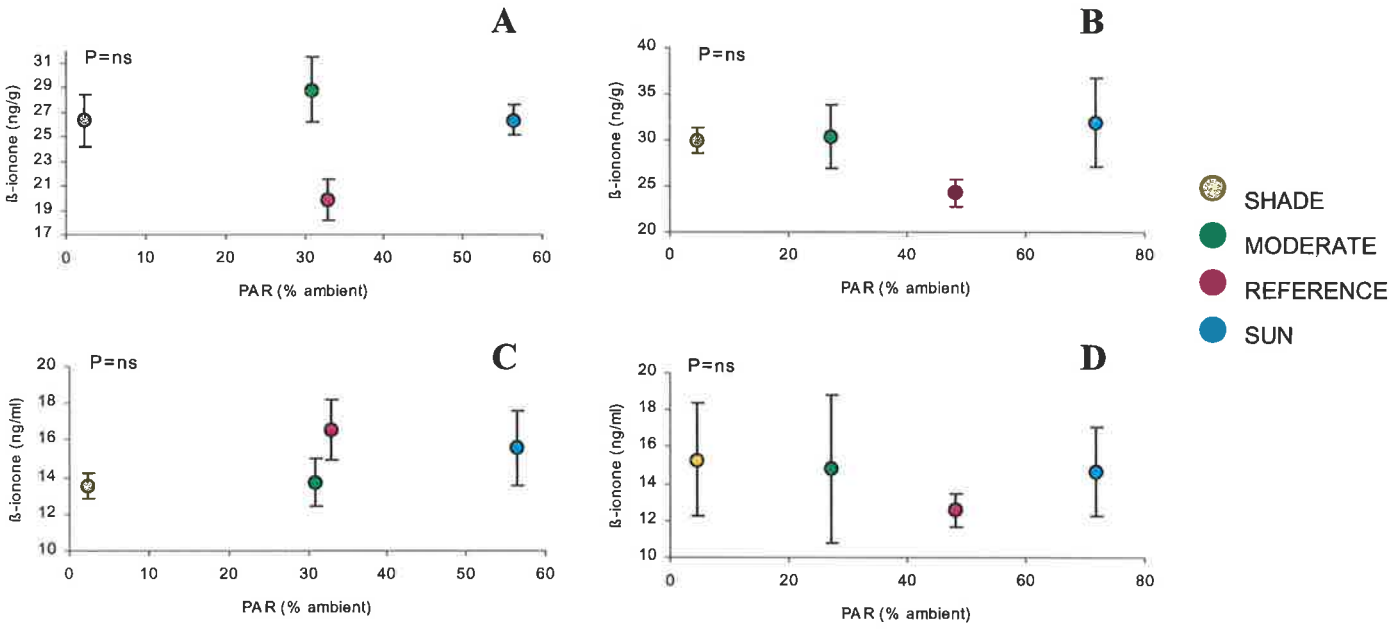


Figure 9.7: The effect of canopy management treatments on bunch exposure and β-ionone concentration in Shiraz grapes and wine grown at Nuriootpa. **A**. 1999/2000 fruit; **B**. 2000/2001 fruit; **C**. 1999/2000 wine; **D**. 2000/2001 wine. (P=ANOVA; n=12; ns = not significant)

9.3.3 Experiment 3: Effect of irrigation (PRD) on the composition of carotenoids in fruit and C_{13}-norisoprenoids in fruit and wine of Cabernet Sauvignon at Langhorne Creek.

This experiment aims to quantify the effect of PRD irrigation on the final carotenoid and C_{13}-norisoprenoid composition of fruit, and the recovery of C_{13}-norisoprenoids in wine. The PRD experiment on Cabernet Sauvignon has already been described in detail in previous chapters. However, for the purpose of this chapter, it is necessary to reiterate that over the two vintages, PRD led to a reduction in canopy size as measured by pruning weight and shoot growth; a relative increase in bunch exposure within the canopy; and a reduction in berry weight (Table 3.4 and Figure 3.8). The implications of changes in these parameters on carotenoid and C_{13}-norisoprenoid composition are discussed in this chapter.

9.3.3.1 The effect of PRD on the carotenoids lutein and β-carotene

The total concentration of the carotenoids lutein and β-carotene in fruit was determined at five different stages of development from approximately 15-16 °Brix until harvest at 23.5 °Brix. Figures 9.8 and 9.9 show the concentration of and lutein and β-carotene in PRD and control fruit during development in 2002/2003. In the 2002/2003 vintage, both carotenoids showed the expected decline in concentration as the fruit matured. For both carotenoids, PRD fruit had a higher concentration throughout development, although this was not statistically significant on all the sampling dates (Figures 9.8, 9.9). Note that the points of significance were at the later stages of development for both carotenoid types (Figures 9.8, 9.9), which is the period during which C_{13}-norisoprenoid increase has been shown to occur (Strauss *et al.* 1987; Shure and Acree, 1994; Baumes *et al.* 2002). This means that the pool size of carotenoid precursors for the formation of C_{13}-norisoprenoid glycosides was potentially increased in PRD-treated berries. For the 2001/2002 season, fluctuations in carotenoid concentration occurred, and a clear decline in the levels of both carotenoids was not observed as the fruit matured (results not shown). It is possible that in 2001/2002 the greatest decrease in the levels of the carotenoids occurred shortly post-veraison, following which levels remained more or less constant or at a slow rate of decline (Razungles *et al.* 1988; Razungles *et al.* 1996). At harvest, however, the concentration of both lutein and β-carotene was significantly higher in PRD fruit in the 2001/2002 vintage (Table 9.4). The changes in the concentration of lutein and β-carotene were not reflected in a change per berry (Table 9.4), due to the decrease in berry weight with PRD.

The results for the carotenoids lutein and β-carotene are in agreement with the findings of Oliveira *et al.* (2003), that an alteration in plant water status has the potential to cause changes in the carotenoid concentration in grape berries. Although the current study only looked at the principal carotenoid types found in *Vitis vinifera* (Razungles *et al.* 1988), it has been shown that the type of changes caused by water deficit in lutein and β-carotene concentration were also reflected in the minor carotenoids i.e. neoxanthin, violaxanthin and luteoxanthin which are derived from β-carotene via the xanthophyll cycle (Baumes *et*

al. 2002; Oliveira *et al.* 2003). As this phenomenon has only recently been observed in grape berries, there is no published information on the possible reasons for the observed changes in carotenoid pools as a result of water deficit. Furthermore, there is little data in the literature on the effect of water stress on carotenoid composition in fruits of other plant species.

Some speculative answers can perhaps be provided through looking at the function of the xanthophyll cycle in plants (Frank *et al.* 1994; Horton *et al.* 1996). The xanthophyll cycle is initiated in plants where photosynthetic tissue is exposed to light intensities in excess of those which can be utilised in photosynthetic electron transport (Horton *et al.* 1996). This allows for the excess light energy to be deactivated, whilst protecting the photodestruction of the photosynthetic apparatus (Frank *et al.* 1994). Two types of xanthophyll cycle have been reported. The primary process occurs through the light-dependant conversion of three oxygenated carotenoids (xanthophylls) in a cyclic reaction, namely interconversion of zeaxanthin, antheraxanthin and violaxanthin (Demmig-Adams *et al.* 1992). A second type of xanthophyll cycle has more recently been discovered involving de-epoxidation of lutein epoxide to lutein in the light, with the reverse process occurring in the dark (Bungard *et al.* 1999; Garcia-Plazaola *et al.* 2003). Water stress in leaves of *Nerium oleander* showed an increase in the xanthophyll cycle pool, and decreases in the yield of chlorophyll fluorescence which indicated increased thermal energy dissipation (Demmig *et al.* 1988).

The tissue of grape berries is only strongly photosynthetic up to veraison, after which chlorophyll fluorescence declines, although some activity remains as the fruit matures (Düring and Davytan, 2002). The decline of carotenoids follows the decline of chlorophyll post-veraison (Razungles *et al.* 1988), including carotenoids of the xanthophyll cycle: zeaxanthin, violaxanthin and antheraxanthin (Düring and Davytan, 2002). However, in sun-adapted berries the pool of xanthophyll pigments remains higher post-veraison than that in shade-adapted fruit (Düring and Davytan, 2002). This indicates that maintenance of the operation of the xanthophyll cycle is important under high light conditions, for the dissipation of excess light energy. It is therefore possible that, under

conditions of water stress, the operation of the xanthophyll cycle is further increased to maintain the capacity of the fruit to protect against photo-oxidative stress. This may also require the maintenance of higher levels of carotenoids in the tissues as a source for xanthophyll cycle pigments. This hypothesis requires further research, but may provide a potential explanation for the changes in carotenoids observed in berries exposed either to water deficit (Oliveira *et al.* 2003) or an induced response through exposure of the roots to drying soil conditions (PRD) as in the current study.

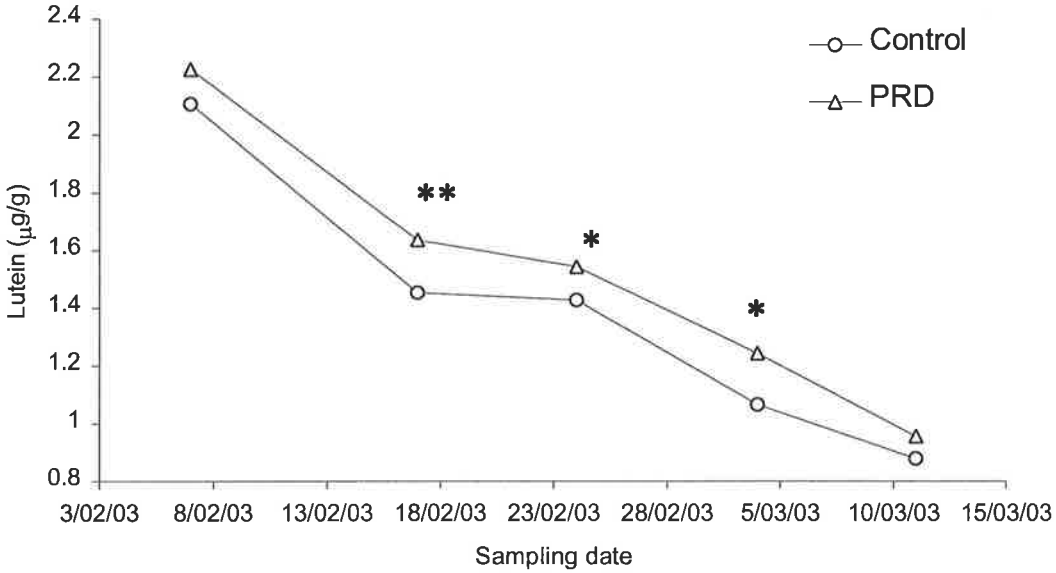


Figure 9.8: Effect of PRD on the concentration of lutein over berry development for the 2001/2002 vintage (ANOVA; n=42; *=P<0.05; **=P<0.01)

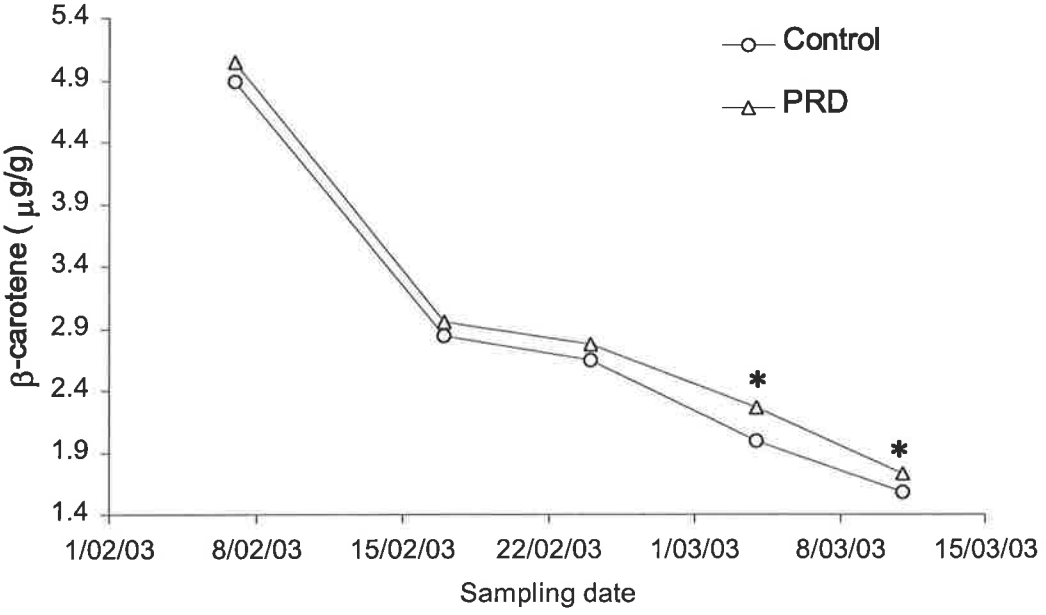


Figure 9.9: Effect of PRD on the concentration of β-carotene over berry development for the 2001/2002 vintage (ANOVA; n=42; *=P<0.05)

Table 9.4: Concentration of carotenoids in PRD-treated Cabernet Sauvignon grown at Langhorne Creek (ANOVA; n=42)

	2001/2002 Vintage				2002/2003 Vintage			
	Control	PRD	% PRD> Control	P	Control	PRD	% PRD> Control	P
Lutein (μg/g)	0.76	0.84	10.0	<0.01	0.70	0.76	8.1	ns
β-carotene (μg/g)	1.54	1.78	13.5	<0.01	1.58	1.73	8.4	<0.05
Lutein (μg/berry)	0.72	0.72	-0.4	ns	0.64	0.62	-2.4	ns
β-carotene (μg/berry)	1.46	1.54	5.3	ns	1.44	1.41	-2.7	ns

9.3.3.2 The effect of PRD on C_{13}-norisoprenoids

The response of C_{13}-norisoprenoids in PRD-treated fruit reflected the changes seen in the carotenoids for both vintages (Table 9.5). Hydrolytically-released β-damascenone and TDN were increased in PRD fruit in both 2001/2002 and 2002/2003, although the magnitude of the increase relative to the control was greater in 2002/2003. β-ionone was not significantly affected by the PRD treatment in 2001/2002, although levels were higher in PRD fruit in 2002/2003. As shown in the other experiments described in this chapter, the final levels of β-ionone show a variable response to environmental factors. TDN was the most significantly affected compound by the PRD treatment. It should be noted that fruit weight decreased in the PRD treatment in both seasons (Table 3.4). When the data for content of C_{13}-norisoprenoids per berry are examined for 2001/2002 (Table 9.6), it is apparent that although their levels were slightly higher per berry with PRD, these differences were not significant in 2002. From the 2001/2002 data, it could be speculated that the relative increase in the concentration of β-damascenone and TDN with PRD is due to the reduction in berry size. A greater proportion of the carotenoid precursors to the C_{13}-norisoprenoids are concentrated in the berry skin (Razungles *et al.* 1988) such that a change in skin to fruit-weight ratio could increase the relative concentration of these compounds per g in smaller fruit. However, both seasons of the study showed a significant, equivalent decrease in fruit weight with the PRD treatment

(Table 3.4). The 2002/2003 data indicate that there was a more significant increase in the concentration of β-damascenone and TDN per gram of fruit, this was also reflected in a significant increase per berry, despite the fruit weight reduction with PRD (Table 9.6). It is therefore more likely that physiological and environmental changes of the PRD experiment are causing the changes seen in the fruit rather than a change in fruit weight alone. Increases in bunch exposure were observed with the PRD treatment in both seasons (Figure 3.8), but in the light of the results of Experiment 2, this is probably not the primary reason for the changes in C_{13}-norisoprenoid concentration. A combination of stress-related signalling within the PRD treated vines (Stoll *et al.* 2000) in conjunction with high light intensities on developing fruit may have activated metabolism within the non-mevalonate pathway of isoprenoid synthesis. This pathway is responsible for the formation of ABA in addition to the isoprenoid compounds discussed in this chapter, and is known to be activated under conditions of water stress (Cowan, 2001). Increased flux within this pathway could result in 1. an increase in ABA synthesised in berry tissues 2. an increase of carotenoid and C_{13}-norisoprenoid compounds 3. potentially indicate increased operation of thermal dissipation mechanisms like the xanthophyll cycle. The increase in C_{13}-norisoprenoids may therefore be an indirect response of the grape berry metabolism to a perceived water stress.

Despite the changes in the levels of β-damascenone in the fruit over both vintages, there was no significant difference in its final concentration in wines made in both vintages (Table 9.7). As expected from the fruit samples, β-ionone concentration in the wine was not affected by PRD. Where a 21% increase in TDN was measured in fruit samples in 2001/2002, this was not reflected in the wine. However, in 2002/2003 where a 32% change per gram homogenate was found, this was reflected in an 18% increase in TDN levels in a wine made from a single representative sample of the treatment. This result indicates that a large change in levels of this compound in fruit can potentially result in its increase in the wine. Similar to what was seen in Experiment 2, the levels of hydrolytically-released TDN in wine did not reflect what was seen in the fruit, and levels were far higher.

Table 9.5: Total C_{13}-norisoprenoid concentration in PRD-treated Cabernet Sauvignon grown at Langhorne Creek (ANOVA; n=42)

	2001/2002 Vintage				2002/2003 Vintage			
	Control	PRD	% PRD> Control	P	Control	PRD	% PRD> Control	P
β-damascenone (ng/g)	30.44	34.94	12.9	<0.05	34.80	45.60	23.7	<0.01
β-ionone (ng/g)	17.11	19.53	12.4	ns	12.57	15.51	19.0	<0.05
TDN (ng/g)	5.90	7.46	20.9	<0.05	4.19	6.25	33.0	<0.01

Table 9.6: Total C_{13}-norisoprenoid content in PRD-treated Cabernet Sauvignon grown at Langhorne Creek (ANOVA; n=42)

	2001/2002 Vintage				2002/2003 Vintage			
	Control	PRD	% PRD> Control	P	Control	PRD	% PRD> Control	P
β-damascenone (ng/berry)	28.17	30.30	7.0	ns	31.20	37.40	16.6	<0.05
β-ionone (ng/berry)	15.99	17.03	6.1	ns	11.35	12.70	10.6	ns
TDN (ng/berry)	5.48	6.44	14.9	ns	3.69	5.11	27.8	<0.05

Table 9.7: Free and hydrolytically-released C_{13}-norisoprenoids in PRD and control Cabernet Sauvignon wines from Langhorne Creek, vintages 2001/2002 and 2002/2003.

	2001/2002 Vintage				2002/2003 Vintage			
	Control	PRD	% PRD> Control	P	Control	PRD	% PRD> Control	P
β-damascenone (ng/ml)	36.00	38.00	5.3	ns	53.39	49.88	-7.0	ns
β-ionone (ng/ml)	16.26	14.82	-9.7	ns	35.22	36.96	4.7	ns
TDN (ng/ml)	122.80	123.10	0.2	ns	74.51	91.54	18.6	<0.01

TDN is slowly released from its precursors during wine ageing (Marais, 2002). The fact that such a large difference between TDN released from precursors in the fruit relative to the wine, indicates that there is an increase in precursors available during the winemaking process. As discussed previously, this is possibly due to the breakdown of carotenoids during fermentation, most likely lutein (Marais, 1992). Although β-damascenone and β-ionone denote positive sensory characteristics in wines (Enzell, 1985), TDN may impart a negative, kerosene-like odour at higher levels (Marais, 2002). This has been mainly studied in Riesling wines, where TDN imparts an important character to the aroma of these wines, but only below a threshold of 20 ppb, over which concentration it can become negative (Marais, 2002). There has been little attention given to the effect of TDN on the aroma and quality of red wines, and the implications of the results of the current study for wine quality are unknown . It should be noted that the reported levels in this study represent hydrolytically-released C_{13}-norisoprenoids, which gives an estimate of the maximum amount of precursor available for release during wine ageing. It is therefore not an exact representation of the aroma or flavour of the wine, but gives an indication of the likely effect of the treatment on flavour or aroma potential in the resultant wines.

9.4 Conclusions

The current study has demonstrated that higher levels of carotenoids in the fruit at harvest can be associated with increased recovery of C_{13}-norisoprenoids. Since the conditions of the experiment are not causing the *de novo* release of C_{13}-norisoprenoids from carotenoids by thermal/hydrolytic degradation (Table 8.1), this must reflect changes in the precursors of these aroma volatiles in the fruit itself. From the current results there was an indication that increased levels of TDN and β-damascenone were produced in response to reduced vine vigour resulting from water deficit or PRD. This response requires the presence of light, as total shading of developing bunches brought very large decreases in both carotenoids and C_{13}-norisoprenoids. However, the amount of light reaching developing fruit did not appear to influence C_{13}-norisoprenoid concentration to the same extent as water deficit. It is therefore conceivable that light exposure does not

operate in isolation to bring about the changes in C_{13}-norisoprenoids, as has been previously thought. Thus, where increases in carotenoids and C_{13}-norisoprenoids are seen in PRD fruit it is likely to be due to a combined effect of increased sun-exposure, and physiological changes resulting from the treatment. In the light of these conclusions, a future research direction could be to investigate the effect of PRD on plant hormones such as ABA and cytokinins in grape berries; and their role in the regulation of secondary metabolism. This may shed light on the control of the pathways of carotenoid and C_{13}-norisoprenoid metabolism. Within this framework, an important area of research is to investigate the effect of water stress on the operation of the xanthophyll cycle in grape berries, both pre- and post-veraison. These studies will allow for PRD to be further optimised to produce fruit and wine with increased flavour/aroma potential. Furthermore, our understanding of the developmental control of C_{13}-norisoprenoid production in fruit is still limited, and there is still opportunity for research into the effects of environmental factors, namely water stress, sunlight and temperature on their concentration on grape berries during fruit maturation.

It is evident that changes in levels of C_{13}-norisoprenoids in fruit may not necessarily be translated into similar changes in the wine. Thus, differences detected in the fruit may be lost in the wine except where these are large. However, the potential still exists to further optimise the levels of β-damascenone and TDN using deficit irrigation strategies such as PRD. The results of the 'reference' treatment of Experiment 2, although they cannot be directly correlated with an irrigation deficit, indicate the potential to greatly increase the final levels of these compounds recovered in wine. Experiment 3 represents data from an experiment where water application was reduced by only 30% in the PRD treatment. Where the water deficit response in grape berries is further optimised, it may be possible to see greater increases in aroma compounds in PRD fruit relative to the controls, and a final improvement in the wine may be seen.

CHAPTER 10: GENERAL DISCUSSION AND CONCLUSIONS

The aim of the current study was to explore the potential effects of the PRD irrigation technique on grape secondary metabolic pathways and their contribution to grape and wine quality. It must be noted that although the intention was to alter only non-hydraulic signalling in grapevines using this irrigation strategy, that some water deficit was experienced by vines in the current study, resulting in hydraulically-mediated physiological changes in both the vine canopy and reproductive growth. Thus, a 'classic' PRD response was not consistently achieved, and this must be taken into account in the conclusions drawn from this study. However, apart from irrigation strategy applied, other factors have the potential to strongly influence both the concentration and proportion of the different groups of secondary metabolic compounds in the grape berry. Thus, the effect of water management on grape secondary pathways was considered together with other factors such as primary carbon metabolism, berry size and bunch exposure, to ascertain their respective contributions to grape and wine quality.

10.1 How does PRD affect secondary metabolism in grape berries?

A summary of some of the potential responses of grape berry metabolism to PRD irrigation is shown in Figure 10.1, drawn from the results of the Langhorne Creek experiment on Cabernet Sauvignon grapevines. In that experiment, a primary response to the PRD treatment was a reduction in berry weight and seed weight from early in fruit development relative to the control treatment. This response was accompanied by increased accumulation of hexose sugars in the PRD treatment in one season of the study (2002) and an increased respiration of malic acid was observed over two seasons. Furthermore, PRD showed the potential to increase the accumulation of anthocyanins and total phenolics in grape berries, measured as concentration per gram berry weight. The increase in the concentration of total phenolics due to PRD was found to be due in part to increased contribution of seed-derived proanthocyanidins and flavan-3-ol monomers. In PRD wines, there was shown to be an improvement in colour that was independent of anthocyanin concentration. Rather, this was shown to be due to increased capacity of anthocyanins in PRD wines to form copigmented or polymeric associations with other

compounds present in the wine. This may be due to a relatively higher contribution of non-anthocyanin phenolic compounds in wines produced from PRD-treated fruit, compared with that made from control treatments. Additionally, PRD increased flux toward isoprenoid secondary metabolites, as estimated from the concentration of the C_{13}-norisoprenoid group of glycosylated flavour/aroma compounds, and their carotenoid precursors.

Thus it was shown that PRD has the potential to influence the concentration of two groups of secondary metabolites: the phenols, including anthocyanins, tannins and other phenolics; and the isoprenoids, including carotenoids and C_{13}-norisoprenoids. As both these groups have been shown to contribute significantly to wine quality in terms of colour, astringency, mouthfeel and aroma/flavour, these results indicate that the PRD irrigation strategy holds the potential to improve wine quality. However, for this to be best optimised, the underlying biochemical basis for these changes in fruit composition in response to PRD must be better understood.

Figure 10.1 Summary of some of the responses of Cabernet Sauvignon grape berries to PRD observed in the current study

Growth and development	Primary metabolism	Secondary metabolism
• Reduction in berry weight • Reduction in seed weight	• Increase in the metabolism of malate • May cause accelerated hexose accumulation	In fruit, may cause: • increased anthocyanin accumulation • increased total phenolics • increased carotenoid levels • increased glycosylated C_{13}-norisoprenoid compounds • increased concentration of seed tannins per gram berry weight In wine, may result in: • increased capacity for copigmentation and polymerisation of wine pigments • increased potential for wine aroma development

10.1.1 The contribution of berry size to the concentration of secondary metabolites

In the current study, the production of smaller berries by Cabernet Sauvignon or Shiraz vines was shown to be a response to carbon limitation. This was either due to an increase in the size of the reproductive sink for photosynthetically-derived carbon, where node number was high in the Nuriootpa Shiraz PRD experiment (Section 2.1.1.1, Chapter 2), for a smaller canopy size over which carbon could be fixed. Or, when berry size was reduced as a direct response to the PRD treatment, as in the Cabernet Sauvignon experiment at Langhorne Creek (Section 2.1.2, Chapter 2), this may have been due to a carbon limitation resulting from a reduction in photosynthesis associated with decreased stomatal conductance due to PRD. But, it could additionally have been caused by a direct hydraulic effect of water deficit induced by the PRD treatment in this experiment, which altered the expression of genes controlling cell growth and development in both berries and seeds.

In both the Cabernet Sauvignon and Shiraz PRD experiments, the opportunity was created for analysis of the contribution of berry size to the concentration of secondary metabolites. Smaller berries have a larger surface area to volume ratio, which means that skin-derived secondary metabolites would be extracted in a smaller volume of must during fermentation, potentially increasing their concentration in the resulting wines. However, the data of the current study have shown that for a range of berry weights, produced either by varying node number per vine or as a response to water stress occurring in the PRD treatment, there is a weak effect of berry size on the concentration of anthocyanins derived from the skin. Thus, smaller berries did not have an increased concentration of anthocyanins. This may be due to a possible increase in skin density or thickness in larger berries relative to small fruit. For the measure of total phenolics, however, a different response was obtained. Where berry size was reduced due to increased node number in the Shiraz experiment, and the additional imposition of PRD did not significantly affect fruit weight, only a weak interaction was found for berry weight and phenolic concentration. However, this relationship was stronger when berry weight was decreased as a direct response to the PRD treatment in Cabernet Sauvignon. Thus decreasing berry size has the potential to increase the concentration of phenolics in

wines. An analysis of the contribution of seed-derived phenolics to the measure of total phenolics showed that seed tannin concentration was increased as berry weight decreased in Cabernet Sauvignon. It is therefore possible that, based on results from the Cabernet Sauvignon experiment, an increased contribution of seed-derived phenolics may be due to a reduction in berry size.

These findings have important implications for our current understanding of the influence of fruit composition on wine quality. If wine colour in red varieties is used as a potential indicator of wine quality, then the winemaking experiment from the current study has shown that using PRD irrigation can improve colour by 10%. Traditionally, this would be thought to be due to increased concentration of anthocyanins in the wine. However, our understanding of the effects of copigmentation and polymerisation of wine pigments has grown in recent years (Boulton, 2001) and the current results confirm that anthocyanin concentration alone is not the primary factor influencing wine colour. Decreased berry size can potentially increase the concentration of non-anthocyanin phenolics, which in turn have the potential to act as intermolecular copigments in wines. This relationship may partly account for results such as those of Freeman (1983), who demonstrated a strong negative correlation between wine colour and berry weight in wines produced from irrigated versus non-irrigated fruit. In that study, however, no significant relationship was detected for berry weight and anthocyanin colour, indicating the contribution of an additional 'unknown' component in the determination of final wine colour.

For the isoprenoid group of secondary metabolites, although a greater proportion of the carotenoids and C_{13}-norisoprenoids are concentrated in the berry skin, they are also present in the berry flesh (Razungles *et al.* 1988). Thus, the potential for a reduction in berry size to bring about an increase in the concentration of isoprenoids would be less probable than for compounds found exclusively in the skin i.e. the anthocyanins. The current study showed that the carotenoids or C_{13}-norisoprenoids per berry were either maintained or increased in response to the PRD treatment on Cabernet Sauvignon (Langhorne Creek) relative to the control treatment. It would be expected that a reduction

in berry weight would result in lowered overall content of isoprenoids per berry due to a smaller berry volume. This demonstrates that the significant increases in carotenoid and C_{13}-norisoprenoid concentration per gram of berry homogenate in response to PRD were not due to the concentrating effect of a reduction in berry weight, but were rather a direct or indirect response of berry metabolism to the PRD treatment. Thus, the current study has shown that for two groups of secondary metabolites, namely the anthocyanins and the isoprenoids, increases in their concentration observed in response to PRD irrigation cannot be attributed to a change in berry size alone. Rather, the response of these groups to PRD irrigation is caused by other factors, which either directly or indirectly affect berry metabolism.

10.1.2 Bunch exposure

A second key question addressed in the current study was the importance of light exposure within the fruiting zone of grapevines in determining final levels of secondary metabolites in harvested fruit. From a range of measures of bunch exposure within the fruiting zone of PRD-irrigated and standard-irrigated vines in the current study, it was evident that PRD irrigation has the potential to significantly increase light incidence within the canopy, most likely due to a decrease in lateral leaf area (Dry, 1997; Stoll, 2000). From the literature, it would be expected that increasing levels of bunch exposure can potentially increase the concentration of anthocyanins, and certain C_{13}-norisoprenoids (TDN) in grape berries (Bergqvist *et al.* 2001, Marais *et al.* 1992a), although carotenoids have been shown to degrade under high light intensities (Marais *et al.* 1991).

10.1.2.1 The response of grape berry anthocyanins to sunlight

In the case of the anthocyanins, total shade was shown to significantly reduce anthocyanin concentration in grape berries relative to fruit which developed within the vine canopy. When the shading treatment was superimposed over PRD and standard irrigation treatments in the Cabernet Sauvignon experiment at Langhorne Creek, the increase in anthocyanin concentration which was obtained in sun-exposed PRD-treated fruit was not found for shaded fruit. This suggests that the increase in anthocyanins under

the PRD treatment was mediated, at least in part, by higher levels of bunch exposure in those vines. However, comparison of fruit sampled from PRD- and control-treated Cabernet Sauvignon grown in pots where berries had the same level of exposure to light showed a large (27%) increase in the anthocyanin content of berry skins, which suggested a response of the anthocyanin metabolic pathway to the PRD treatment that was independent of bunch exposure. Closer examination of the composition of anthocyanins, in terms of the relative proportion of anthocyanidin types, and the pattern of glycosidation showed that the response of the anthocyanins to the PRD treatment was not typical of that expected for increased exposure to sunlight. Instead of showing a decrease in the proportion of the coumarate forms of anthocyanins, a response previously shown for fruit under increased irradiation (Haselgrove *et al.* 2000), anthocyanins under the PRD treatment showed a shift in the relative contribution of anthocyanidin types in field-grown vines. Non-malvidin anthocyanidins were significantly increased by PRD at the expense of malvidin, although the effect on malvidin was small.

A conclusion from this result is that the response of grape berry anthocyanins to PRD requires sunlight, as demonstrated by the shading experiment, but is not exclusively mediated by increased levels of sunlight reaching PRD-treated fruit. Rather, signals produced by the PRD treatment appear to have a direct effect on anthocyanin metabolism, which can occur even where berries receive equivalent levels of sunlight during their development. In the presence of high levels of sun exposure in the case of field-grown vines, it appears that the distribution of the anthocyanins into a diverse range of anthocyanidin types would increase the effectiveness of the berry skin for photoprotection by absorption of a variety of wavelengths of radiation, but also appears to increase the radical scavenging capacity of the fruit in the protection against oxidative stress. This appears to be a response to increased potential for oxidative damage within the fruit under high levels of light incidence, which would be exacerbated by the oxidative conditions induced by a water deficit.

10.1.2.3 The response of grape berry isoprenoids to sunlight

The current study has shed some light on our current thinking about the response of C_{13}-norisoprenoids and carotenoids to sunlight. In Shiraz fruit grown at Nuriootpa, it became evident that total shading of bunches from berry set produced grapes and wines with very low levels of C_{13}-norisoprenoids. However, only a small amount of sunlight penetration to the canopy interior was required to restore C_{13}-norisoprenoid levels. This was shown through a canopy management experiment where three different levels of bunch exposure were compared (Section 2.1.1.2, Chapter 2). There was little difference in the levels of the C_{13}-norisoprenoids β-damascenone and β-ionone in fruit sampled from shaded canopies and highly exposed canopies. However, TDN showed a significant response to higher levels of irradiation which has been shown in previous studies (Marais *et al.* 1992a). The aim of the current study was to explore the influence of irrigation strategy on C_{13}-norisoprenoid metabolism, and to explore the potential contribution of bunch exposure to the observed response. Interestingly, a deficit irrigation treatment on Shiraz (Section 2.1.1.2, Chapter 2) significantly increased levels of both β-damascenone and TDN, even though bunch exposure was equivalent to levels observed for a moderately-exposed canopy. This response showed that these C_{13}-norisoprenoids can be affected by a metabolic response to water stress alone, and are not necessarily regulated by external factors such as sunlight. The PRD irrigation experiment on Cabernet Sauvignon at Langhorne Creek additionally showed that over two seasons, C_{13}-norisoprenoid concentration could be increased in grape berries. In the light of the results of the canopy trial on Shiraz, this response is not likely to be due to the slight increase (30%) in sunlight incidence of the fruiting zone of PRD vines. Rather, there appears to be a metabolic response of the isoprenoid pathway to the PRD treatment directly, due to changes in hydraulic and/or non-hydraulic signals produced within the plant.

An interesting finding was that carotenoid concentration was increased in response to the PRD treatment. Traditionally, increases in C_{13}-norisoprenoid concentration have been associated with increased degradation of carotenoid precursors, leading to a reduction in the total pool of carotenoids (Marais *et al.* 1991, Baumes *et al.* 2002). This increase in the pool size of carotenoid precursor in response to water limitation was recently shown by

Oliveira *et al.* (2003), where a non-irrigated treatment caused a 60% increase in carotenoid concentration compared with an irrigated control. This result, in conjunction with the findings of the current study shows that deficit irrigation has the potential to increase flux in the isoprenoid pathway, presumably toward increased synthesis of the stress hormone ABA via the carotenoids (Lichtenthaler, 1999). This response to stress conditions experienced by the vine has the potential to increase the pool size of precursors to carotenoid-derived flavour compounds, which include the C_{13}-norisoprenoids. This has important implications for flavour development in grapes and wines, and deficit irrigation may become a significant tool to manipulate wine flavour in the future.

10.1.3 Hydraulic and/or non-hydraulic plant signalling

In the current study, there has been an attempt to distinguish between biochemical responses of secondary metabolism within the grape berry which occur as a direct response to water deficit experienced by the grapevine from those which are indirect, i.e. due to a restriction in berry growth and development or due to changes in bunch microclimate (bunch exposure). A key finding has been that the water-deficit induced accumulation of secondary metabolites in berries could be mediated by the PRD treatment alone, and was independent of the level of bunch exposure to sunlight. This suggests a direct, biochemically-mediated response to signals produced by the PRD treatment.

The most well-known, and well-documented response of vines to PRD, is the increase of ABA in roots and xylem sap in response to partial drying of the root system (Stoll *et al.* 2000). This is of particular significance, as it has recently been shown that ABA levels in fruit increase in response to water deficit, independent of the stage of berry development (Antolin *et al.* 2003). As yet, the effect of PRD on berry ABA has not been ascertained, but there is a strong possibility that some of the metabolic responses observed in the current study are mediated, at least in part, by alterations in the levels of this plant hormone. In the current study, the PRD treatment did result in a measure of water deficit being experienced by the vines, as determined by decreased leaf and stem water potential.

Therefore the involvement of hydraulic signals, in addition to non-hydraulic signals, cannot be ruled out in these experiments.

An important point to consider at this stage is that both non-hydraulic and hydraulic responses to some extent involve changes in the metabolism of ABA, building up in roots or leaves respectively (Davies *et al.* 1986, Cowan *et al.* 1982). It is therefore conceivable that a water deficit may affect ABA concentration in grape berries, whether the stress is of a magnitude sufficient to mediate a hydraulic response, or a classic PRD system which would produce only non-hydraulic signals in the roots, without affecting leaf water status or berry weight. Increases in anthocyanin concentration of berry skins has been shown in previous studies where PRD did not affect leaf water potential nor cause an alteration in berry weight, and a non-hydraulic signal would be the most likely factor responsible for the effect (Dry, 1997; Stoll, 2000). However, a similar response has been shown for anthocyanin content of berry skins in the current study where vines experienced a mild water deficit using the PRD technique. This has also been shown to occur in response to a severe water deficit, where non-irrigated vines produced berries with higher anthocyanin concentration in the skins compared with an irrigated control (Esteban *et al.* 2001). Presumably, these responses would be mediated by both hydraulic and non-hydraulic signals. This shows that metabolic pathways controlling the pool size of secondary metabolites can respond to varying levels of water deficit.

It is conceivable that these changes can occur in response to elevated levels of ABA in berry flesh and skins. The anthocyanin pathway in grape berry cells has been shown to be strongly upregulated by the exogenous application of ABA (Hiratsuka *et al.* 2001, Kim *et al.* 1997). Furthermore, the non-mevalonate pathway of isoprenoid metabolism is responsible for the formation of ABA in plant tissues, and may be activated when plants are under environmental stress (Cowan, 2001). Presumably, increases in the pools of intermediates toward ABA synthesis would be upregulated where ABA is actively synthesised by plant cells, which may account for some of the changes in berry carotenoid pools shown in the current study. Although the response of carotenoid pools to the PRD treatment was small, this may have resulted in increased flux toward ABA

synthesis in the grape berries. Further analysis of the effect of deficit irrigation strategies, including PRD, on ABA metabolism in grape berries should be an important focus of future research, in order to account for some of the changes in secondary metabolism which have been observed in the current study. Amongst the various plant signals which influence plant metabolism, ABA is a very likely candidate for the mediation of the observed responses. However, the magnitude of water stress and the type of stress, i.e. an hydraulic or a non-hydraulic response, may bring about very different effects on secondary metabolic pathways.

10.2 Yield versus quality – the limitations of the PRD technique

A second objective in the current study was to explore the limitation of the PRD technique in terms of crop load and water-use efficiency. For previous studies of the application of PRD in different grape varieties and in different localities, it was shown that PRD could be implemented where irrigation was reduced up to 50% relative to conventional practice, without a significant yield penalty (Dry *et al.* 2000c). The current study has shown that there are a number of factors which may influence the response of vines to a PRD-type irrigation strategy, which may affect the final yield and quality of the crop at harvest.

10.2.1 Irrigation cycling

The responses to the PRD irrigation strategy shown in the current study bring to light some important questions surrounding the application of PRD as a deficit irrigation strategy. It was shown that the PRD treatment resulted in a hydraulic water deficit relative to the control treatment, where the PRD treatment had half the irrigation volume applied compared with the control treatment. This result is different from what has previously been found for the technique, where no change in leaf water potential was observed in response to PRD (Dry, 1997; Stoll, 2000; Stoll *et al.* 2000). This resulted in a yield penalty in some instances of up to 25% relative to the control treatment, which was associated with a reduction in berry size. As the effectiveness of PRD as an irrigation strategy could be dependent upon soil type, this may become an increasingly important consideration in the choice of irrigation strategy by grapegrowers in the future

(Kriedemann and Goodwin, 2003). In the current study, PRD irrigation was implemented in sandy loam soils in both the Cabernet Sauvignon (Langhorne Creek) and Shiraz (Nuriootpa) experiments. Such soils have been suggested to be the best medium for this irrigation strategy compared with soils of a higher clay content (Kriedemann and Goodwin, 2003). Despite this, a 'classic' PRD response was not achieved for either site, even where irrigation scheduling was rigorous, and planned according to closely-monitored levels of soil moisture. The question which needs to be addressed is the length of the PRD cycle. Under the conditions of the current study, the potential exists that soil water content decreased rapidly in the soils due to rapid drainage of irrigation water together with a high usage of available soil water by roots. Thus, under these conditions, an extended PRD cycle time could have led to excessively dry soil on the 'dry' side of the PRD treatment, such that the vines experienced a hydraulically-mediated water deficit. As this holds the potential to reduce yield and berry size in addition to vine canopy size, the applicability of PRD must be considered as a 'cost-benefit' scenario. PRD has the potential to increase WUE of vines, as has been shown in the current study, but if there is a significant yield penalty associated with the treatment, there would be no net gain in WUE.

Furthermore, it is known from a previous study by Loveys *et al.* (1998) that the production of ABA signals from roots in drying soil is transient, and decreases as the soil continues to dry. There is a strong likelihood that the non-hydraulic PRD response is dependent upon maintenance of plant signals like ABA from the roots, and thus required the soil to remain in the early stages of drying following an irrigation event. The problem observed in the current study may be due to a long cycle time in rapidly drying soil, in which the pulse of ABA had come and gone quickly and was not sustained. By shortening the cycle times it may be possible amplify the number of ABA pulses achievable from the roots, maintaining a lowered rate of leaf stomatal conductance and additionally reducing the possibility of affecting leaf water relations.

10.2.2 Node number per vine

The comparison of three different pruning levels superimposed on standard and PRD irrigation strategies allowed for the limitations of irrigation techniques at different cropping levels to be explored. Standard practice for the Barossa Valley region is a 60 node per vine pruning level. As would be expected, increasing node number per vine resulted in an increase in yield. Where PRD received half the irrigation volume of the control treatment, a general observation was that yield and berry size were not detrimentally altered by the PRD irrigation strategy when node number (bunch number) per vine was low, i.e. at 30 and 60 nodes per vine. However, when node number per vine was increased to 120 nodes, the vines showed increased sensitivity to PRD irrigation, in terms of both reproductive and vegetative growth. Thus, there was a reduction in both yield and vegetative growth in PRD vines at high node number, although this was not statistically significant when analysed across the three pruning levels. No change in fruit secondary metabolites was observed at higher node number per vine, although in one season, PRD significantly improved anthocyanin and phenolic concentration across all pruning levels. This means that using the PRD technique, growers might expect to see an improvement in quality, provided that vines were not over-cropped. Where PRD is combined with a large bunch number per vine, e.g. in the case of minimal pruning, and irrigation is limited as in the Barossa typically to 1 ML/ha, growers might expect to see a yield penalty, but see little change in fruit quality compared to PRD-irrigated vines pruned to lower node numbers. It appears that 'vine balance', i.e. the partitioning of structural carbohydrate between vegetative and reproductive growth, became problematic where node number per vine was high. Thus, there was a limitation of carbon resources for partitioning over a large reproductive sink, which was exacerbated under PRD irrigation, such that a reduction in both vegetative and reproductive growth occurred.

10.3 Relevance of the current findings to the wine industry

The current study has shown that the application of a mild water deficit to grapevines has the potential to alter the metabolism of secondary compounds in fruit. As many of these secondary compounds are important to the determination of final wine quality, this may demonstrate that growers have the potential to enhance the quality of fruit and wines through deficit irrigation strategies, including PRD. Despite the potential limitations surrounding the application of PRD in different regions, soil types and at different cropping levels (Kriedemann and Goodwin, 2003), the technique still holds the advantage of providing a continuous and mild water stress to vines, and additionally has the potential to improve fruit quality (Dry *et al.* 2003c). Using PRD would allow growers to avoid the potential deleterious effect of other deficit irrigation strategies such as RDI, which may result in a devastating yield penalty if incorrectly managed. Furthermore, if a slight yield reduction is associated with increased fruit quality when PRD is used commercially, then the net result may be increased return per ha.

This is an important consideration where there is currently an increased drive within the Australian wine industry to produce fruit of improved quality. This drive is set against a background of a rapidly expanding wine production base, which could potentially lead to a glut of low-quality, low-profit wines if viticultural practices are not improved in the immediate future. This study has compared PRD and conventional irrigation where both irrigation strategies have used water volumes at the lowest limit (approximately 1 ML/ha) for the viticultural regions where the experiments were based. Thus, the margin for the improvement of grape and wine quality through the effect of deficit irrigation on secondary metabolites was small. However, in regions where far higher irrigation volumes are used e.g. 6 ML/ha or more in vineyards of e.g. Riverland, Sunraysia and Riverina, the potential to restrict water use and boost grape and wine quality may be far greater than the levels shown in the current study. This question will become increasingly important in the future, in the light of recent restrictions imposed on water use in South Australian vineyards.

10.4 Suggestions for future research

A number of key issues have arisen from the results of the current study:

1. A very important question is the role of ABA in the regulation of secondary pathways. Are the responses due to increased ABA in grape berries throughout fruit growth and development; or is there a potential 'switching on' of certain secondary pathways by increased ABA at certain key developmental stages? We know ABA is increased in fruit experiencing water deficit from recent reports (Antolin *et al.* 2003) but as yet, the timing and potential alteration of its accumulation pattern in fruit in response to water deficit remains unresolved. Within this question, a separation of the effects of water deficit versus a 'classic' PRD-type response involving solely non-hydraulic signals needs to be achieved. Both hydraulic and non-hydraulic plant signals are mediated to some extent through the metabolism of ABA, but the effects of these respective signals on grape berry ABA levels remains unclear.

2. A key finding in the current study was that wine colour could be improved using the PRD irrigation technique, even where anthocyanin levels in fruit and wines were unchanged. Increases in the contribution of 'total phenolics' to wine colour, possibly through increased intermolecular copigmentation and polymerisation of coloured pigments appears to be responsible for this phenomenon. The concept of 'total phenolics' is currently based on a measure of wine absorbance at 280 nm, and therefore includes anthocyanins, simple phenolics, flavonols, flavan-3-ols and condensed tannins. Each component of this diverse group of phenolic compounds has the potential to contribute to the copigmentation effect observed in wines at different levels. Future research of this topic should include a detailed analysis to determine which phenolic compounds are increased by the PRD irrigation technique. The current study showed that seed tannins can be increased in fruit, but as yet, their potential contribution as wine copigments is poorly understood.

3. A third issue which needs to be addressed is the limitation of region and soil type on the application of the PRD technique. Before PRD is presented to the wine industry as a possible tool for the improvement of grape and wine quality, its limitations in different winegrowing regions of Australia need to be explored on an ongoing basis. A range of studies has been conducted in different regions of Australia in recent years, and some have not demonstrated the expected response to PRD irrigation (Kriedemann and Goodwin, 2003; S. Pudney, pers comm.). Despite this, the technique holds strong potential for the manipulation of grape and wine quality, together with an increasing need for improved water use efficiency of a limited water supply within the wine industry. Thus, research into the applicability of PRD as an irrigation strategy should continue, perhaps in conjunction with other deficit irrigation strategies such as RDI.

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APPENDIX A: EXPERIMENTAL DESIGN

Figure A: Randomised-block experimental design Nuriootpa Shiraz PRD and pruning experiment

Vine	Buffer Row 17	Buffer Row 18	Buffer Row 19	
41	3	2	1	REP 5
40	3	2	1	
39	3	2	1	
38	3	2	1	
37	1	3	2	
36	1	3	2	
35	1	3	2	
34	1	3	2	
33	1	3	2	REP 4
32	1	3	2	
31	1	3	2	
30	1	3	2	
29	2	1	3	
28	2	1	3	
27	2	1	3	
26	2	1	3	
25	2	1	3	REP 3
24	2	1	3	
23	2	1	3	
22	2	1	3	
21	3	2	1	
20	3	2	1	
19	3	2	1	
18	3	2	1	
17	2	3	1	REP 2
16	2	3	1	
15	2	3	1	
14	2	3	1	
13	2	1	3	
12	2	1	3	
11	2	1	3	
10	2	1	3	
9	3	2	1	REP 1
8	3	2	1	
7	3	2	1	
6	3	2	1	
5	1	3	2	
4	1	3	2	
3	1	3	2	
2	1	3	2	
1	Buffer	Buffer	Buffer	

Irrigation Mainplot	
	standard
	PRD

Pruning Sub-plot	
1	30 node
2	60 node
3	120 node

APPENDIX B: EXPERIMENTAL DESIGN

		Vine plot (two vines per plot)						
Block 1	Control	1	2	3	4	5	6	7
	PRD	1	2	3	4	5	6	7
Block 2	PRD	1	2	3	4	5	6	7
	Control	1	2	3	4	5	6	7
Block 3	PRD	1	2	3	4	5	6	7
	Control	1	2	3	4	5	6	7

Figure B: Langhorne Creek Cabernet Sauvignon Experimental Design

APPENDIX C: TEMPERATURE IN SHADE BOXES

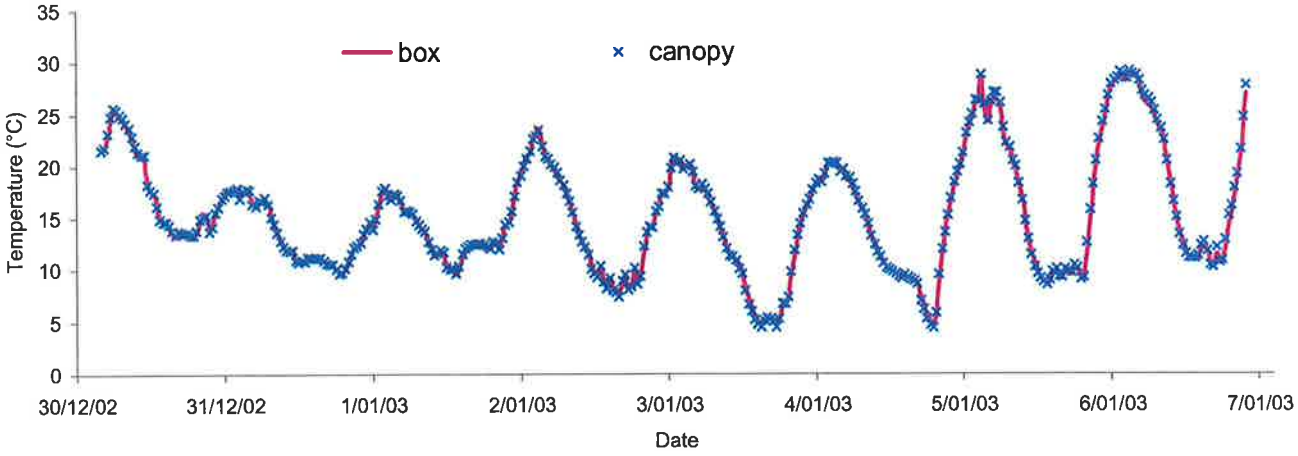

Figure C1: Comparison of temperature between bunches in shade boxes and bunches within the grapevine canopy over one week at Langhorne Creek in the 2003 vintage.

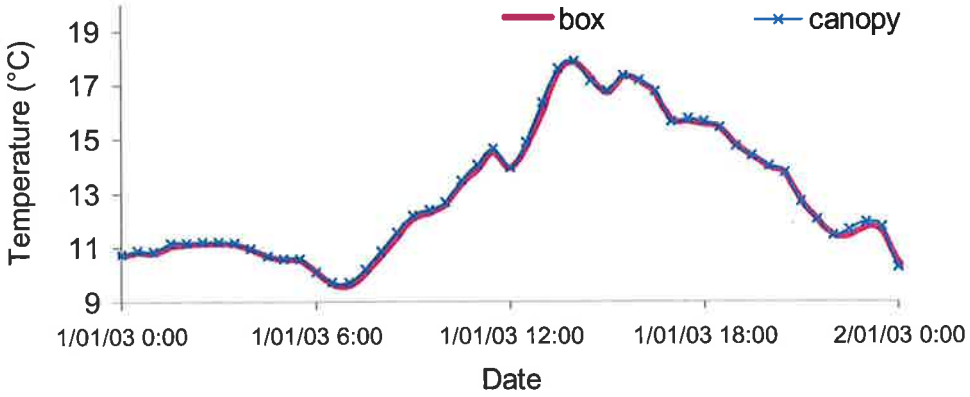

Figure C2: Diurnal changes in the temperature of box-shaded and exposed bunches at Langhorne Creek in the 2003 vintage.

APPENDIX D: TEMPERATURE, PRECIPITATION AND RELATIVE HUMIDITY AT LANGHORNE CREEK, SA.

APPENDIX D1 (2001): Max or Min °C = mean ambient temperature; Rain = precipitation (mm); RH am/pm = relative humidity at 9 am/3 pm

Date	Max°C	Min°C	Rain	RH am	RH pm	Date	Max°C	Min°C	Rain	RH am	RH pm	Date	Max°C	Min°C	Rain	RH am	RH pm	Date	Max°C	Min°C	Rain	RH am	RH pm
1/1/2001	40.2	14.2	0.0	25.0	12.0	1/2/2001	39.1	11.1	0.0	31.0	10.0	1/3/2001	30.3	8.6	0.0	56.0	31.0	1/4/2001	21.1	10.6	0.2	68.0	68.0
2/1/2001	37.1	25.2	0.0	32.0	28.0	2/2/2001	41.2	26.0	0.0	27.0	18.0	2/3/2001	24.6	9.2	0.0	71.0	43.0	2/4/2001	19.7	7.0	0.0	66.0	48.0
3/1/2001	31.0	11.9	0.0	72.0	38.0	3/2/2001	28.8	20.0	0.0	72.0	68.0	3/3/2001	25.4	13.1	0.0	76.0	59.0	3/4/2001	25.9	5.7	0.0	55.0	31.0
4/1/2001	37.1	18.9	0.0	27.0	26.0	4/2/2001	20.0	16.9	0.0	84.0	75.0	4/3/2001	23.3	15.9	0.0	66.0	53.0	4/4/2001	26.6	6.8	0.0	56.0	56.0
5/1/2001	23.4	15.5	0.0	66.0	54.0	5/2/2001	27.1	16.3	0.0	64.0	45.0	5/3/2001	27.7	13.7	0.0	80.0	54.0	5/4/2001	28.9	8.8	0.0	75.0	38.0
6/1/2001	23.2	12.4	0.0	68.0	54.0	6/2/2001	36.7	17.2	0.0	45.0	36.0	6/3/2001	37.4	14.9	0.0	47.0	21.0	6/4/2001	33.2	10.2	0.0	36.0	18.0
7/1/2001	24.4	16.7	0.0	77.0	59.0	7/2/2001	40.0	20.9	0.0	48.0	54.0	7/3/2001	35.9	16.2	0.0	32.0	20.0	7/4/2001	34.2	18.1	0.0	21.0	19.0
8/1/2001	26.0	14.6	0.0	70.0	52.0	8/2/2001	39.9	24.7	17.0	31.0	22.0	8/3/2001	35.7	14.0	0.0	72.0	20.0	8/4/2001	19.8	12.4	0.6	56.0	45.0
9/1/2001	34.4	16.1	0.0	60.0	31.0	9/2/2001	23.0	18.0	0.0	82.0	61.0	9/3/2001	36.5	16.4	0.0	28.0	15.0	9/4/2001	20.4	10.0	0.2	63.0	55.0
10/1/2001	40.2	18.6	0.0	35.0	31.0	10/2/2001	23.8	16.4	0.4	64.0	51.0	10/3/2001	24.3	20.7	0.0	66.0	74.0	10/4/2001	20.8	8.8	0.0	73.0	59.0
11/1/2001	25.3		0.0	63.0	53.0	11/2/2001	31.9	12.6	0.0	74.0	27.0	11/3/2001	20.2	12.4	0.0	61.0	46.0	11/4/2001	19.9	10.9	0.0	71.0	53.0
12/1/2001				73.0	22.0	12/2/2001	29.9	17.4	0.0	72.0	63.0	12/3/2001	19.9	13.7	0.0	82.0	45.0	12/4/2001	19.9	3.8	0.0	49.0	31.0
13/1/2001	43.5	14.7	0.0	49.0	16.0	13/2/2001	23.3	16.3	6.0	79.0	48.0	13/3/2001	20.7	8.7	2.0	63.0	47.0	13/4/2001	24.2	3.9	0.0	43.0	26.0
14/1/2001	36.6	21.4	0.0	32.0	32.0	14/2/2001	21.6	15.5	0.0	81.0	54.0	14/3/2001	26.5	7.8	0.0	70.0	30.0	14/4/2001	26.1	8.8	0.0	24.0	19.0
15/1/2001	25.0	19.3	0.0	69.0	62.0	15/2/2001	22.1	12.8	0.2	70.0	48.0	15/3/2001	33.5	9.0	0.0	27.0	18.0	15/4/2001	28.9	12.5	0.0	24.0	48.0
16/1/2001	26.5	13.1	0.0	56.0	33.0	16/2/2001	24.5	10.9	0.0	66.0	42.0	16/3/2001	27.6	19.9	0.0	70.0	87.0	16/4/2001	22.1	12.8	0.0	80.0	57.0
17/1/2001	24.1	13.1	0.0	66.0	46.0	17/2/2001	35.1	10.2	0.0	51.0	20.0	17/3/2001	20.2	11.6	7.0	67.0	57.0	17/4/2001	27.8	9.0	0.0	63.0	36.0
18/1/2001	23.9	8.8	0.0	59.0	45.0	18/2/2001	40.3	16.8	0.0	26.0	21.0	18/3/2001	22.2	10.2	0.8	85.0	58.0	18/4/2001	32.3	14.9	0.0	25.0	19.0
19/1/2001	37.8	12.4	0.0	58.0	17.0	19/2/2001	39.7	25.6	0.0	19.0	18.0	19/3/2001	23.1	8.5	0.4	90.0	42.0	19/4/2001	18.4	12.9	0.0	55.0	63.0
20/1/2001	38.2	19.8	0.0	22.0	58.0	20/2/2001	40.4	17.6	0.0	25.0	12.0	20/3/2001	27.7	9.2	0.0	43.0	67.0	20/4/2001	16.8	8.1	0.0	59.0	48.0
21/1/2001	27.2	16.4	0.0	75.0	49.0	21/2/2001	27.9	19.5	0.0	81.0	66.0	21/3/2001	15.8	11.1	6.0	67.0	90.0	21/4/2001	17.3	7.1	0.0	55.0	55.0
22/1/2001	32.7	15.9	0.0	57.0	37.0	22/2/2001	23.0	15.0	0.0	64.0	48.0	22/3/2001	18.5	13.0	2.0	89.0	65.0	22/4/2001	16.0	12.0	15.0	92.0	76.0
23/1/2001	39.9	19.3	0.0	48.0	40.0	23/2/2001	23.5	13.9	0.0	78.0	62.0	23/3/2001	25.0	8.9	0.0	87.0	40.0	23/4/2001	17.7	11.9	2.0	71.0	65.0
24/1/2001	38.1	19.7	0.0	79.0	33.0	24/2/2001	22.4	13.9	0.0	72.0	57.0	24/3/2001	21.1	13.6	2.0	76.0	54.0	24/4/2001	18.7	12.1	0.0	56.0	50.0
25/1/2001	36.9	19.7	2.0	68.0	48.0	25/2/2001	28.0	18.2	0.2	79.0	49.0	25/3/2001	20.3	10.2	2.0	62.0	76.0	26/4/2001	22.3	14.5	0.0	69.0	56.0
26/1/2001	26.3	19.9	0.2	85.0	51.0	26/2/2001	24.3	16.1	1.0	77.0	52.0	26/3/2001	21.2	13.7	3.0	73.0	53.0	27/4/2001	18.2	13.0	1.0	69.0	54.0
27/1/2001	28.0	12.9	0.0	65.0	44.0	27/2/2001	24.8	13.5	0.0	78.0	44.0	27/3/2001	19.4	13.2	0.0	65.0	61.0	28/4/2001	16.6	5.4	0.0	70.0	45.0
28/1/2001	24.3	10.9	0.0	56.0	41.0	28/2/2001	24.5	14.7	0.0	60.0	57.0	28/3/2001	19.4	12.6	0.0	60.0	46.0	29/4/2001	17.1	3.4	0.2	73.0	50.0
29/1/2001	24.8	11.8	0.0	63.0	45.0							29/3/2001	18.6	6.7	0.0	56.0	47.0	30/4/2001	20.8	4.9	0.0	73.0	42.0
30/1/2001	23.8	14.3	0.0	61.0	44.0							30/3/2001	23.5	6.0	0.0	67.0	39.0						
31/1/2001	28.5	8.3	0.0	56.0	25.0							31/3/2001	23.8	6.8	0.0	65.0	44.0						
Jan 01	30.9	15.7	2.2	58.4	39.5	**Feb 01**	20.5	16.6	24.8	80.7	43.9	**Mar 01**	24.8	11.9	25.2	85.1	48.5	**Apr 01**	22.5	9.7	19.2	58.3	45.9
1/5/2001	21.6	7.6	0.0	94.0	50.0	1/6/2001	13.9	6.4	0.0	82.0	66.0	1/7/2001	17.1	5.4	0.0	80.0	55.0	1/8/2001	14.2	7.6	7.0	73.0	66.0
2/5/2001	22.9	8.6	0.2	69.0	40.0	2/6/2001	18.5	4.9	0.0	44.0	49.0	2/7/2001	18.4	4.6	0.0	76.0	46.0	2/8/2001	14.1	6.1	0.0	65.0	53.0
3/5/2001	22.3	11.0	0.0	79.0	42.0	3/6/2001	19.7	7.1	0.0	87.0	64.0	3/7/2001	18.1	3.5	0.0	78.0	46.0	3/8/2001	16.1	2.4	0.0	66.0	46.0
4/5/2001	20.7	13.7	0.0	79.0	60.0	4/6/2001	22.7	6.1	0.2	84.0	37.0	4/7/2001	18.6	2.9	0.0	72.0	47.0	4/8/2001	19.4	4.7	0.0	55.0	61.0
5/5/2001	20.2	8.3	0.0	81.0	58.0	5/6/2001	19.7	8.6	0.2	68.0	72.0	5/7/2001	13.4	8.3	7.0	80.0	78.0	5/8/2001	15.3	10.8	5.0	71.0	68.0
6/5/2001	19.0	8.6	0.2	81.0	60.0	6/6/2001	15.5	13.0	14.0	94.0	91.0	6/7/2001	14.2	5.3	0.6	94.0	68.0	6/8/2001	15.0	8.5	0.8	65.0	60.0
7/5/2001	21.9	7.1	0.2	79.0	44.0	7/6/2001	15.9	8.8	6.0	85.0	73.0	7/7/2001	13.6	8.4	2.0	90.0	86.0	7/8/2001	13.1	5.5	0.2	78.0	77.0
8/5/2001	23.8	7.5	0.0	51.0	36.0	8/6/2001	16.0	13.0	3.0	87.0	79.0	8/7/2001	15.9		6.0	93.0	83.0	8/8/2001	15.2	7.9	5.0	75.0	61.0
9/5/2001	17.2	12.9	1.0	92.0	87.0	9/6/2001	16.7	10.3	2.0	77.0	71.0	9/7/2001	13.3	5.4	0.2	96.0	79.0	9/8/2001	19.2	8.2	0.0	54.0	40.0
10/5/2001	16.3	11.7	5.0	81.0	70.0	10/6/2001	15.9	8.1	0.2	95.0	74.0	10/7/2001	13.4	7.4	0.0	96.0	78.0	10/8/2001	20.4	8.4	0.0	40.0	38.0
11/5/2001	19.7	6.9	0.2	93.0	61.0	11/6/2001	16.7	11.0	7.0	93.0	79.0	11/7/2001	11.6	3.6	0.2	91.0	79.0	11/8/2001	20.5	6.7	1.0	64.0	44.0
12/5/2001	19.6	8.8	0.0	90.0	49.0	12/6/2001	16.8	9.0	0.0	93.0	68.0	12/7/2001	13.9	7.5	6.0	96.0	76.0	12/8/2001	17.1	6.6	0.0	75.0	59.0
13/5/2001	25.7	7.1	0.2	74.0	40.0	13/6/2001	16.5	10.8	1.0	86.0	76.0	13/7/2001	16.9	6.2	0.0	82.0	59.0	13/8/2001	18.3	5.7	0.0	91.0	72.0
14/5/2001	26.5	14.0	0.0	77.0	37.0	14/6/2001	14.3	9.2	1.0	73.0	77.0	14/7/2001	15.6	7.0	0.2	78.0	69.0	14/8/2001	20.4	4.8	0.2	78.0	46.0
15/5/2001	18.9	10.8	1.0	57.0	62.0	15/6/2001	13.3	8.6	0.0	81.0	70.0	15/7/2001	15.1	9.1	0.8	78.0	60.0	15/8/2001	22.6	11.7	0.0	29.0	33.0
16/5/2001	16.7	11.3	4.0	74.0	75.0	16/6/2001	12.8	9.3	0.0	82.0	70.0	16/7/2001	14.3	8.1	1.0	77.0	58.0	16/8/2001	22.1	15.2	0.0	49.0	66.0
17/5/2001	13.3	9.0	8.0	86.0	90.0	17/6/2001	13.0	4.9	0.0	81.0	64.0	17/7/2001	15.0	6.8	0.2	81.0	80.0	17/8/2001	14.4	7.8	8.0	68.0	76.0
18/5/2001	14.0	9.8	21.0	90.0	63.0	18/6/2001	14.2	1.4	0.2	90.0	62.0	18/7/2001	14.4	4.5	0.0	86.0	71.0	18/8/2001	14.1	9.0	5.0	75.0	73.0
19/5/2001	13.7	9.1	0.6	87.0	67.0	19/6/2001	16.1	3.5	0.0	76.0	75.0	19/7/2001	13.6	5.7	1.0	89.0	91.0	19/8/2001	16.1	9.5	0.0	73.0	81.0
20/5/2001	15.5	10.6	0.0	70.0	64.0	20/6/2001	16.8	8.2	0.0	77.0	74.0	20/7/2001	14.2	8.3	3.0	76.0	75.0	20/8/2001	14.8	10.8	2.0	65.0	76.0
21/5/2001	17.4	6.9	0.2	74.0	69.0	21/6/2001	16.6	12.5	0.2	81.0	75.0	21/7/2001	14.6	4.1	0.0	94.0	55.0	21/8/2001	15.2	7.6	0.2	65.0	83.0
22/5/2001	16.4	8.5	0.0	89.0	63.0	22/6/2001	15.3	11.9	2.0	77.0	68.0	22/7/2001	16.0	1.8	0.0	85.0	66.0	22/8/2001	14.8	9.1	3.0	68.0	73.0
23/5/2001	16.8	4.8	0.0	85.0	53.0	23/6/2001	14.5	10.6	0.0	74.0	73.0	23/7/2001	14.0	7.3	0.2	92.0	62.0	23/8/2001	15.0	8.8	0.2	77.0	70.0
24/5/2001	18.7	5.2	0.2	77.0	55.0	24/6/2001	17.7	8.7	0.2	74.0	68.0	24/7/2001	13.6	3.5	0.0	86.0	79.0	24/8/2001	19.6	3.4	0.2	66.0	44.0
25/5/2001	19.5	5.9	0.0	65.0	49.0	25/6/2001	17.3	11.1	0.0	78.0	69.0	25/7/2001	15.0	3.2	0.2	95.0	69.0	25/8/2001	17.0	11.2	2.0	77.0	82.0
26/5/2001	16.8	10.2	2.0	83.0	64.0	26/6/2001	14.7	10.3	0.6	78.0	62.0	26/7/2001	16.3	5.4	0.0	90.0	50.0	26/8/2001	12.6	6.6	11.0	88.0	76.0
27/5/2001	15.9	9.2	10.0	77.0	79.0	27/6/2001	14.8	4.5	0.0	81.0	71.0	27/7/2001	14.8	8.5	0.0	87.0	69.0	27/8/2001	13.9	7.1	13.0	73.0	70.0
28/5/2001	15.1	8.4	11.0	84.0	63.0	28/6/2001	17.1	6.7	0.0	71.0	54.0	28/7/2001	13.8	9.7	0.0	70.0	66.0	28/8/2001	15.8	6.6	2.0	88.0	74.0
29/5/2001		7.6	2.0	69.0	61.0	29/6/2001	16.5	5.5	0.0	75.0	50.0	29/7/2001	13.3	3.7	0.2	90.0	75.0	29/8/2001	16.1	8.1	9.0	77.0	67.0
30/5/2001	14.0	9.1	0.0	88.0	67.0	30/6/2001	15.6	4.1	0.0	75.0	58.0	30/7/2001	19.1	2.2	0.2	92.0	49.0	30/8/2001	17.6	5.8	0.4	81.0	55.0
31/5/2001	14.8	4.9	0.0	88.0	54.0							31/7/2001	20.3	7.4	0.0	46.0	48.0	31/8/2001	16.8	3.6	0.0	74.0	60.0
May 01	16.5	8.9	67.0	78.8	59.1	**Jun 01**	16.2	8.3	37.8	80.0	68.0	**Jul 01**	15.2	5.8	29.0	84.4	66.9	**Aug 01**	16.7	7.6	75.2	69.1	63.0
1/9/2001	14.6	8.1	6.0	90.0	78.0	1/10/2001	16.8	12.0	4.0	78.0	71.0	1/11/2001	22.0	7.7	0.0	78.0	61.0	1/12/2001	21.9	5.8	0.0	49.0	41.0
2/9/2001	16.1	9.9	9.0	89.0	84.0	2/10/2001	15.9	5.3	0.8	61.0	51.0	2/11/2001	19.0	12.2	0.0	65.0	57.0	2/12/2001	29.3	13.1	6.0	75.0	49.0
3/9/2001	15.1	5.5	1.0	79.0	81.0	3/10/2001	16.6	8.4	0.4	69.0	89.0	3/11/2001	26.8	8.8	0.0	67.0	32.0	3/12/2001	15.5	13.0	4.0	94.0	78.0
4/9/2001	14.0	8.0	2.0	85.0	75.0	4/10/2001	15.9	8.3	2.0	68.0	63.0	4/11/2001	29.5	11.0	0.0	60.0	42.0	4/12/2001	20.3	12.5	4.0	87.0	65.0
5/9/2001	16.1	2.6	2.0	69.0	55.0	5/10/2001	18.6	4.3	0.2	80.0	56.0	5/11/2001	21.2	16.7	0.6	87.0	94.0	5/12/2001	24.2	7.2	0.2	67.0	43.0
6/9/2001	21.6	4.8	0.0	66.0	57.0	6/10/2001	14.2	9.9	3.0	68.0	77.0	6/11/2001	17.5	12.2	16.0	78.0	56.0	6/12/2001	24.6	13.8	8.0	96.0	63.0
7/9/2001	21.7	11.2	7.0	55.0	67.0	7/10/2001	15.7	6.5	4.0	81.0	70.0	7/11/2001	17.4	8.2	0.0	50.0	48.0	7/12/2001	17.1	11.5	5.0	94.0	66.0
8/9/2001	13.6	7.2	27.0	78.0	85.0	8/10/2001	15.7	8.5	4.0	79.0	69.0	8/11/2001	24.4	9.9	0.0	69.0	38.0	8/12/2001	18.6	9.7	5.0	61.0	47.0
9/9/2001	17.2	9.7	1.0	68.0	59.0	9/10/2001	21.9	3.8	0.2	62.0	45.0	9/11/2001	18.5	9.3	0.0	69.0	55.0	9/12/2001	19.8	8.4	0.0	63.0	51.0
10/9/2001	21.5	5.4	0.2	74.0	48.0	10/10/2001	22.4	9.2	0.0	41.0	48.0	10/11/2001	16.1	9.5	0.0	59.0	70.0	10/12/2001	18.6	10.4	0.0	48.0	57.0
11/9/2001	18.6	11.7	0.0	75.0	75.0	11/10/2001	16.5	7.0	2.0	60.0	55.0	11/11/2001	15.3	6.6	4.0	72.0	51.0	11/12/2001	17.1	11.1	1.0	91.0	60.0
12/9/2001	17.3	11.7	0.6	87.0	74.0	12/10/2001	16.3	10.0	3.0	78.0	76.0	12/11/2001	16.4	8.2	5.0	82.0	84.0	12/12/2001	20.2	12.6	0.2	66.0	51.0
13/9/2001	16.4	6.6	0.2	74.0	63.0	13/10/2001	17.1	9.0	0.6	75.0	64.0	13/11/2001	18.7	11.0	5.0	84.0	60.0	13/12/2001	21.8	11.9	0.0	68.0	55.0
14/9/2001	17.3	7.0	0.2	75.0	63.0	14/10/2001	17.1	7.6	2.0	57.0	53.0	14/11/2001	22.3	9.5	0.6	69.0	46.0	14/12/2001	19.4	11.5	0.0	72.0	59.0
15/9/2001	21.0	7.2	0.2	64.0	48.0	15/10/2001	20.8	6.7	0.2	55.0	47.0	15/11/2001	26.2	7.4	0.0	59.0	67.0	15/12/2001	21.0	12.4	0.0	63.0	51.0
16/9/2001	19.5	9.8	0.0	73.0	79.0	16/10/2001	20.3	8.9	0.0	79.0	67.0	16/11/2001	31.1	7.4	0.0	77.0	26.0	16/12/2001	18.9	12.5	0.0	68.0	55.0
17/9/2001	17.3	6.1	1.0	71.0	56.0	17/10/2001	19.0	8.8	0.4	87.0	65.0	17/11/2001	30.2	14.5	0.0	35.0	46.0	17/12/2001	21.1	7.9	0.0	60.0	47.0
18/9/2001	23.7	2.4	0.0	67.0	41.0	18/10/2001	14.8	10.3	1.0	66.0	63.0	18/11/2001	18.4	11.0	5.0	80.0	58.0	18/12/2001	24.7	7.6	0.0	53.0	37.0
19/9/2001	27.2	9.1	0.0	47.0	40.0	19/10/2001	15.5	8.1	3.0	73.0	59.0	19/11/2001	17.9	9.0	0.2	51.0	49.0	19/12/2001	33.6	9.4	0.0	43.0	21.0
20/9/2001	17.7	13.4	0.0	80.0	66.0	20/10/2001	20.1	6.5	0.0	55.0	42.0	20/11/2001	24.2	9.2	0.0	72.0	32.0	20/12/2001	21.3	15.4	0.0	78.0	57.0
21/9/2001	21.1	7.2	0.0	95.0	65.0	21/10/2001	25.9	4.0	0.0	32.0	25.0	21/11/2001	26.7	8.0	0.0	67.0	33.0	21/12/2001	22.9	10.0	0.0	67.0	55.0
22/9/2001	20.0	9.0	0.2	88.0	79.0	22/10/2001	21.3	12.0	0.0	35.0	74.0	22/11/2001	32.2	12.9	0.0	40.0	23.0	22/12/2001	24.5	10.5	0.0	57.0	45.0
23/9/2001	29.2	11.6	0.0	51.0	35.0	23/10/2001	23.1	14.6	3.0	74.0	43.0	23/11/2001	22.2	13.1	12.0	89.0	51.0	23/12/2001	24.0	13.2	0.0	65.0	50.0
24/9/2001	21.1	11.2	14.0	84.0	74.0	24/10/2001	17.9	12.4	0.6	83.0	67.0	24/11/2001	20.8	14.3	0.0	66.0	49.0	24/12/2001	21.4	10.1	0.0	52.0	46.0
25/9/2001	14.5	9.3	7.0	78.0	67.0	25/10/2001	17.6	10.9	5.0	61.0	65.0	25/11/2001	20.0	13.3	0.0	64.0	64.0	25/12/2001	21.7	13.4	0.6	60.0	30.0
26/9/2001	16.5	7.5	1.0	58.0	54.0	26/10/2001	17.0	8.7	0.2	70.0	62.0	26/11/2001	18.4	11.1	0.6	71.0	75.0	26/12/2001	19.4	11.0	5.0	57.0	47.0
27/9/2001	19.6	2.7	0.0	76.0	57.0	27/10/2001	19.9	7.6	2.0	56.0	52.0	27/11/2001	19.8	12.1	1.0	79.0	58.0	27/12/2001	23.0	11.8	0.0	65.0	48.0
28/9/2001	25.3	5.8	0.0	54.0	36.0	28/10/2001	17.9	9.1	0.0	60.0	46.0	28/11/2001	24.1	10.8	0.2	64.0	46.0	28/12/2001	28.9	9.6	0.0	66.0	25.0
29/9/2001	27.8	10.6	0.0	40.0	50.0	29/10/2001	18.5	8.6	0.0	62.0	49.0	29/11/2001	21.2	10.3	0.0	62.0	50.0	29/12/2001	32.6	9.9	0.0	33.0	57.0
30/9/2001	22.1	12.1	0.0	66.0	66.0	30/10/2001	19.0	12.0	0.0	73.0	61.0	30/11/2001	19.2	11.8	0.0	63.0	53.0	30/12/2001	21.9	12.0	0.0	48.0	36.0
						31/10/2001	19.5	12.2	0.2	81.0	69.0							31/12/2001	21.1	9.6	0.0	66.0	51.0
Sep 01	19.5	8.1	79.6	71.2	62.6	**Oct 01**	18.3	8.7	41.8	66.4	60.1	**Nov 01**	21.9	10.6	50.2	67.5	52.5	**Dec 01**	22.3	10.0	39.0	65.6	49.8

APPENDIX D: TEMPERATURE, PRECIPITATION AND RELATIVE HUMIDITY AT LANGHORNE CREEK, SA.

APPENDIX D2 (2002): Max or Min °C = mean ambient temperature; Rain = precipitation (mm); RH am/pm = relative humidity at 9 am/3 pm

Date	Max°C	Min°C	Rain	RH am	RH pm	Date	Max°C	Min°C	Rain	RH am	RH pm	Date	Max°C	Min°C	Rain	RH am	RH pm	Date	Max°C	Min°C	Rain	RH am	RH pm
1/1/2002	20.9	13.4	0.8	86.0	47.0	1/2/2002	26.0	13.5	0.0	78.0	54.0	1/3/2002	19.2	12.7	2.0	76.0	64.0	1/4/2002	31.9	9.6	0.0	44.0	23.0
2/1/2002	20.6	11.0	1.0	60.0	41.0	2/2/2002	19.1	15.8	0.0	74.0	65.0	2/3/2002	21.7	12.6	0.0	67.0	62.0	2/4/2002	27.3		0.0	33.0	74.0
3/1/2002	21.7	11.4	0.2	63.0	46.0	3/2/2002	19.7	12.7	0.4	52.0	46.0	3/3/2002	20.1	12.3	0.0	71.0	70.0	3/4/2002				74.0	66.0
4/1/2002	22.9	11.2	0.0	63.0	46.0	4/2/2002	21.3	11.4	0.0	52.0	49.0	4/3/2002	27.7	8.8	0.0	80.0	42.0	4/4/2002	17.7	12.5	0.4	46.0	59.0
5/1/2002	34.0	7.6	0.0	48.0	20.0	5/2/2002	23.3	8.0	0.0	73.0	50.0	5/3/2002	33.9	10.5	0.2	48.0	25.0	5/4/2002	19.5	4.9	0.2	45.0	77.0
6/1/2002	29.3	17.9	0.0	32.0	87.0	6/2/2002	29.3	10.5	0.0	64.0	47.0	6/3/2002	25.5	11.8	0.0	51.0	55.0	6/4/2002	20.1	8.9	0.0	59.0	
7/1/2002	21.0	12.2	1.0	56.0	51.0	7/2/2002	20.4	12.0	0.0	79.0	66.0	7/3/2002	21.4	12.0	0.0	79.0	58.0	7/4/2002	19.1	8.2	0.0	58.0	37.0
8/1/2002	19.8	12.7	0.2	70.0	58.0	8/2/2002	18.5	12.9	1.0	72.0	85.0	8/3/2002	20.5	10.7	0.0	58.0	43.0	8/4/2002	27.8	13.3	1.0	32.0	60.0
9/1/2002	30.4	6.9	0.2	58.0	20.0	9/2/2002	21.6	13.4	0.4	82.0	62.0	9/3/2002	27.6	6.9	0.0	75.0	33.0	9/4/2002	31.0	8.5	0.0	23.0	78.0
10/1/2002	39.8	12.1	0.0	21.0	11.0	10/2/2002	19.9	12.3	0.4	72.0	48.0	10/3/2002	34.3	10.4	0.0	50.0	20.0	10/4/2002	23.6	12.1	0.2	66.0	93.0
11/1/2002	28.0	16.7	0.0	62.0	51.0	11/2/2002	21.6	5.3	0.0	67.0	42.0	11/3/2002	22.4	14.1	0.0	66.0	42.0	11/4/2002	27.7	11.0	0.0	45.0	94.0
12/1/2002	20.1	10.2	0.0	61.0	46.0	12/2/2002	29.5	7.5	0.0	66.0	27.0	12/3/2002	21.2	8.6	0.0	66.0	51.0	12/4/2002	27.4	13.8	0.0	67.0	94.0
13/1/2002	21.4	13.9	0.0	66.0	52.0	13/2/2002	36.0	10.9	0.0	35.0	15.0	13/3/2002	21.8	9.1	0.0	78.0	50.0	13/4/2002	18.5	14.0	5.0	78.0	74.0
14/1/2002	23.4	14.1	0.0	68.0	47.0	14/2/2002	39.2	14.6	0.0	39.0	11.0	14/3/2002	22.2	8.6	0.0	78.0	54.0	14/4/2002	18.9	13.7	0.2	50.0	75.0
15/1/2002	24.6	11.2	0.0	65.0	49.0	15/2/2002	33.4	22.0	0.0	38.0	48.0	15/3/2002	27.2	7.7	0.0	77.0	27.0	15/4/2002	21.2	6.0	0.0	40.0	76.0
16/1/2002	24.6	11.9	0.0	65.0	47.0	16/2/2002	22.8	12.7	0.0	62.0	42.0	16/3/2002	31.4	10.3	0.0	52.0	16.0	16/4/2002	20.0	6.9	0.0	59.0	69.0
17/1/2002	23.0	12.7	0.0	64.0	45.0	17/2/2002	24.9	9.7	0.0	67.0	40.0	17/3/2002	35.8	13.6	0.0	49.0	23.0	17/4/2002	22.4	7.0	0.0	44.0	71.0
18/1/2002	32.6	9.2	0.0	58.0	23.0	18/2/2002	29.8	12.2	0.0	69.0	32.0	18/3/2002	30.3	19.4	0.0	15.0	63.0	18/4/2002	26.6	9.1	0.0	25.0	51.0
19/1/2002	39.0	14.2	0.0	34.0	15.0	19/2/2002	38.2	13.2	0.0	31.0	25.0	19/3/2002	24.7	14.2	0.0	69.0	41.0	19/4/2002	30.0	15.0	0.0	28.0	36.0
20/1/2002	34.5	21.4	0.0	66.0	31.0	20/2/2002	27.7	15.7	0.0	57.0	39.0	20/3/2002	23.0	13.7	0.0	71.0	51.0	20/4/2002	27.9	15.7	0.0	20.0	67.0
21/1/2002	21.2	15.5	14.0	90.0	71.0	21/2/2002	22.6	11.4	0.0	51.0	50.0	21/3/2002	22.5	11.0	0.0	78.0	55.0	21/4/2002	24.0	9.5	0.0	36.0	57.0
22/1/2002	22.0	15.0	2.0	69.0	57.0	22/2/2002	21.2	13.6	0.0	65.0	55.0	22/3/2002	27.8	10.7	0.0	81.0	29.0	22/4/2002	26.2	11.1	0.0	31.0	53.0
23/1/2002	24.3	9.3	0.0	65.0	38.0	23/2/2002	23.6	14.9	0.0	74.0	56.0	23/3/2002	32.8	7.2	0.0	48.0	11.0	23/4/2002	30.7	10.4	0.0	25.0	43.0
24/1/2002		11.7	0.0	74.0	37.0	24/2/2002	30.0	14.3	0.0	74.0	39.0	24/3/2002	35.0	13.5	0.0	39.0	19.0	24/4/2002	24.8	20.5	0.0	69.0	74.0
25/1/2002	35.9	11.9	0.0	57.0	18.0	25/2/2002	34.7	11.9	0.0	44.0	46.0	25/3/2002	21.9	11.8	0.0	70.0	54.0	25/4/2002	18.9	13.8	2.0	52.0	67.0
26/1/2002	33.8	15.3	0.0	33.0	29.0	26/2/2002	23.3	12.5	0.0	73.0	57.0	26/3/2002	18.9	12.0	20.0	85.0	64.0	26/4/2002	17.6	9.9	0.0	58.0	75.0
27/1/2002	25.3	19.4	0.6	74.0	69.0	27/2/2002	21.0	13.0	0.0	59.0	47.0	27/3/2002	19.1	14.1	1.0	86.0	66.0	27/4/2002	18.9	5.3	0.0	43.0	73.0
28/1/2002	22.2	10.5	0.2	63.0	59.0	28/2/2002	20.2	11.6	0.0	65.0	53.0	28/3/2002	18.3	15.0	0.0	88.0	80.0	28/4/2002	20.1	4.1	0.0	39.0	72.0
29/1/2002	23.4	14.9	0.2	85.0	64.0							29/3/2002	19.2	14.6	0.0	76.0	70.0	29/4/2002	22.0	5.1	0.0	32.0	67.0
30/1/2002	24.6	10.9	0.0	75.0	60.0							30/3/2002	18.9	13.0	0.0	66.0	56.0	30/4/2002	22.5	5.6	0.0	31.0	78.0
31/1/2002	24.1	10.9	0.0	69.0	54.0							31/3/2002	26.5	7.6	0.0	67.0	33.0						
Jan 02	26.3	12.8	20.4	62.6	44.8	Feb 02	25.7	12.5	2.2	61.9	46.3	Mas 02	24.9	11.6	23.2	66.5	46.0	Apr 02	23.7	10.2	9.0	45.3	66.7
1/5/2002	23.8	8.5	0.0	41.0	72.0	1/6/2002	15.2	6.1	3.0	72.0	86.0	1/7/2002	15.6	6.4	0.0	53.0	36.0	1/8/2002	14.5	9.0	0.0	83.0	76.0
2/5/2002	26.8	8.0	0.0	35.0	60.0	2/6/2002	17.7	3.3	0.2	40.0	82.0	2/7/2002	16.0	2.6	0.0	37.0	54.0	2/8/2002	14.7	3.0	0.2	78.0	54.0
3/5/2002	28.5	9.0	0.0	26.0	38.0	3/6/2002	18.7	3.9	0.0	33.0	72.0	3/7/2002	13.8	7.2	9.0	86.0	81.0	3/8/2002	13.2	7.9	6.0	75.0	76.0
4/5/2002	28.5	9.5	0.0	23.0	27.0	4/6/2002	19.4	5.1	0.0	30.0	72.0	4/7/2002	15.8	8.9	2.0	77.0	72.0	4/8/2002	15.1	6.6	1.0	73.0	54.0
5/5/2002	26.3	12.3	0.0	29.0	37.0	5/6/2002	20.1	6.7	0.0	41.0	46.0	5/7/2002	15.5	12.3	3.0	79.0	66.0	5/8/2002	17.4	5.8	0.0	42.0	40.0
6/5/2002	28.4	10.4	0.0	23.0	27.0	6/6/2002	20.3	9.1	0.0	46.0	53.0	6/7/2002	15.2	10.4	0.6	84.0	77.0	6/8/2002	16.8	10.3	6.0	81.0	81.0
7/5/2002	29.0	11.1	0.0	21.0	24.0	7/6/2002	17.8	11.2	0.0	59.0	74.0	7/7/2002	15.1	6.4	0.0	90.0	62.0	7/8/2002	16.8	12.7	0.2	82.0	78.0
8/5/2002	29.0	16.7	0.0	21.0	91.0	8/6/2002	15.2	9.9	14.0	82.0	71.0	8/7/2002	18.6	10.9	0.0	41.0	38.0	8/8/2002	17.8	6.7	0.0	80.0	60.0
9/5/2002		15.6	5.0	68.0	85.0	9/6/2002	16.9	10.3	3.0	82.0	79.0	9/7/2002	16.8	11.5	3.0	76.0	58.0	9/8/2002	20.1	4.8	0.0	76.0	43.0
10/5/2002	21.2	10.0	0.0	64.0	75.0	10/6/2002	16.9	12.9	0.2	81.0	73.0	10/7/2002	14.1	4.2	0.0	74.0	45.0	10/8/2002	23.3	7.1	0.0	35.0	29.0
11/5/2002	25.8	7.9	0.0	28.0	31.0	11/6/2002	15.7	12.5	0.0	81.0	72.0	11/7/2002	19.4	2.4	0.0	44.0	32.0	11/8/2002	20.2	11.4	3.0	83.0	81.0
12/5/2002	22.8	11.9	0.0	54.0	67.0	12/6/2002	18.2	13.2	0.2	76.0	81.0	12/7/2002	18.9	5.6	0.8	100.0	40.0	12/8/2002	13.1	7.5	9.0	73.0	76.0
13/5/2002		11.5	0.0	66.0	85.0	13/6/2002	15.3	10.9	20.0	74.0	62.0	13/7/2002	19.4	9.1	2.0	91.0	74.0	13/8/2002	13.5	6.8	2.0	86.0	79.0
14/5/2002	19.8	6.3	0.2	41.0	78.0	14/6/2002	16.8	9.7	3.0	85.0	73.0	14/7/2002	14.8	7.1	3.0	78.0	73.0	14/8/2002	13.1	3.6	1.0	97.0	69.0
15/5/2002	24.3	9.1	0.8	77.0	76.0	17/6/2002	15.4	6.9	0.4		77.0	15/7/2002	15.3	7.0	0.0	72.0	49.0	15/8/2002	14.5	1.7	0.2	90.0	54.0
16/5/2002	18.0	13.2	2.0	45.0	50.0	18/6/2002	13.3	3.6	0.0	92.0	50.0	16/7/2002	15.9	9.1	0.2	82.0	84.0	16/8/2002	16.2	1.0	0.0	85.0	46.0
17/5/2002	17.4	10.3	0.0	42.0	82.0	19/6/2002	15.2	0.6	0.0	70.0	56.0	17/7/2002	15.4	9.5	0.4	82.0	75.0	17/8/2002	13.7	1.7	0.0	88.0	64.0
18/5/2002	15.2	8.4	0.6	60.0	70.0	20/6/2002	15.7	9.3	0.0	94.0	56.0	18/7/2002	17.1	8.7	0.0	91.0	66.0	18/8/2002	14.9	2.1	0.0	79.0	45.0
19/5/2002	12.7	7.7	18.0	82.0	78.0	21/6/2002	15.9	6.9	0.2	79.0	77.0	19/7/2002	16.8	7.2	0.0	49.0	46.0	19/8/2002	15.6	4.6	0.0	81.0	56.0
20/5/2002	15.8	8.6	19.0	80.0	66.0	22/6/2002	15.8	9.4	0.2	81.0	73.0	20/7/2002	15.7	10.2	0.0	73.0	64.0	20/8/2002	15.2	7.1	0.4	91.0	69.0
21/5/2002	14.3	11.2	0.0	72.0	92.0	23/6/2002	15.2	7.1	0.0	92.0	68.0	21/7/2002	15.6	6.3	0.0	69.0	55.0	21/8/2002	17.0	3.0	0.0	66.0	45.0
22/5/2002	15.5	10.0	1.0	68.0	98.0	24/6/2002	16.8	4.7	0.0	69.0	53.0	22/7/2002	15.4	5.9	0.0	71.0	71.0	22/8/2002	13.9	4.6	0.0	81.0	69.0
23/5/2002		10.5	0.0	77.0	83.0	25/6/2002	16.8	9.5	0.0	80.0	54.0	23/7/2002	17.5	9.2	0.0	49.0	63.0	23/8/2002	12.7	1.2	2.0	75.0	44.0
28/5/2002	15.1	2.6	0.2	59.0	89.0	26/6/2002	15.7		0.0	77.0	64.0	24/7/2002	14.8	10.1	0.0	83.0	57.0	24/8/2002	13.1	-0.7	0.0	80.0	49.0
29/5/2002	16.3	2.6	0.0	47.0	66.0	27/6/2002	13.7	10.3	2.0	85.0	67.0	25/7/2002	16.1	3.6	0.0	67.0	68.0	25/8/2002	16.5	-0.1	0.0	81.0	31.0
30/5/2002	16.7	4.6	0.0	69.0	93.0	28/6/2002	12.2	6.9	7.0	72.0	69.0	26/7/2002	16.7	8.3	0.4	86.0	64.0	26/8/2002	20.9	1.7	0.0	65.0	29.0
31/5/2002	15.3	8.5	4.0	69.0	85.0	29/6/2002	12.1	5.3	0.0	74.0	60.0	27/7/2002	22.5	4.1	0.0	66.0	39.0	27/8/2002	22.2	6.8	0.0	33.0	24.0
						30/6/2002	12.7	0.5	0.2	83.0	57.0	28/7/2002	13.6	10.2	5.0	95.0	92.0	28/8/2002	17.7	11.2	0.0	77.0	59.0
												29/7/2002	13.7	6.7	12.0	79.0	75.0	29/8/2002	15.1	5.2	0.2	80.0	64.0
												30/7/2002	15.7	4.0	0.2	91.0	65.0	30/8/2002	17.7	8.4	0.0	61.0	46.0
												31/7/2002	19.1	4.9	0.0	72.0	48.0	31/8/2002	18.6	6.8	3.0	80.0	63.0
May 02	21.1	9.4	50.8	50.6	68.3	Jun 02	16.1	7.6	53.6	71.5	67.0	Jul 02	16.3	7.4	41.6	73.6	61.6	Aug 02	18.3	5.5	34.2	75.4	57.3
1/9/2002	20.2	5.5	0.0	84.0	39.0	1/10/2002	25.0	3.1	0.0	29.0	20.0	1/11/2002	31.4	4.5	0.0	66.0	17.0	1/12/2002	30.1	7.3	0.0	61.0	21.0
2/9/2002	23.6	9.7	0.0	29.0	45.0	2/10/2002	28.4	9.5	0.0	18.0	21.0	2/11/2002	26.6	18.3	0.4	32.0	49.0	2/12/2002	30.7	17.1	0.0	40.0	47.0
3/9/2002	17.6	10.9	8.0	77.0	86.0	3/10/2002	18.4	13.2	0.0	80.0	72.0	3/11/2002	20.7	10.6	0.2	55.0	47.0	3/12/2002	21.3	8.8	0.0	53.0	61.0
4/9/2002	16.4	10.6	1.0	77.0	63.0	4/10/2002	12.8	9.2	4.0	92.0	92.0	4/11/2002	18.4	9.1	0.0	49.0	43.0	4/12/2002	18.4	12.2	0.2	64.0	55.0
5/9/2002	21.4	4.9	0.4	60.0	34.0	5/10/2002	15.1	11.2	1.0	88.0	80.0	5/11/2002	23.6	5.6	0.0	58.0	25.0	5/12/2002	18.0	13.7	0.4	90.0	48.0
6/9/2002	16.3	7.8	0.4	68.0	63.0	6/10/2002	23.1	6.3	0.0	75.0	32.0	6/11/2002	31.0	5.8	0.0	27.0	16.0	6/12/2002	18.5	11.3	1.0	59.0	40.0
7/9/2002	16.0	11.3	1.0	73.0	71.0	7/10/2002	19.9	12.9	0.2	63.0	72.0	7/11/2002	33.6	14.0	0.0	16.0	14.0	7/12/2002	26.4	6.1	0.0	59.0	23.0
8/9/2002	17.4	7.5	0.4	80.0	68.0	8/10/2002	15.1	7.5	8.0	51.0	46.0	8/11/2002	29.3	9.9	0.0	41.0	33.0	8/12/2002	22.9	10.2	0.0	43.0	55.0
9/9/2002	14.5	9.3	0.0	69.0	59.0	9/10/2002	15.2	5.8	0.2	70.0	62.0	9/11/2002	25.2	8.4	0.0	74.0	52.0	9/12/2002	17.2	8.4	0.4	62.0	62.0
10/9/2002	14.1	5.4	1.0	77.0	66.0	10/10/2002	17.3	4.0	0.0	67.0	42.0	10/11/2002	24.0	13.2	0.0	73.0	40.0	10/12/2002	18.7	10.0	0.0	47.0	46.0
11/9/2002	17.0	2.2	0.4	68.0	31.0	11/10/2002	23.5	3.4	0.0	64.0	26.0	11/11/2002	37.5	9.2	0.0	34.0	18.0	11/12/2002	20.0	13.1	0.0	63.0	51.0
12/9/2002	21.5	1.3	0.0	33.0	24.0	12/10/2002	27.2	6.9	0.0	25.0	30.0	12/11/2002	32.7	20.5	0.4	82.0	72.0	12/12/2002	18.9	13.2	0.0	74.0	53.0
13/9/2002	22.5	6.6	0.0	41.0	29.0	13/10/2002	17.5	9.1	0.0	61.0	43.0	13/11/2002	19.1	8.1	0.2	48.0	41.0	13/12/2002	21.1	13.3	0.0	59.0	43.0
14/9/2002	27.5	9.2	0.0	29.0	21.0	14/10/2002	19.1	9.4	0.0	74.0	60.0	14/11/2002	20.5	6.3	0.0	64.0	50.0	14/12/2002	32.5	7.3	0.0	61.0	23.0
15/9/2002	27.3	12.0	0.0	30.0	29.0	15/10/2002	25.2	6.9	0.0	56.0	36.0	15/11/2002	23.6	12.8	0.0	59.0	31.0	15/12/2002	36.3	13.7	0.0	30.0	20.0
16/9/2002	16.1	12.0	2.0	81.0	61.0	16/10/2002	22.4	8.5	0.0	68.0	45.0	16/11/2002		7.6	0.0	30.0	15.0	16/12/2002	33.5	14.6	0.0	73.0	33.0
17/9/2002	15.1	5.9	0.8	64.0	67.0	17/10/2002	20.3	13.1	0.0	70.0	47.0	17/11/2002	36.8	17.7	0.0	16.0	14.0	17/12/2002	34.1	17.3	0.0	66.0	39.0
18/9/2002	16.9	11.6	0.0	80.0	77.0	18/10/2002	31.2	7.6	0.0	27.0	58.0	18/11/2002	21.3		0.0	70.0	52.0	18/12/2002	28.9	16.0	0.0	85.0	41.0
19/9/2002	16.6	11.1	0.0	72.0	67.0	19/10/2002	19.8	12.7	0.0	52.0	62.0	19/11/2002	20.4	12.3	0.0	75.0	60.0	19/12/2002	34.2	16.6	0.0	72.0	42.0
20/9/2002	22.3	5.5	0.0	68.0	37.0	20/10/2002	19.2	7.8	0.0	66.0	56.0	20/11/2002	18.9	13.4	0.2	83.0	68.0	20/12/2002	29.9	14.6	0.0	75.0	30.0
21/9/2002	20.5	7.6	0.0	82.0	67.0	21/10/2002	22.0	5.6	0.0	61.0	43.0	21/11/2002	26.2	4.5	0.2	57.0	29.0	21/12/2002	36.5	12.6	0.0	72.0	27.0
22/9/2002	16.6	11.3	0.0	68.0	68.0	22/10/2002	28.6		0.0	23.0	65.0	22/11/2002	25.0	6.1	0.0	71.0	31.0	22/12/2002	21.2	16.1	0.0	74.0	49.0
23/9/2002	22.3	10.5	0.0	73.0	45.0	23/10/2002	17.3	9.3	1.8	69.0	47.0	23/11/2002	37.7	11.8	0.0	23.0	18.0	23/12/2002	19.9	11.6	0.0	49.0	46.0
24/9/2002	18.3	7.4	0.0	68.0	59.0	24/10/2002	18.4	9.4	0.4	57.0	51.0	24/11/2002	26.3	19.6	0.4	75.0	71.0	24/12/2002	21.1	12.3	0.0	65.0	53.0
25/9/2002	17.7	8.2	0.0	70.0	64.0	25/10/2002	18.0	6.4	3.0	76.0	56.0	25/11/2002	21.5	14.8	5.0			25/12/2002	22.6	13.3	0.0	65.0	53.0
26/9/2002	17.8	3.3	0.2	60.0	38.0	26/10/2002	16.9	8.4	0.2	45.0	44.0	26/11/2002	17.8	12.2	21.0	97.0	64.0	26/12/2002	25.0	10.7	0.0	72.0	48.0
27/9/2002	18.6	3.9	0.0	39.0	54.0	27/10/2002	18.5	6.2	0.0	69.0	51.0	27/11/2002	18.4	12.0	0.6	71.0	73.0	27/12/2002	26.3	11.8	0.0	71.0	36.0
28/9/2002	14.3	5.3	4.0	87.0	57.0	28/10/2002	25.8	5.4	0.0	74.0	27.0	28/11/2002	25.4	14.2	0.0	79.0	50.0	28/12/2002	38.1	12.2	0.0	30.0	15.0
29/9/2002	15.3	7.1	4.0	77.0	58.0	29/10/2002	32.9	6.5	0.0	22.0	14.0	29/11/2002	22.0	11.3	0.0	75.0	61.0	29/12/2002	35.4	25.7	0.0	22.0	47.0
30/9/2002	17.5	7.0	0.0	67.0	47.0	30/10/2002	19.9	14.0	0.0	73.0	57.0	30/11/2002	19.7	10.9	0.0	54.0	45.0	30/12/2002	33.8	17.5	0.0	47.0	73.0
						31/10/2002	18.5	8.1	0.0	60.0	43.0							31/12/2002	21.6	16.5	2.4	72.0	49.0
Sep 02	18.6	7.7	23.6	65.0	53.1	Oct 02	21.0	8.2	15.8	58.9	45.4	Nov 02	25.3	11.2	25.6	56.8	41.3	Dec 02	26.3	13.1	4.4	60.5	42.9

APPENDIX D: TEMPERATURE, PRECIPITATION AND RELATIVE HUMIDITY AT LANGHORNE CREEK, SA.

APPENDIX D3 (2003): Max or Min °C = mean ambient temperature; Rain = precipitation (mm); RH am/pm = relative humidity at 9 am/3 pm

Date	Max°C	Min°C	Rain	RH am	RH pm	Date	Max°C	Min°C	Rain	RH am	RH pm	Date	Max°C	Min°C	Rain	RH am	RH pm	Date	Max°C	Min°C	Rain	RH am	RH pm
1/1/2003	19.9	12.0	0.2	63.0	41.0	1/2/2003	22.3	12.3	0.0	79.0	53.0	1/3/2003	18.2	10.1	0.2	74.0	52.0	1/4/2003	27.8	11.4	0.2	80.0	28.0
2/1/2003	23.4	12.7	0.2	70.0	51.0	2/2/2003	24.3	10.8	0.0	71.0	45.0	2/3/2003	19.5	9.3	0.0	68.0	51.0	2/4/2003	22.3	15.3	0.0	91.0	71.0
3/1/2003	23.7	12.2	0.0	77.0	56.0	3/2/2003	35.2	11.9	0.0	32.0	20.0	3/3/2003	19.1	11.8	0.0	80.0	60.0	3/4/2003	18.5	12.5	0.0	58.0	45.0
4/1/2003	23.8	10.2	0.0	84.0	36.0	4/2/2003	41.3	17.6	0.2	24.0	19.0	4/3/2003	19.7	11.1	0.0	84.0	57.0	4/4/2003	18.7	4.7	0.0	65.0	34.0
5/1/2003	34.8	10.5	0.0	66.0	17.0	5/2/2003	22.0	19.0	0.0	83.0	71.0	5/3/2003	23.2	8.4	0.0	93.0	45.0	5/4/2003	26.2	5.7	0.0	73.0	27.0
6/1/2003	38.0	15.2	0.0	25.0	15.0	6/2/2003	24.8	14.7	0.0	71.0	48.0	6/3/2003	28.1	9.2	0.0	81.0	24.0	6/4/2003	28.5	8.2	0.0	41.0	27.0
7/1/2003	36.2	18.3	0.0	17.0	73.0	7/2/2003	22.1	14.6	0.0	81.0	60.0	7/3/2003	29.2	11.4	0.0	35.0	48.0	7/4/2003	27.5	12.9	8.0	27.0	28.0
8/1/2003	21.0	12.8	0.0	40.0	48.0	8/2/2003	23.4	13.3	0.0	69.0	53.0	8/3/2003	20.7	12.4	0.0	69.0	46.0	8/4/2003	16.9	13.8	0.0	88.0	78.0
9/1/2003	22.8	14.1	0.0	53.0	40.0	9/2/2003	24.3	9.6	0.0	75.0	38.0	9/3/2003	21.6	7.5	0.0	74.0	45.0	9/4/2003	19.4	8.4	0.0	80.0	46.0
10/1/2003	26.2	10.8	0.0	70.0	34.0	10/2/2003	22.6	11.4	0.0	73.0	45.0	10/3/2003	23.5	8.2	0.0	87.0	56.0	10/4/2003	19.6	9.3	5.0	75.0	92.0
11/1/2003	35.1	12.6	0.0	43.0	18.0	11/2/2003	28.7	11.4	0.0	75.0	33.0	11/3/2003	25.7	12.2	0.0		52.0	11/4/2003	21.9	8.1	0.0		58.0
12/1/2003	39.2	14.8	0.0	21.0	13.0	12/2/2003	31.7	11.2	0.0	69.0	31.0	12/3/2003	33.9	12.1	0.0	77.0	24.0	12/4/2003	25.5	7.1	1.0	92.0	43.0
13/1/2003	32.3	26.0	0.0	17.0	35.0	13/2/2003	26.5	12.7	0.0	82.0	48.0	13/3/2003	23.9	12.9	0.0	81.0	70.0	13/4/2003	21.5	12.4	3.0	60.0	69.0
14/1/2003	21.3	13.8	0.0	53.0	43.0	14/2/2003	30.2	10.9	0.0	71.0	28.0	14/3/2003	20.0	14.2	0.0	65.0	48.0	14/4/2003	19.3	13.8	2.0	86.0	69.0
15/1/2003	22.8	12.3	0.0	56.0	45.0	15/2/2003	21.3	13.9	12.0	81.0	72.0	15/3/2003	19.0	11.7	0.2	87.0	67.0	15/4/2003	17.3	14.3	0.0	85.0	67.0
16/1/2003	29.9	12.2	0.0	72.0	29.0	16/2/2003	22.3	15.7	0.0	96.0	81.0	16/3/2003	24.8	8.1	0.0	91.0	42.0	16/4/2003	17.4	5.3	0.0	73.0	41.0
17/1/2003	38.3	13.7	0.0	33.0	15.0	17/2/2003	23.5	13.6	0.0	73.0	45.0	17/3/2003	34.3	10.7	0.0	43.0	20.0	17/4/2003	18.7	4.6	0.0	75.0	39.0
18/1/2003	25.6	16.5	0.0	75.0	50.0	18/2/2003	24.4	12.7	0.0	66.0	29.0	18/3/2003	36.8	16.0	7.0	27.0	20.0	18/4/2003	22.7	5.1	0.0	68.0	29.0
19/1/2003	40.4	13.2	0.0	47.0	18.0	19/2/2003	27.9	15.0	26.0	66.0	27.0	19/3/2003	23.6	13.5	4.0	92.0	76.0	19/4/2003	24.7	6.8	0.0	60.0	29.0
20/1/2003	34.3	23.3	3.0	83.0	51.0	20/2/2003	21.3	16.0	5.0		91.0	20/3/2003	19.4	11.9	1.0	91.0	52.0	20/4/2003	25.5	8.2	0.0	67.0	29.0
21/1/2003	23.0	14.4	0.2	68.0	54.0	21/2/2003	24.0	17.3	0.4	97.0	79.0	21/3/2003	17.4	12.0	0.2	80.0	61.0	21/4/2003	25.2	7.5	0.0	76.0	57.0
22/1/2003	24.2	14.1	0.0	72.0	48.0	22/2/2003	22.3	16.9	0.2		82.0	22/3/2003	18.1	11.8	0.0	92.0	69.0	22/4/2003	26.1	7.3	0.0	61.0	26.0
23/1/2003	29.5	9.0	0.0	60.0	25.0	23/2/2003	25.9	16.1	0.0		74.0	23/3/2003	19.0	11.1	0.2	79.0	44.0	23/4/2003	26.6	9.8	0.0	67.0	40.0
24/1/2003	40.4	14.4	0.0	32.0	14.0	24/2/2003	26.3	16.4	1.0	100.0	71.0	24/3/2003	25.5	7.4	0.0	99.0	33.0	24/4/2003	23.0	7.4	0.0	87.0	48.0
25/1/2002			0.0	20.0	17.0	25/2/2003	19.0	16.2	0.0		87.0	25/3/2003	23.4	8.1	0.0	85.0	58.0	25/4/2003	23.2	6.2	0.0	81.0	55.0
26/1/2003	21.5	18.0	0.2	78.0	70.0	26/2/2003	22.8	14.2	0.0	88.0	66.0	26/3/2003	23.5	13.3	0.0	91.0	48.0	26/4/2003	26.5	9.2	0.0	64.0	33.0
27/1/2003	24.5	13.5	0.0	68.0	36.0	27/2/2003	21.9	14.5	1.0	80.0	61.0	27/3/2003	22.6	12.8	0.0		56.0	27/4/2003	27.4	9.1	0.0	56.0	26.0
28/1/2003	31.6	13.3	0.0	57.0	20.0	28/2/2003	18.5	13.4	0.4	75.0	62.0	28/3/2003	20.9	12.3	0.0	93.0	66.0	28/4/2003	22.6	12.9	0.0	36.0	57.0
29/1/2003	38.7	16.9	9.0	35.0	22.0							29/3/2003	21.3	9.9	0.0	96.0	60.0	29/4/2003	20.3	8.3	0.4	76.0	50.0
30/1/2003	23.6	14.6	0.2	92.0	42.0							30/3/2003	20.5	11.6	0.0	79.0	53.0	30/4/2003	16.6	9.7	18.0	92.0	89.0
31/1/2003	21.5	12.6	0.0	60.0	48.0							31/3/2003	22.6	7.5	0.0	95.0	47.0						
Jan 03	28.9	14.3	13.0	54.4	36.3	Feb 03	25.0	14.0	46.2	74.0	54.3	Mar 03	23.2	11.0	12.6	78.9	56.0	Apr 03	22.6	9.2	37.6	70.3	47.7
1/2/2003	15.1	7.4	0.0	92.0	69.0	1/2/2003	19.5	12.6	0.0	48.0	74.0	1/3/2003	13.6	7.7	0.2		90.0	1/4/2003	15.4	2.7	0.2	68.0	43.0
2/2/2003	17.2	10.1	0.0	71.0	64.0	2/2/2003	17.6	10.8	3.0	71.0	82.0	2/3/2003	14.6	6.9	0.0		78.0	2/4/2003	17.1	6.1	0.0	81.0	53.0
3/2/2003	17.6	13.2	0.2	83.0	83.0	3/2/2003	17.0	11.3	0.8	78.0	65.0	3/3/2003	16.0	4.9	2.0	95.0	84.0	3/4/2003	17.3	6.5	0.0	33.0	28.0
4/2/2003	16.4	7.6	0.0	78.0	50.0	4/2/2003	17.1	10.4	27.0			4/3/2003	15.8	7.4	0.2	66.0	74.0	4/4/2003	18.5	10.5	9.0	32.0	32.0
5/2/2003	22.6	5.0	0.0	62.0	22.0	5/2/2003	15.6	6.9	3.0	79.0	71.0	5/3/2003	17.8	6.6	0.0	69.0	42.0	5/4/2003	14.9	9.2	0.8	83.0	56.0
6/2/2003	23.2	6.2	0.0	52.0	29.0	6/2/2003	14.0	10.9	13.0	85.0	89.0	6/3/2003	17.5	5.4	0.0	47.0	41.0	6/4/2003	14.4	9.5	1.0	90.0	69.0
7/2/2003	23.7	8.4	0.0	67.0	32.0	7/2/2003	15.3	9.5	0.0	67.0	57.0	7/3/2003	16.8	8.4	0.0	85.0	73.0	7/4/2003	15.4	6.5	0.0	82.0	74.0
8/2/2003	23.5	9.0	0.0	47.0	30.0	8/2/2003	15.4	6.4	0.0	86.0	72.0	8/3/2003	15.5	7.8	2.0	86.0	77.0	8/4/2003	14.7	7.4	9.0	85.0	79.0
9/2/2003	25.7	12.5	1.0	70.0	29.0	9/2/2003	13.7	4.6	0.0	73.0	54.0	9/3/2003	15.1	5.9	0.0	91.0	66.0	9/4/2003	12.2	5.0	0.0	83.0	62.0
10/2/2003	15.1	12.7	7.0	96.0	96.0	10/2/2003	16.0	6.4	0.0	71.0	49.0	10/3/2003	17.1	7.3	0.0	76.0	50.0	10/4/2003	9.0	1.2	6.0	78.0	67.0
11/2/2003	17.8	12.4	0.8		82.0	11/2/2003	16.7	5.1	0.0	75.0	40.0	11/3/2003	19.3	5.0	15.0	60.0	38.0	11/4/2003	13.9	4.3	0.0	96.0	85.0
12/2/2003	19.1	10.4	0.2	96.0	79.0	12/2/2003	14.8	7.5	2.0	81.0	61.0	12/3/2003	14.7	9.3	0.0	86.0	65.0	12/4/2003	20.1	7.5	4.0	86.0	89.0
13/2/2003	20.0	9.9	0.2		66.0	13/2/2003	14.9	8.9	0.2	80.0	78.0	13/3/2003	14.8	6.6	0.0	87.0	76.0	13/4/2003	14.3	8.8	5.0	90.0	88.0
14/2/2003	17.7	8.7	0.0		80.0	14/2/2003	14.3	9.8	2.0	77.0	76.0	14/3/2003	16.9	6.1	0.0	74.0	46.0	14/4/2003	12.4	7.0	0.0	84.0	65.0
15/2/2003	17.8	8.4	0.0		82.0	15/2/2003	14.6	6.8	0.0	92.0	68.0	15/3/2003	17.1	12.5	2.0	43.0	42.0	15/4/2003	13.3	3.0	0.0	76.0	49.0
16/2/2003	18.2	9.2	0.0	75.0	91.0	16/2/2003	15.6	9.6	0.0	83.0	79.0	16/3/2003	13.6	8.6	0.0	99.0	71.0	16/4/2003	14.5	1.2	0.0	72.0	54.0
17/2/2003	19.0	12.4	0.4	82.0	81.0	17/2/2003	15.2	10.1	1.0	85.0	84.0	17/3/2003	14.1	5.2	0.0	76.0	67.0	17/4/2003	15.3	4.4	3.0	63.0	76.0
18/2/2003	18.7	14.1	4.0	80.0	83.0	18/2/2003	14.5	10.3	2.0		81.0	18/3/2003	13.9	4.2	0.0	84.0	69.0	18/4/2003	11.8	4.3	0.0	86.0	57.0
19/2/2003	16.7	13.9	17.0	75.0	81.0	19/2/2003	14.9	9.7	0.0		72.0	19/3/2003	10.8	0.4	0.0		79.0	19/4/2003	12.9	0.3	0.2	87.0	55.0
20/2/2003	18.3	10.8	3.0	85.0	71.0	20/2/2003	14.4	9.2	0.0		74.0	20/3/2003	13.7	1.6	0.0	94.0	70.0	20/4/2003	10.5	2.1	5.0	66.0	62.0
21/2/2003	15.6	10.3	0.0	81.0	78.0	21/2/2003	14.6	9.5	4.0	79.0	83.0	21/3/2003	17.4	4.2	5.0	87.0	70.0	21/4/2003	15.3	7.2	0.2	82.0	68.0
22/2/2003	16.0	5.8	0.4	71.0	89.0	22/2/2003	13.6	9.6	2.0	84.0	74.0	22/3/2003	15.7	10.9	0.0	80.0	61.0	22/4/2003	17.1	5.8	4.0	83.0	61.0
23/2/2003	16.5	9.2	0.4	84.0		23/2/2003	13.6	6.1	0.0	87.0	72.0	23/3/2003	14.2	8.8	7.0	82.0	76.0	23/4/2003	11.4	7.8	23.0	98.0	
24/2/2003	16.8	10.5	10.0	100.0	84.0	24/2/2003	13.7	4.1	0.0	84.0	73.0	24/3/2003	13.4	7.7	10.0	74.0	74.0	24/4/2003	14.5	9.5	2.0	85.0	74.0
25/2/2003	15.4	11.0	0.6	83.0	85.0	25/2/2003	12.5	0.7	0.0		76.0	25/3/2003	11.6	7.4	3.0	76.0	68.0	25/4/2003	13.2	9.3	2.0	82.0	76.0
26/2/2003	15.2	8.9	0.0	69.0	89.0	26/2/2003	17.3	3.5	10.0	80.0	57.0	26/3/2003	12.3	6.3	0.0	79.0	59.0	26/4/2003	13.6	6.2	0.6	81.0	73.0
27/2/2003	14.5	9.8	0.2	71.0	95.0	27/2/2003	16.6	9.6	7.0	91.0	74.0	27/3/2003	15.7	3.3	0.0	63.0	45.0	27/4/2003	15.1	7.9	0.2	82.0	73.0
28/2/2003	16.7	4.3	0.0	67.0	87.0	28/2/2003	16.0	10.0	4.0	95.0	95.0	28/3/2003	15.8	9.9	7.0	48.0	86.0	28/4/2003	16.6	5.6	0.0	74.0	48.0
29/2/2003	17.7	4.3	0.2	63.0	79.0	29/2/2003	16.5	11.9	0.2	87.0	71.0	29/3/2003	12.5	7.4	1.0	81.0	72.0	29/4/2003	19.1	6.8	11.0	39.0	34.0
30/2/2003	20.7	4.8	0.0	41.0	58.0	30/2/2003	14.0	11.0	0.0	96.0	87.0	30/3/2003	11.8	6.3	0.0	85.0	70.0	30/4/2003	14.7	6.9	7.0	70.0	61.0
31/2/2003	21.2	8.2	0.0	51.0	76.0							31/3/2003	13.4	3.5	0.0	89.0	69.0	31/4/2003	12.9	8.3	4.0	84.0	74.0
May 03	18.4	9.3	45.6	73.1	70.7	Jun 03	15.3	8.4	81.2	80.6	72.0	Jul 03	14.9	6.6	54.4	77.9	66.1	Aug 03	14.6	6.1	97.2	76.8	62.9
1/3/2003	13.0	5.5	5.0	79.0	91.0	1/10/2003	16.8	7.8	1.0	86.0	72.0	1/11/2003	16.5	8.7	0.0	58.0	54.0	1/12/2003	18.9	14.0	0.2	85.0	52.0
2/9/2003	13.9	7.4	0.2	81.0	80.0	2/10/2003	14.5	10.0	1.0	83.0		2/11/2003	16.9	8.6	0.0	69.0	58.0	2/12/2003	27.2	8.3	0.0	63.0	35.0
3/9/2003	14.6	9.0	0.2	80.0	71.0	3/10/2003	14.4	8.4	0.0	93.0	81.0	3/11/2003	19.8	4.2	0.0	64.0	44.0	3/12/2003	24.8	9.2	0.2	65.0	52.0
4/9/2003	19.4	2.5	0.0	74.0	38.0	4/10/2003	14.8	9.1	0.0	83.0	69.0	4/11/2003	27.3	5.6	0.0	67.0	19.0	4/12/2003	19.9	12.2	0.0	83.0	69.0
5/9/2003	17.3	11.2	11.0	72.0	68.0	5/10/2003	16.2	4.6	0.0	70.0	66.0	5/11/2003	27.1	9.2	0.0	61.0	23.0	5/12/2003	19.3	12.0	0.0	52.0	43.0
6/9/2003	15.5	9.4	0.2		77.0	6/10/2003	16.5	4.0	2.0	70.0	61.0	6/11/2003	19.3	10.2	0.0	80.0	67.0	6/12/2003	19.9	9.4	0.0	57.0	45.0
7/9/2003	15.9	4.3	0.0	90.0	71.0	7/10/2003	16.8	5.1	0.4	72.0	65.0	7/11/2003	19.0	11.7	0.0	68.0	52.0	7/12/2003	26.9	8.1	0.0	60.0	39.0
8/9/2003	19.0	7.9	0.0	75.0	62.0	8/10/2003	14.2	9.7	2.0	80.0	69.0	8/11/2003	19.7	9.6	0.0	63.0	48.0	8/12/2003	31.4	10.7	0.0	79.0	27.0
9/9/2003	18.4	11.2	0.0	79.0	72.0	9/10/2003	14.5	5.1	0.0	58.0	52.0	9/11/2003	19.9	10.4	0.0	75.0	52.0	9/12/2003	39.9	12.2	0.0	24.0	12.0
10/9/2003	16.8	7.7	0.6	83.0	87.0	10/10/2003	13.4	7.8	0.0	68.0	58.0	10/11/2003	20.9	9.0	0.0	74.0	48.0	10/12/2003	23.2	15.9	0.0	58.0	45.0
11/9/2003	19.3	5.3	2.0	95.0	66.0	11/10/2003	14.2	1.2	0.0	63.0	55.0	11/11/2003	21.4	10.1	0.0	70.0	51.0	11/12/2003	22.9	14.2	0.2	66.0	44.0
12/9/2003	14.9	6.8	0.0	74.0	53.0	12/10/2003	16.5	2.7	0.0	65.0	53.0	12/11/2003	21.6	12.7	0.0	73.0	42.0	12/12/2003	21.5	11.3	0.0	51.0	65.0
13/9/2003	15.7	6.6	16.0	39.0	80.0	13/10/2003	23.0	4.4	0.0	63.0	27.0	13/11/2003	30.8	6.9	0.0	56.0	17.0	13/12/2003	23.0	13.4	0.0	67.0	56.0
14/9/2003	12.6	7.0	1.0	67.0	76.0	14/10/2003	27.2	5.7	0.0	31.0	17.0	14/11/2003	35.4	10.5	0.0	21.0	12.0	14/12/2003	29.2	14.1	0.0	62.0	30.0
15/9/2003	14.6	7.7	3.0	70.0	71.0	15/10/2003	19.0	13.2	0.0	64.0	54.0	15/11/2003	37.8	19.2	0.0	13.0	60.0	15/12/2003	37.7	12.1	0.0	35.0	10.0
16/9/2003	15.3	10.6	0.0	77.0	80.0	16/10/2003	19.3	7.0	0.0	65.0	58.0	16/11/2003	20.3	13.3	1.0	68.0	48.0	16/12/2003	40.8	17.8	0.0	14.0	8.0
17/9/2003	16.3	11.8	0.2	78.0	78.0	17/10/2003	25.5	5.2	0.0	57.0	27.0	17/11/2003	30.8	11.0	0.0	49.0	17.0	17/12/2003	23.2	17.8	0.0	81.0	59.0
18/9/2003	16.6	11.5	0.0	87.0	78.0	18/10/2003	32.1	8.3	0.0	31.0	20.0	18/11/2003	32.1	18.4	0.0	82.0	38.0	18/12/2003	28.0	16.9	19.0	62.0	46.0
19/9/2003	18.2	8.0	0.0	65.0	70.0	19/10/2003	16.8	7.9	4.0	44.0	47.0	19/11/2003	37.3	17.0	0.0	51.0	26.0	19/12/2003	30.2	16.1	0.0	49.0	87.0
20/9/2003	22.8	4.0	0.6	74.0	49.0	20/10/2003	19.7	11.2	0.0	74.0	58.0	20/11/2003	24.5	16.5	0.0	84.0	61.0	20/12/2003	26.2	16.8	2.0	86.0	65.0
21/9/2003	22.2	11.1	0.0	71.0	61.0	21/10/2003	17.0	11.7	0.0	94.0	64.0	21/11/2003	21.8	10.7	1.0	74.0	52.0	21/12/2003	26.9	16.5	0.0	76.0	67.0
22/9/2003	25.5	7.7	0.0	43.0	74.0	22/10/2003	18.2	7.6	0.0	54.0	46.0	22/11/2003	16.4	11.1	0.0	69.0	62.0	22/12/2003	21.4	14.7	0.0	77.0	61.0
23/9/2003	23.6	6.2	2.0	61.0	37.0	23/10/2003	16.9	10.9	0.0	58.0	58.0	23/11/2003	17.5	8.9	0.0	83.0	62.0	23/12/2003	25.2	13.8	0.0	58.0	38.0
24/9/2003	17.4		0.2	69.0	49.0	24/10/2003	17.4	8.4	3.0	53.0	52.0	24/11/2003	18.9	8.9	0.0			24/12/2003		10.1	0.0	37.0	11.0
25/9/2003			3.0	79.0	72.0	25/10/2003	15.1	10.2	0.0	76.0	71.0	25/11/2003	25.2	7.3	0.0	65.0	33.0	25/12/2003	24.8	11.8	0.0	57.0	55.0
26/9/2003	14.7	7.9	0.0	62.0	55.0	26/10/2003	14.8	4.8	3.0	85.0	53.0	26/11/2003	30.4	9.4	0.0	48.0	14.0	26/12/2003	21.8	15.1	0.0	56.0	44.0
27/9/2003	14.1	5.9	0.8	59.0	43.0	27/10/2003	19.7	4.0	5.0	62.0	35.0	27/11/2003	33.0	10.7	0.0	25.0	13.0	27/12/2003	23.0	11.2	0.0	54.0	34.0
28/9/2003	19.2	0.8	0.0	42.0	57.0	28/10/2003	21.4	9.5	4.0	73.0	72.0	28/11/2003	33.5	15.9	0.0	17.0	14.0	28/12/2003	34.3	9.3	0.0	46.0	13.0
29/9/2003	13.6	5.0	12.0	54.0	50.0	29/10/2003	13.5	8.5	11.0	74.0	83.0	29/11/2003	32.3	15.0	0.4	54.0	21.0	29/12/2003	35.9	19.0	0.0	11.0	36.0
30/9/2003	16.6	0.9	9.0	62.0	30.0	30/10/2003	19.1	4.8	1.0	70.0	44.0	30/11/2003	20.1	14.8	0.0	70.0	69.0	30/12/2003	32.0	25.1		28.0	55.0
						31/10/2003	15.7	8.1	0.4	80.0	51.0							31/12/2003	29.4	13.5		31.0	38.0
Sep 03	17.1	7.2	67.0	70.4	64.9	Oct 03	17.9	7.3	37.8	67.7	54.6	Nov 03	24.9	11.2	2.4	60.4	40.7	Dec 03	27.0	13.6	21.6	55.8	43.2

APPENDIX E: TEMPERATURE, PRECIPITATION AND RELATIVE HUMIDITY AT NURIOOTPA, SA.

APPENDIX E1: (2000): Max or Min °C = mean ambient temperature; Rain = precipitation (mm); RH am/pm = relative humidity at 9 am/3 pm

Date	Max°C	Min°C	Rain	RH am	RH pm	Date	Max°C	Min°C	Rain	RH am	RH pm	Date	Max°C	Min°C	Rain	RH am	RH pm	Date	Max°C	Min°C	Rain	RH am	RH pm
1/1/2000	20.6	5.5	0	50	31	1/2/2000	38.2	14.6	0	22	5	1/3/2000	36.2	19.6	0	36	21	1/4/2000	20	7.7	0	84	55
2/1/2000	26.9	10.3	0	49	20	2/2/2000	40.5	22.5	0	16	4	2/3/2000	38	20	0	42	23	2/4/2000	25.6	12.2	0	63	44
3/1/2000	30	13.7	0	33	28	3/2/2000	39	16	0	18	14	3/3/2000	29	19.8	0	69	47	3/4/2000	25.6	5.8	0	64	30
4/1/2000	20.7	9.5	0	43	32	4/2/2000	40	21.1	0	41	18	4/3/2000	23.7	11.6	0	72	35	4/4/2000	18.3	14.1	0.8	89	62
5/1/2000	21.5	5	0	46	37	5/2/2000	28.9	19.3	0	59	37	5/3/2000	23.7	12	0	70	46	5/4/2000	17.5	12	6.8	80	54
6/1/2000	23.9	10	0	59	32	6/2/2000	26.6	11.7	0	58	21	6/3/2000	21.7	14	0	78	49	6/4/2000	19.9	7.8	0	70	37
7/1/2000	26.3	9.7	0	58	33	7/2/2000	31.8	13.8	0	44	18	7/3/2000		10.3	0	76	40	7/4/2000	22.2	9.9	0	50	32
8/1/2000	31.3	13.7	0	43	11	8/2/2000	37.4	18.4	0	26	18	8/3/2000	27.5	10.3	0	68	39	8/4/2000	24.5	11.1	0	42	31
9/1/2000	31.4	17.8	0	33	21	9/2/2000	40	16.1	0	25	14	9/3/2000	25.9	13.9	0	58	41	9/4/2000	26.4	14.2	0	58	48
10/1/2000	34.8	15.1	0	28	13	10/2/2000	39	26.5	0	26	21	10/3/2000	30.4	12.2	0	83	22	10/4/2000	26.4	15.8	0.4	67	37
11/1/2000	36	20.2	0	37	17	11/2/2000	24.5	14.4	1.2	85	38	11/3/2000	32.3	13.8	0	56	20	11/4/2000	24.5	12.4	0	59	52
12/1/2000	35.2	23.2	0	20	8	12/2/2000	23.9	11.3	0	58	31	12/3/2000	33.6	19.4	0	43	21	12/4/2000	18.3	15.3	16.2	94	94
13/1/2000	35.2	21.2	0	20	13	13/2/2000	25.2	11.1	0	49	20	13/3/2000	30.8	18.8	0	44	73	13/4/2000	23.5	15.5	0	86	64
14/1/2000	34.8	20.8	0	18	12	14/2/2000	31.5	9.1	0	36	30	14/3/2000	22.4	11.4	0	57	43	14/4/2000	20.1	16.9	17.8	87	82
15/1/2000	36	21.3	0	33	13	15/2/2000	32.4	16.6	0	26	13	15/3/2000	25.7	10.9	0	64	32	15/4/2000	20.9	9.6	0	82	55
16/1/2000	38.3	23	0	31	16	16/2/2000	35.6	13.8	0	31	16	16/3/2000	27.7	12.3	0	68	36	16/4/2000	19.2	9.5	0	85	59
17/1/2000	38	25.8	0	40		17/2/2000	34.7	15.8	0	34	14	17/3/2000	38	14.6	0	36	17	17/4/2000	17.9	8.3	0.6	84	60
18/1/2000	35.6	26.8	0			18/2/2000	35.9	17.7	0	35	14	18/3/2000	24.3	17.5	0	74	51	18/4/2000	18.6	5.9	0	86	42
19/1/2000	26.4	17.1	0	76		19/2/2000	36	23.2	0	48	14	19/3/2000	21.5		0	81	57	19/4/2000	20.8	4.5	0	73	39
20/1/2000	23.2	16.3	0	69		20/2/2000	21.4	16.2	23.4	91	89	20/3/2000	20.1	11.6	0.4	63	50	20/4/2000	18.3	4.7	0	83	59
21/1/2000	21.6	13.6	0	75	42	21/2/2000	22.5	17.5	21.4	96	96	21/3/2000	23.7	10.6	0	61	30	21/4/2000	17.1	3.9	0	80	59
22/1/2000	21.9	11.4	0	55	40	22/2/2000	24.8	18.7	17.6	81	70	22/3/2000	28.1	11.7	0	41	45	22/4/2000	15.5	5.2	0	59	
23/1/2000	22.5	8.3	0	50	23	23/2/2000	26.3	18.4	1.2	87	67	23/3/2000	15.8	8.6	17.2	68	56	23/4/2000	15.1	5.7	0	63	51
24/1/2000	17.7	13.2	0	41	55	24/2/2000	30.3	17.8	0	76	55	24/3/2000	18.6	7.5	0	65	43	24/4/2000	18.6	9	0	85	48
25/1/2000	30	13.9	0.8	46	21	25/2/2000	31.4	19.6	0	76	49	25/3/2000	24	7.7	0	56	28	25/4/2000	18.6	7.5	0	57	36
26/1/2000	20.9	16.4	0	66	60	26/2/2000	26.2	19.4	3.4	84	60	26/3/2000	26.3		0	57	25	26/4/2000	21.9	3.7	0	50	28
27/1/2000	21.9	12.3	0	59	35	27/2/2000	27.1	15.2	0	73	47	27/3/2000	23.2	10.6	0	56	35	27/4/2000	24.5	8.5	0	55	37
28/1/2000	22.9	10.7	0	59	33	28/2/2000	31.8	15.5	0	33	23	28/3/2000	20.9	14.9	0.8	98	51	28/4/2000	26	14	0	55	36
29/1/2000	25.9	9.7	0	61	24	29/2/2000	31.5	20.4	0	41	29	29/3/2000	22	6.1	0.6	83	46	29/4/2000	27.1	18.3	0	51	32
30/1/2000	29.6	12	0	64	24							30/3/2000	19.1	8	0	80	58	30/4/2000	15.9	10.9	16.8	76	50
31/1/2000	34.2	14.5	0	41	10							31/3/2000	21	7.9	0	84	43						
Jan-00	28.2	14.8	0.8	46.8	28.1	Feb-00	31.5	16.9	68.2	50.9	32.6	Mar-00	25.8	12.7	19.0	64.1	39.5	Apr-00	21.0	10.0	59.4	70.6	48.0
1/5/2000	17.9	3.8	0	80	52	1/6/2000	11.6	5.8	0.2	99	66	1/7/2000	10.8	7.9	0	88	77	1/8/2000	17.1	3.5	0	71	37
2/5/2000	18.6	5.1	0	67	50	2/6/2000	12.1	5	0	91	86	2/7/2000	11.7	4.3	0	79	71	2/8/2000	18.3	1.7	0	62	38
3/5/2000	16.7	10.1	0	84	62	3/6/2000	13	0.8	0	84	84	3/7/2000	13.6	6.7	0	79	66	3/8/2000	17.6	1.4	0	54	30
4/5/2000	16.9	5	0	92	68	4/6/2000	11.1		6.4	94	84	4/7/2000	16.5	4.9	0	82	57	4/8/2000	15.7	6.3	0	46	47
5/5/2000	15.7	4.9	1.8	93	66	5/6/2000	11.5	3.5	8	99	89	5/7/2000	16	4.6	0	68	47	5/8/2000	16.7	4.9	0	66	54
6/5/2000	14.6	10	0.4	67	70	6/6/2000	12.6	7.5	0.4	99	79	6/7/2000	15	9.5	0.6	95	89	6/8/2000	18.2	7	0	65	51
7/5/2000	17.7	3.2	0	92	58	7/6/2000	12.5	7.2	0.2	99	71	7/7/2000	13.8	5.4	3	99	63	7/8/2000	17.4	7.1	0.4	86	58
8/5/2000	18.1	1.8	0	83	46	8/6/2000	14	7.7	0.4	98	73	8/7/2000	12	4.8	1.2	72	82	8/8/2000	12.1	8.2	0.2	90	81
9/5/2000	19.9	1.8	0	78	45	9/6/2000	14.3	9.3	0.4	95	57	9/7/2000	11.6	2.8	1.4	98	72	9/8/2000	12.7	7.8	5.8	90	65
10/5/2000	19.2	4.7	0	64	44	10/6/2000	10.1	4.5	0	78	72	10/7/2000	12.6	-0.1	1	100	74	10/8/2000	13.8	6.5	7.4	90	70
11/5/2000	21.7	10.5	0	58	41	11/6/2000	13.5	5.3	0	73	60	11/7/2000	13.5	0.4	0	82	49	11/8/2000	13.1	-0.2	0	87	52
12/5/2000	20.6	10.3	0	53	85	12/6/2000	13.8	-0.2	0	85	60	12/7/2000	13.8	6.4	0.8	92	69	12/8/2000	11.2	0.1	0	82	61
13/5/2000	15.3	10.2	7.4	87	84	13/6/2000	13	1.5	0	86	56	13/7/2000	12.3	2.5	0.4	88	69	13/8/2000	11.6	3.9	0	89	71
14/5/2000	14.6	9.2	4	97	67	14/6/2000	15.4	2.8	0.2	76	43	14/7/2000	12	2.9	0	72	73	14/8/2000	14.1	2.2	0	81	55
15/5/2000	15.7	6.3	1.2	95	79	15/6/2000	15.4	0.8	0	63	42	15/7/2000	15.8	6.1	4.2	82	45	15/8/2000	11.6	5.6	13.2	87	57
16/5/2000	16.1	8	1.8	98	74	16/6/2000	13.3	9.7	0	59	96	16/7/2000	17	4.1	0.2	93	60	16/8/2000	11.3	0.7	0.4	82	54
17/5/2000	14.1	7.3	0	96	84	17/6/2000	14.7	1.1	4	99	59	17/7/2000	18	4	0	77	46	17/8/2000	12.8	4.6	0	63	44
18/5/2000	16	3.4	2.8	94	62	18/6/2000	13.8	2.2	0.2	89	60	18/7/2000	15.4	8.9	1.2	77	62	18/8/2000		5.5	0	52	50
19/5/2000	15.6	4.8	0	78	59	19/6/2000	17.5	4.4	0	72	45	19/7/2000	11.2	5	16	95	60	19/8/2000			0		
20/5/2000	15.7	10.3	0.4	95	92	20/6/2000	15.5		0.4	72	54	20/7/2000	13.6	6.4	0.4	65	52	20/8/2000			0.6		
21/5/2000	14.6	7.5	0.4	74	57	21/6/2000	13	2.3	0.2	79	68	21/7/2000	12.6	6.3	19	80	70	21/8/2000	16		0		48
22/5/2000	15.6	-0.1	0	89	52	22/6/2000	13.5	8	9.6	90	86	22/7/2000	16.1	7.8	0.4	62	52	22/8/2000	10	6.3	4.8	77	83
23/5/2000	15.9	0.6	0	72	35	23/6/2000	14.5	3.3	4	99	74	23/7/2000	17.9	6.3	0	48	48	23/8/2000	12.8	6.8	11	97	71
24/5/2000	16.7	1.6	0	66	61	24/6/2000	16.3	7.1	0	82	56	24/7/2000	14	9.3	0.6	83	66	24/8/2000	12.6	3	3.2	97	68
25/5/2000	16.4	10	0.4	97	61	25/6/2000	17.3	1.5	0	80	56	25/7/2000	10.5	6.5	3.8	95	83	25/8/2000	12.4	5.9	1	89	77
26/5/2000	11.2	7.1	3.2	93	85	26/6/2000	13.5		0	69	69	26/7/2000	10.2	2.1	10.6	91	79	26/8/2000	13.9	6.8	3	91	55
27/5/2000	9.8	2.8	22.4	89	76	27/6/2000	12.7	8.9	7.8	96	63	27/7/2000	11.4	2.4	1.6	99	81	27/8/2000	14.1	4.6	0.6	75	46
28/5/2000	12.1	3.3	8.2	82	68	28/6/2000	9.4	6.8	4.8	92	81	28/7/2000	11.5	1.9	0.8	96	61	28/8/2000	13.4	8.5	5.8	84	57
29/5/2000	12.8	8.3	1	89	80	29/6/2000	13	4.3	11.6	97	74	29/7/2000	12.8	1	0	86	61	29/8/2000	13.6	6.1	0.8	95	58
30/5/2000	12.2	5	0.6	80	71	30/6/2000	13.4	3.3	0.2	97	76	30/7/2000	14.9	1.7	0	75	48	30/8/2000	15	5.4	0	61	52
31/5/2000	13.1	3.1	0.2	99	76							31/7/2000	16.1	3.9	0	75	44	31/8/2000	15.2	8.6	0	87	56
May-00	15.8	5.8	56.2	83.3	65.4	Jun-00	13.5	4.6	59.0	86.3	67.3	Jul-00	13.7	4.7	67.2	82.9	63.1	Aug-00	14.1	5.0	58.2	78.4	56.8
1/9/2000	14.5	3.7	0	53	93	1/10/2000	14.1	7.4	1.4	59	33	1/11/2000	19.7	11.8	0.6	76	50	1/12/2000	23.5	5	0	44	29
2/9/2000	15.1	7	13	86	65	2/10/2000	14.5	3.1	0	71	61	2/11/2000	19	12.3	0	77	60	2/12/2000	30.8	11	0	29	12
3/9/2000	13.8	4.9	2.2	72	51	3/10/2000	21.1	1.1	0	68	31	3/11/2000	21.1	8.4	0	64	44	3/12/2000	29.8	14.3	0	38	22
4/9/2000	17.5	4.4	0	62	58	4/10/2000	26.3	9.2	0	33	15	4/11/2000	21.6	9.3	0	62	38	4/12/2000	34.5	12.8	0	42	18
5/9/2000	11.9	8.2	2	83	63	5/10/2000	31.3	7.3	0	22	7	5/11/2000	21.1	9.5	0	60	37	5/12/2000	31.5	11.7	0	27	22
6/9/2000	15.9	6.9	0.8	88	60	6/10/2000	26.6	11.5	0	84	33	6/11/2000	22.6	8	0	56	24	6/12/2000	27.1	10.1	0	42	15
7/9/2000	15	9	19	93	61	7/10/2000	13.6	13	0.8	88	91	7/11/2000	28.7	12.8	0	25	15	7/12/2000	29.8	13.3	0	40	20
8/9/2000	14.2	8.9	8.4	88	67	8/10/2000	17	2.6	5.6	75	41	8/11/2000	23.7	18.2	0.2	21	86	8/12/2000	25.3	10.1	0	55	26
9/9/2000	14.2	9.2	4.2	90	64	9/10/2000	17.1	2	0	55	32	9/11/2000	22.1	13.5	11.6	90	63	9/12/2000	31	11.3	0	46	13
10/9/2000	14.1	7.9	1	93	92	10/10/2000	17.7	5	0	70	42	10/11/2000	24	13.1	0	73	46	10/12/2000	36.4	14	0	11	10
11/9/2000	13.3	8.7	2.2	65	62	11/10/2000	21.5	0.9	0	50	28	11/11/2000	21.3	13.8	8.6	85	65	11/12/2000	34.2	16.7	0	21	23
12/9/2000	15	7.1	0.4	80	72	12/10/2000	21.7	7.9	0	29	27	12/11/2000	22	13.8	0	89	59	12/12/2000	26.9	11.6	0	57	31
13/9/2000	20.4	4	0.2	59	43	13/10/2000	14.1	8	7	65	41	13/11/2000		13.4	0	87	42	13/12/2000	25.9	9.1	0	64	35
14/9/2000	16.8	11	0	62	71	14/10/2000	14.8	4.1	0.4	72	68	14/11/2000	27.1		0	69	37	14/12/2000	25.4	10.3	0	42	22
15/9/2000		2.8	0	86	48	15/10/2000	16.7	10.8	7.4	89	63	15/11/2000	28.1	15.9	0	63	31	15/12/2000	30.8	11.8	0	37	14
16/9/2000	24.8		0	45	50	16/10/2000	20.2	8.9	0.2	62	37	16/11/2000	28.8	14.5	0	66	33	16/12/2000	36.1	16.1	0	20	10
17/9/2000	15.1	7.7	0	87	70	17/10/2000	21.2	12.4	0	38	49	17/11/2000	30.8	17.9	0	61	48	17/12/2000	32.5	14.9	0	22	11
18/9/2000	15.9	10.5	0	81	61	18/10/2000	14.6	12.7	14.4	96	81	18/11/2000	33.9	18.6	0	52	23	18/12/2000	32.2	15.3	1.6	48	27
19/9/2000	18	3.2	0	73	54	19/10/2000	15.7	8.4	15.4	67	61	19/11/2000	33.8	18.1	0	49	46	19/12/2000	35.7	13.1	0	37	16
20/9/2000	22.5	7.6	0.2	78	46	20/10/2000	19.4	8.3	0	75	62	20/11/2000	25.2	11.2	0.8	65	48	20/12/2000	36.2	15.1	0	34	12
21/9/2000	17.5	5.1	0	81	52	21/10/2000	24.8	9.2	0	59	39	21/11/2000	24.9	12.9	0	69	31	21/12/2000		16.8	0	44	68
22/9/2000	19.9	3.5	0	71	47	22/10/2000	25.3	12.5	0	46	31	22/11/2000	27.6	11	0	59	25	22/12/2000	21.2	16.8	5.6	97	54
23/9/2000	19.9	8.7	0	72	82	23/10/2000	20.8	13.5	0	80	62	23/11/2000	29.5	13.6	0	55	22	23/12/2000	23.2	8.9	0.2	59	34
24/9/2000	29.3	14.2	0.4	71	27	24/10/2000	17.6	12.7	0	89	65	24/11/2000	31.7	14.2	0	48	22	24/12/2000	23.6	14.9	0.6	83	58
25/9/2000	23.6	13.1	0.4	65	57	25/10/2000	20.3	8.4	0.2	65	44	25/11/2000	35.4	15.2	0	24	15	25/12/2000	22.9	8.1	0	64	41
26/9/2000	21.8	12.8	0	75	60	26/10/2000	17.4	5.7	0	59	60	26/11/2000	37.4	22.6	0	22	17	26/12/2000	22.4	10.1	0	59	44
27/9/2000	19.4	8.2	0	86	81	27/10/2000	18.1	10	0	49	46	27/11/2000	38.4	23.4	0	27	11	27/12/2000	20	11.4	0	55	38
28/9/2000	25.4	9.5	2.2	65	37	28/10/2000	17.9	3.8	0	42	23	28/11/2000	29.2	19.6	0	30	67	28/12/2000	20.7	7.4	0	55	33
29/9/2000	17.3	12.7	0.6	56	48	29/10/2000	20	6.7	0	57	27	29/11/2000	25.7	11.8	0	57	29	29/12/2000	25.3	8.3	0	46	18
30/9/2000	18.4	9	0	70	54	30/10/2000	22.1	8.1	0	50	20	30/11/2000	22.4	8	0	58	33	30/12/2000	29.1	12.9	0	45	20
Sep-00	17.9	7.8	113.4	76.1	60.0	Oct-00	19.3	7.7	111.8	62.9	44.8	Nov-00	26.3	13.7	89.0	56.8	36.7	Dec-00	28.0	11.9	66.2	46.5	27.5

APPENDIX E: TEMPERATURE, PRECIPITATION AND RELATIVE HUMIDITY AT NURIOOTPA, SA.

APPENDIX E2: (2001): Max or Min °C = mean ambient temperature; Rain = precipitation (mm); RH am/pm = relative humidity at 9 am/3 pm

Date	Max°C	Min°C	Rain	RH am	RH pm	Date	Max°C	Min°C	Rain	RH am	RH pm	Date	Max°C	Min°C	Rain	RH am	RH pm	Date	Max°C	Min°C	Rain	RH am	RH pm
1/1/2001	37.6	11.7	0.2	20	9	1/2/2001	35.5	18.9	0	25	10	1/3/2001	31.1	13	0	48	16	1/4/2001	22.2	8.6	0	58	45
2/1/2001	37.8	25.1	0	30	24	2/2/2001	38.2	23.6	0	27	14	2/3/2001	27.3	12.4	0	58	23	2/4/2001	20	9.3	0	60	33
3/1/2001	34.1	11.5	0	50	20	3/2/2001	36.4	17.1	0	69	31	3/3/2001	31.4	14.6	0	48	11	3/4/2001	26.5	10	0	48	21
4/1/2001	36.5	19.8	0	27	21	4/2/2001	29.4	18.7	0	59	46	4/3/2001	26.8	13.6	0	69	23	4/4/2001	26.5	5.6	0	43	19
5/1/2001	28.5	14.7	0	59	28	5/2/2001	30.1	15.6	0.2	61	44	5/3/2001	32.5	15.3	0	57	28	5/4/2001	29.6	7.2	0	55	14
6/1/2001	29.1	13.1	0	50	10	6/2/2001	36.1	19.3	0	54	34	6/3/2001	34.1	20.4	0	37	18	6/4/2001	30.5	8.6	0	26	12
7/1/2001	29.3	13	0	65	32	7/2/2001	39.8	25	0	62	27	7/3/2001	33.8	18.7	0	25	17	7/4/2001	32.2	14.1	0	17	13
8/1/2001	31	15.6	0	65	22	8/2/2001	40.6	23.5	0	32	15	8/3/2001	33.7	15.1	0	41	19	8/4/2001	17.2	11.2	13	68	44
9/1/2001	36.6	18.8	0	39	18	9/2/2001	26.3	21.4	0	85	60	9/3/2001	34	14.9	0	32	26	9/4/2001	19.1	5.8	0.2	74	49
10/1/2001	40.7	24.2	0	22	13	10/2/2001	26.7	14.3	0	72	38	10/3/2001	31	17.3	0	32	49	10/4/2001	20.2	9.4	0.2	77	43
11/1/2001	37.3	18.4	0	29	17	11/2/2001	33.3	14.1	0	53	26	11/3/2001	25.7	11.5	0	61	30	11/4/2001	20.8	6.7	0	84	47
12/1/2001	34.4	17.5	0	30	5	12/2/2001	30.2	19	0	62	68	12/3/2001	21.9	9.4	0	58	42	12/4/2001	17.4	7	0	53	23
13/1/2001	43.5	23.5	0	24	10	13/2/2001	23.1	16.1	2.8	95	50	13/3/2001	23	3.7	0	65	38	13/4/2001	22.3	6.7	0	39	20
14/1/2001	41.3	21.1	0	14	12	14/2/2001	24	13.7	9.6	90	50	14/3/2001	27	10.1	0	57	39	14/4/2001	24.7	4.4	0	24	12
15/1/2001	27.2	20.4	0	69	47	15/2/2001	23.9	11	0.2	67	29	15/3/2001	29.9	10.4	0			15/4/2001	27.9	5.1	0	25	10
16/1/2001	29.3	14.5	0	43	21	16/2/2001	27.4	11.1	0	62	26	16/3/2001	23.2	16.9	1.4	84	66	16/4/2001	22.4	5	0	72	46
17/1/2001	32.4	14.9	0	50	20	17/2/2001	32.5	13.9	0	40	19	17/3/2001	18.8	8.3	13	83	47	17/4/2001	27.4	6.6	0	53	32
18/1/2001	31.5	12.5	0	43	9	18/2/2001	37.6	20.2	0	24	16	18/3/2001	22.4	8.1	0	89	45	18/4/2001	31.6	12.1	0	28	13
19/1/2001	35.6	17	0	50	15	19/2/2001	38.7	22.4	0	22	12	19/3/2001	25.2	11.4	0	73	34	19/4/2001	19.1	12.2	0	55	43
20/1/2001	38.4	20.5	0	18	12	20/2/2001	37.4	15.8	0	20	8	20/3/2001	26.3	9.3	0	47	60	20/4/2001	14.5	5.3	0	56	40
21/1/2001	38.4	19.2	0	60	23	21/2/2001	30.4	16.2	0	22	38	21/3/2001	13.7	8.7	13.6	83	94	21/4/2001	16.5	1.2	0	49	35
22/1/2001	41.6	20.3	0	41	12	22/2/2001	30.5	12.5	0	67	17	22/3/2001	19.2	10.6	1.2	97	61	22/4/2001	14.6	10.1	5	97	76
23/1/2001	42.6	25	0	25	9	23/2/2001	32.1	9.5	0	48	13	23/3/2001	24.6	7.1	0	95	42	23/4/2001	15.8	10.1	0.4	73	56
24/1/2001	38.7	24.8	0.2	72	71	24/2/2001	28.8	12.9	0	68	30	24/3/2001	20	12.2	1.4	90	48	24/4/2001	17.5	4.9	0	67	40
25/1/2001	36.3	22.4	1.8	64	61	25/2/2001	29.3	14.8	2.4	65	39	25/3/2001	20.1	9.9	0.6	62	45	25/4/2001	21.4	5.6	0	57	32
26/1/2001	25.4	19.3	11	93	49	26/2/2001	30.6	17.8	0	65	39	26/3/2001	18.9	12.1	1.2	90	61	26/4/2001	20.9	8.5	0	76	42
27/1/2001	23.7	10.9	0	71	50	27/2/2001	27	12	0	72	29	27/3/2001	18.3	9.6	0.4	84	54	27/4/2001	18.1	11.1	0.6	87	40
28/1/2001	26.6	10.4	0	66	25	28/2/2001	27.6	9.7	0	58	29	28/3/2001	19.3	11.4	0	55	36	28/4/2001	15.6	7.2	0	70	41
29/1/2001	29.6	12.4	0	62	18							29/3/2001	19.9	8	0	59	38	29/4/2001	16.5	6.2	0	64	43
30/1/2001	26.8	13.2	0	54	29							30/3/2001	24.6	8.2	0	61	27	30/4/2001	21.3	2.7	0	64	39
31/1/2001	29.6	13.5	0	44	22							31/3/2001	27.8	8.1	0	50	17						
Jan-01	33.9	17.4	13.2	46.7	23.7	Feb-01	31.5	16.4	15.2	55.2	30.7	Mar-01	25.3	11.6	32.8	63.6	39.5	Apr-01	21.7	7.6	10.4	57.3	34.1
1/5/2001	20.9	5	0	66	38	1/6/2001	13.8	4.8	0	74	58	1/7/2001	15.5	0.2	0	84	50	1/8/2001	12.6	3.9	1.4	80	54
2/5/2001	21.5	5.3	0	63	33	2/6/2001	19	3.5	0	48	38	2/7/2001	17	2.1	0	84	53	2/8/2001	13.8	-0.7	0	86	53
3/5/2001	19.6	10.6	0	56	41	3/6/2001	19.2	3.9	0	84	46	3/7/2001	15.9	1.5	0	68	45	3/8/2001	14.9	0.1	0	71	44
4/5/2001	21	11.5	0	69	43	4/6/2001	21.5	4.6	0	72	27	4/7/2001	18	3.7	0	68	43	4/8/2001	18.2	3.2	0	60	43
5/5/2001	20.3	3.5	0	64	40	5/6/2001	18.3	10.7	0	73	68	5/7/2001	10.1	7	7.8	93	95	5/8/2001	13.6	9.4	8.2	92	59
6/5/2001	18.2	2.1	0.2	99	50	6/6/2001	15.5	11.8	4	89	94	6/7/2001	12.3	3.1	14.8	84	57	6/8/2001	11.9	4.2	3.4	90	58
7/5/2001	20.8	5.2	0	69	40	7/6/2001	13.5	9.3	7.4	89	76	7/7/2001	11.1	1.8	0	97	88	7/8/2001	11.4	4.2	0.4	70	79
8/5/2001	23.4	3.3	0	59	24	8/6/2001	14	10.7	17	94	81	8/7/2001	13.6	5.4	1.2	99	85	8/8/2001	12.5	5.7	10.6	82	59
9/5/2001	18.5	6	0	66	69	9/6/2001	14.7	10.4	4	97	69	9/7/2001	13.8	6.6	0.2	93	75	9/8/2001	17.2	3.3	0.2	52	36
10/5/2001	18.7	9.8	0.2	81	49	10/6/2001	14.3	8.7	0.2	95	70	10/7/2001	13	6.6	0	94	79	10/8/2001	19.3	5.6	0	69	63
11/5/2001	19.7	3.1	0	69	41	11/6/2001	14	10.2	17.4	95	92	11/7/2001	9.6	7.6	2	83	94	11/8/2001	18.2	4.6	0.8	60	43
12/5/2001	21.5	9.1	0	62	39	12/6/2001	13.7	10.7	0	99	88	12/7/2001	12.2	7.9	4.4	98	76	12/8/2001	14.9	5.9	0.4	99	62
13/5/2001	25.5	8.4	0	47	25	13/6/2001			1.6	96	69	13/7/2001	14.8	2.4	0.4	96	64	13/8/2001	17.1	1.3	0	89	56
14/5/2001	26.1	15.2	0	27	18	14/6/2001	12.8		3.8		56	14/7/2001	13.8	6.1	0.4	90	67	14/8/2001	19.3	1.4	0	73	32
15/5/2001	18.4	7.5	0	55	67	15/6/2001		1.7	0	97	65	15/7/2001	13.3	6.8	0.4	95	67	15/8/2001	20.8	5.5	0	57	31
16/5/2001	14.2	9.2	3.2	87	67	16/6/2001	10.3		0		69	16/7/2001	12.5	8	3.2	97	69	16/8/2001	17.2	12.2	0	30	85
17/5/2001	11.7	6.9	7	88	85	17/6/2001	12.4	6.8	0	80	60	17/7/2001	13.4	5.7	2	96	70	17/8/2001	11.7	5.9	12.2	83	95
18/5/2001	12.7	8.8	12.6	79	61	18/6/2001	12.6	-0.6	0	92	69	18/7/2001	10.8	4.8	1.2	99	74	18/8/2001	11.8	7.8	4.2	69	75
19/5/2001	11.5	1.9	0.2	91	66	19/6/2001	12.4	-1.2	0	93	82	19/7/2001	12.7	4.6	0	88	69	19/8/2001	14.4	7.9	0	95	86
20/5/2001	15.1	0.9	0	86	54	20/6/2001	15.1	6.4	0.2	95	80	20/7/2001	13	7.6	0.2	73	62	20/8/2001	12.7	9	2.4	87	72
21/5/2001	17.1	1.7	0	80	46	21/6/2001	14.1	10.6	6	95	94	21/7/2001	13	6.5	0	78	72	21/8/2001	11.9	4.7	2.4	87	82
22/5/2001	16.6	3.4	0	87	49	22/6/2001	12.2	10.4	1.2	86	75	22/7/2001	13.9	1.4	0	92	59	22/8/2001	13	8.1	5.4	88	63
23/5/2001	16.5	2.3	0	78	51	23/6/2001	12	9.6	0	93	77	23/7/2001	12.3	-0.1	0	95	72	23/8/2001	13.5	2.8	0	93	56
24/5/2001	17	1.3	0	82	45	24/6/2001	15.8	4.3	0	82	60	24/7/2001	12	3.5	0.2	73	64	24/8/2001	17.1	3.5	0.8	65	40
25/5/2001	19.3	4.7	0	70	39	25/6/2001	16.4	4.8	0	68	44	25/7/2001	13	4.1	0	87	67	25/8/2001	11.4	9.9	0.2	87	92
26/5/2001	13.7	10.2	2	94	87	26/6/2001	13.7	8.9	1	86	72	26/7/2001	13.4	3.3	0.2	99	70	26/8/2001	10.2	4.9	12.8	88	82
27/5/2001	13	7.7	17.2	91	75	27/6/2001	14.3	6.4	0	78	63	27/7/2001	14	4.6	0	94	65	27/8/2001	12.9	4.3	6.4	82	69
28/5/2001	14.2	4.4	11	80	69	28/6/2001	15.1	1.5	0	83	60	28/7/2001	12.9	2.9	0.8	82	59	28/8/2001	14.3	8.3	2	97	66
29/5/2001	13.4	1	0	87	65	29/6/2001	14.8	0	0	85	47	29/7/2001	13.6	0.6	0	93	67	29/8/2001	15.1	5.8	3	96	57
30/5/2001	13.4	7.3	0	77	53	30/6/2001	14.1	-0.3	0	79	56	30/7/2001	18.5	1	0	87	45	30/8/2001	15.4	3.3	0	80	55
31/5/2001	12.9	5.6	0	65	51							31/7/2001	19.1	4.4	0	54	43	31/8/2001	13.9	6.3	0	64	83
May-01	17.6	5.9	53.6	73.4	51.6	Jun-01	14.6	6.2	63.8	85.6	66.8	Jul-01	13.6	4.2	39.4	87.5	66.6	Aug-01	14.6	5.2	77.2	78.1	62.3
1/9/2001	12	8	5.2	92	92	1/10/2001	16	10.9	3	81	81	1/11/2001	23	8.4	0.4	91	41	1/12/2001	24.6	4.2	0	60	27
2/9/2001	13.1	6.4	24.8	94	92	2/10/2001	14.3	6.9	2.4	81	67	2/11/2001	21.4	10.1	0	71	41	2/12/2001	30.2	13.3	1.4	30	30
3/9/2001	15	3.9	1.2	81	89	3/10/2001	15.6	8.2	6.6	88	65	3/11/2001	24.6	8.8	0	69	32	3/12/2001	19.3	12.9	0.4	77	55
4/9/2001	14.3	8	2.6	74	58	4/10/2001		6.8	0	70	71	4/11/2001	28.8	13.4	0	38	43	4/12/2001	22.7	10.8	0	73	40
5/9/2001	14.8	0.9	0	76	58	5/10/2001	18.8		0		47	5/11/2001	21	16.5	0.8	87	92	5/12/2001	22.5	10.9	0	58	32
6/9/2001	18.8	4.2	0	68	46	6/10/2001	11.9	8	2.4	91	82	6/11/2001	17.6	12.7	5.2	81	49	6/12/2001	25.5	13.7	5.2	91	52
7/9/2001	20.8	11.3	5.2	58	46	7/10/2001	15.5	5.6	2.6	94	50	7/11/2001	16.9	7.4	0	52	34	7/12/2001	17.5	11.8	0	75	74
8/9/2001	11.6	6	11.6	85	87	8/10/2001	16.8	6.8	0.8	70	47	8/11/2001	23.8	10.3	0	66	32	8/12/2001	19.3	6	0.8	65	35
9/9/2001	15.2	7.7	9.6	88	64	9/10/2001	20.8	7.8	0	57	28	9/11/2001	18.3	5.9	0	65	41	9/12/2001	20	7.9	0	51	39
10/9/2001	20.4	4.8	0	60	39	10/10/2001	18	12.2	0.4	33	58	10/11/2001	16.2	8.1	0	63	44	10/12/2001	18.7	4.9	0.2	72	46
11/9/2001	17.1	11.5	0.2	66	68	11/10/2001	14.6	5.9	4.2	76	65	11/11/2001	14.5	1.1	0	65	69	11/12/2001	19.5	10.6	0.4	84	50
12/9/2001	16	6.8	0	86	71	12/10/2001	15.2	8.2	2.2	80	59	12/11/2001	15.4	6.1	1.6	85	80	12/12/2001	25	8.3	0	58	32
13/9/2001	15.7	8	0	85	64	13/10/2001	15.8	7.4	2.2	69	86	13/11/2001	20.8	7.6	0.2	67	43	13/12/2001	26.9	10.9	0	67	30
14/9/2001	16.4	4.7	0	83	59	14/10/2001	15.6	2.6	1.2	64	60	14/11/2001	22.9	8.3	0	74	33	14/12/2001	23.7	12.5	0	66	34
15/9/2001	20.8	3.8	0	69	43	15/10/2001	21.1	2.4	0	55	32	15/11/2001	27.2	8.4	0	38	19	15/12/2001	24.6	9.6	0	59	27
16/9/2001	17.2	10.8	0	57	66	16/10/2001	21.5	9.9	0	69	44	16/11/2001	31.6	10.4	0	38	15	16/12/2001	23.3	9.7	0	59	34
17/9/2001	16.4	5.9	0	63	52	17/10/2001	16.5	9	0	82	57	17/11/2001	30.4	15	0	26	15	17/12/2001	25.6	9	0	55	26
18/9/2001	21.9	5.3	0	45	38	18/10/2001	14.7	9.7	2.4	84	51	18/11/2001	16.3	11.4	5.6	78	42	18/12/2001	28.2	11.6	0	41	21
19/9/2001	26.7	9.5	0	52	26	19/10/2001	15	3.1	0	52	44	19/11/2001	18.9	5.7	0.2	48	30	19/12/2001	34.7	11.5	0	25	10
20/9/2001	18.6	10.9	0	79	63	20/10/2001	18.6	4.8	0	54	32	20/11/2001	23.9	7.6	0	56	25	20/12/2001	24.6	16.2	0	60	46
21/9/2001	23.1	10.3	0	72	45	21/10/2001	25.1	6.8	0	31	11	21/11/2001	27.1	11.6	0	46	21	21/12/2001	23.9	7.1	0	64	24
22/9/2001	22.8	14.6	0	63	76	22/10/2001	19.1	14.4	0.2	61	56	22/11/2001	29.3	15.7	0	34	18	22/12/2001	26.5	7.7	0	59	23
23/9/2001	27.5	12.3	1	43	37	23/10/2001	21.4	13.6	6.2	88	56	23/11/2001	21.2	11.9	14.6	90	43	23/12/2001	24.2	14	0	49	31
24/9/2001	14.8	8.4	23.5	96	74	24/10/2001	17	10.1	3.2	73	55	24/11/2001	20.8	12.6	0	71	42	24/12/2001	21.5	11.3	0	57	39
25/9/2001	13.4	8.5	9.6	84	54	25/10/2001	16.4	9.5	6	80	54	25/11/2001	19.3	11.1	1	68	59	25/12/2001	18.8	11.1	1.4	80	42
26/9/2001	15.1	3.7	0.4	61	54	26/10/2001	16.7	9.4	7.8	80	59	26/11/2001	19.8	11.1	0.4	92	50	26/12/2001	19.7	8.8	0.8	62	28
27/9/2001	17.8	6.1	0	65	45	27/10/2001	18.5	2.8	0.8	66	41	27/11/2001	20.5	7.4	0	82	46	27/12/2001	21.8	10.4	0	66	50
28/9/2001	24	8.3	0	35	30	28/10/2001	18.6	9.9	0	65	41	28/11/2001	25.6	9.5	0	58	28	28/12/2001	30	11.2	0	58	13
29/9/2001	27.6	10.5	0	35	26	29/10/2001	17.2	4.4	0	64	44	29/11/2001	22.2	10	0	65	38	29/12/2001	33.7	11.4	0	29	17
30/9/2001	20	17.6	0	49	71	30/10/2001	17	9.2	0.8	92	58	30/11/2001	18.5	9.4	2	85	31	30/12/2001	21.4	10.9	0	58	33
						31/10/2001	17.6	11.1	1	93	71							31/12/2001	22.6	8.6	0	56	78
Sep-01	18.2	8.0	84.9	88.7	57.8	Oct-01	17.3	7.9	58.4	71.4	53.3	Nov-01	22.0	9.8	32.0	65.0	39.9	Dec-01	23.9	10.2	10.6	60.1	36.1

APPENDIX E: TEMPERATURE, PRECIPITATION AND RELATIVE HUMIDITY AT NURIOOTPA, SA.

APPENDIX E3: (2002): Max or Min °C = mean ambient temperature; Rain = precipitation (mm); RH am/pm = relative humidity at 9 am/3 pm

Date	Max °C	Min °C	Rain	RH am	RH pm	Date	Max °C	Min °C	Rain	RH am	RH pm	Date	Max °C	Min °C	Rain	RH am	RH pm	Date	Max °C	Min °C	Rain	RH am	RH pm
1/1/2002	19	11,6	2,2	89	55	1/2/2002	33,1	10,2	0	48	16	1/3/2002	21	5,8	0	70	47	1/4/2002	29,1	8,4	0	44	23
2/1/2002	19,2	10,4	2,2	63	37	2/2/2002	21,9	10,3	0	61	65	2/3/2002	23,1	7	0	76	41	2/4/2002	29	11,7	0	38	26
3/1/2002	21	3,8	0,6	66	35	3/2/2002	19,3	10,8	0	60	30	3/3/2002	22,3	12,1	0	80	50	3/4/2002	26,5	12,7	0	41	52
4/1/2002	25,2	5,9	0	57	27	4/2/2002	22,9	10	0	56	31	4/3/2002	29	12,8	0	67	28	4/4/2002	18,1	9,9	0	55	32
5/1/2002	33,4	10,6	0	43	8	5/2/2002	26,1	10,6	0	59	28	5/3/2002	32,9	12,6	0	44	16	5/4/2002	20,6	7,2	0	50	34
6/1/2002	23,9	14,2	0	22	71	6/2/2002	27,8	8,3	0	58	32	6/3/2002	31,5	12,7	0	43	11	6/4/2002	23,5	11,4	0	59	33
7/1/2002	19,3	12,1	2,6	67	51	7/2/2002	22,3	12,2	0	79	48	7/3/2002	24,7	12,6	0	67	29	7/4/2002	24,4	13	0	37	39
8/1/2002	22,1	10,5	0,2	66	39	8/2/2002	18,9	10,4	0	63	54	8/3/2002	21,7	9,5	0	64	31	8/4/2002	27,6	14,7	0	38	63
9/1/2002	31,9	9,4	0	52	14	9/2/2002	21,9	12,3	0,2	70	52	9/3/2002	27,8	10,7	0	56	21	9/4/2002	28,9	6	0	47	50
10/1/2002	39,4	12,3	0	17	6	10/2/2002	19,8	10,7	0	73	34	10/3/2002	33,2	9,9	0	38	12	10/4/2002	26,3	11,2	0	60	61
11/1/2002	24,1	15,1	0	60	30	11/2/2002	23,3	8,3	0	59	25	11/3/2002	22,3	12,3	0	62	30	11/4/2002	32,1	10,4	0,2	71	23
12/1/2002	22,8	8,6	0	54	28	12/2/2002	30,2	11	0	58	14	12/3/2002	23,6	5	0	76	29	12/4/2002	27,6	15,3	0	49	50
13/1/2002	25,3	10,2	0	58	31	13/2/2002	35	10,9	0	30	8	13/3/2002	24	10,9	0	64	32	13/4/2002	18,9	12,6	2	83	72
14/1/2002	27,3	11,8	0	58	30	14/2/2002	38,1	15,4	0	21	18	14/3/2002	25,5	11,2	0	76	23	14/4/2002	19,2	12,2	0	69	48
15/1/2002	29,1	12,9	0	58	28	15/2/2002	29,5	21,3	0	29	32	15/3/2002	28	11,9	0	60	19	15/4/2002	21,7	8,8	0	48	35
16/1/2002	29,4	14,6	0	43	19	16/2/2002	23,6	9,3	0	55	25	16/3/2002	30,4	7,9	0	48	10	16/4/2002	23,2	7,4	0	48	30
17/1/2002	30,2	12,3	0	63	21	17/2/2002	27,6	10,9	0	61	27	17/3/2002	35,3	10,5	0	20	10	17/4/2002	25,8	6,9	0	55	21
18/1/2002	33,9	14,4	0	35	10	18/2/2002	30,7	14	0	53	22	18/3/2002	32,6	14,2	0	45	12	18/4/2002	26,9	8,2	0	34	20
19/1/2002	36,7	15,1	0	13	11	19/2/2002	37,1	18,4	0	22	18	19/3/2002	24,4	14,3	0	71	31	19/4/2002	28,3	13,2	0	37	23
20/1/2002	33,3	20,8	0	42	24	20/2/2002	22	15,5	0	67	43	20/3/2002	23,1	6,4	0	81	39	20/4/2002	25,5	16,9	0	30	42
21/1/2002	20,2	16,4	10,6	94	81	21/2/2002	20,9	12	0	58	38	21/3/2002	25,7	6,7	0	80	34	21/4/2002	24,2	9,9	0	60	37
22/1/2002	23,3	14,7	13,6	86	48	22/2/2002	23,3	8,2	0	70	37	22/3/2002	25,7	12,6	0	62	27	22/4/2002	27,8	14,5	0	40	22
23/1/2002	26,5	11,7	0	58	35	23/2/2002	27,4	12,7	0	85	35	23/3/2002	31,5	3,8	0	37	7	23/4/2002	29,4	6,6	0	52	20
24/1/2002	30,4	13,8	0	56	26	24/2/2002	31,4	13,6	0	63	17	24/3/2002	34,4	11,3	0	31	11	24/4/2002	20,6	12,6	0	61	60
25/1/2002	35	17	0	40	21	25/2/2002	33,8	17,2	0	34	14	25/3/2002	21	12,8	0	76	46	25/4/2002	16,9	14	1,6	89	73
26/1/2002	35,6	17,3	0	31	16	26/2/2002	30,9	15,5	0	32	19	26/3/2002	18,3	10,6	21,6	88	52	26/4/2002	18,7	4,6	0	64	41
27/1/2002	25,4	19,9	0	74	72	27/2/2002	23,7	12,3	0	68	25	27/3/2002	19,5	11,1	0	82	54	27/4/2002	18,2	4,6	0	68	32
28/1/2002	23,8	12	0	68	38	28/2/2002	23	10,7	0	69	34	28/3/2002	21,5	12,6	0	82	55	28/4/2002	18,7	4	0	58	30
29/1/2002	26,5	12,7	0	77	36							29/3/2002	22,5	12,4	0	75	44	29/4/2002	22,1	2,4	0	39	18
30/1/2002	30,3	11,3	0	52	25							30/3/2002	21,7	8,7	0	63	30	30/4/2002	22,1	1,8	0	49	23
31/1/2002	26,8	11,6	0	65	22							31/3/2002	25,2	10,9	0	58	27						
Jan-02	27,6	12,7	32,6	55,7	32,6	Feb-02	26,6	12,3	0,2	55,7	30,6	Mar-02	25,9	10,4	21,6	82,6	29,3	Apr-02	24,1	9,8	3,8	52,4	37,8
1/5/2002	23,7	6,1	0	57	34	1/6/2002	13,1	2,6	1,2	98	85	1/7/2002	13,7	-1,9	0,2	72	31	1/8/2002	14,1	3,1	0	85	67
2/5/2002	24,7	12,3	0	51	36	2/6/2002	15,9	0,6	0,2	80	40	2/7/2002	15	-0,2	0	28	42	2/8/2002	12	2,9	0	81	66
3/5/2002	26,5	13,1	0	37	21	3/6/2002	16,8	0,1	0	89	31	3/7/2002	12,2	6,3	1,4	98	58	3/8/2002	10,8	6,3	14,2	80	68
4/5/2002	26,7	8,9	0	33	18	4/6/2002	18,3	1,1	0	64	30	4/7/2002	13,8	7,9	4,8	98	73	4/8/2002	13,3	1,6	1,4	99	56
5/5/2002	26,5	5,1	0	38	20	5/6/2002	18,1	2,8	0	56	34	5/7/2002	12,4	8,9	5,8	96	85	5/8/2002	15,1	2,9	0	71	49
6/5/2002	27,5	5,8	0	37	17	6/6/2002	17,7	10,5	0	50	50	6/7/2002	12,8	7,4	9	90	92	6/8/2002	14,1	7,9	1,2	89	68
7/5/2002	27,1	5,5	0	43	14	7/6/2002	16,8	7,1	0	60	33	7/7/2002	13,6	8,1	2,8	100	66	7/8/2002	14,5	8,8	0	84	62
8/5/2002	27,8	7,5	0	21	11	8/6/2002	13	7,2	1,4	87	72	8/7/2002	16,7	5,5	0	38	32	8/8/2002	16,7	2,2	0	78	56
9/5/2002	18,8	12	1,6	91	74	9/6/2002	14,9	9	2,2	97	83	9/7/2002	13,3	9,9	0,2	96	52	9/8/2002	18,7	3,1	0	80	36
10/5/2002	21,2	7,8	3,4	99	47	10/6/2002	15,4	10,6	4,6	88	70	10/7/2002	12,9	-0,9	0,8	80	49	10/8/2002	22,2	4,7	0	52	21
11/5/2002	23,2	8,3	0	72	26	11/6/2002	15,8	10,5	0	73	62	11/7/2002	19,4	2,5	0	53	20	11/8/2002	14,3	10,6	0	69	58
12/5/2002	23,8	9,3	0	35	31	12/6/2002	17,1	12,2	0	61	52	12/7/2002	18,6	2,9	0	92	31	12/8/2002	11	5,6	6,4	73	69
13/5/2002	16,3	11	1,2	82	63	13/6/2002	13,6	8,9	6,4	82	66	13/7/2002	10,1	6	0	74	88	13/8/2002	11,9	0,3	0,4	95	66
14/5/2002	17,8	8,6	0	56	44	14/6/2002	14,2	6,3	4,4	70	79	14/7/2002	12,9	2,3	9	82	68	14/8/2002	11,4	0,4	0	89	61
15/5/2002	21,8	10,8	0,4	83	90	15/6/2002	15,5	6,9	10	88	74	15/7/2002	13,8	4,9	0	78	55	15/8/2002	13,4	1,3	0,2	71	48
16/5/2002	15,8	11,4	3,6	94	47	16/6/2002	15	9,1	3,4	96	65	16/7/2002	14	4,7	0,6	82	61	16/8/2002	15	1	0	69	33
17/5/2002	15,3	2,9	2	84	49	17/6/2002	14,9	6,2	0,2	99	59	17/7/2002	13,6	7,7	1,8	97	75	17/8/2002	13,9	4,4	0	66	54
18/5/2002	13,1	6,4	0,2	74	66	18/6/2002	11,5	5,3	0	82	44	18/7/2002	16,4	8,2	1	89	56	18/8/2002	13,1	4,2	0	69	45
19/5/2002	11,5	6	34,2	95	83	19/6/2002	13,9	0,8	0,2	61	44	19/7/2002	14,9	5,8	0	74	52	19/8/2002	13	-1,7	0	79	47
20/5/2002	14,6	7	22	88	55	20/6/2002	10,9	5,7	0	85	96	20/7/2002	13,7	9,7	0	94	49	20/8/2002		-0,3	0	91	52
21/5/2002	13,1	8,9	0	89	59	21/6/2002	14	5,9	3,6	97	71	21/7/2002	13,8	0,1	0	89	49	21/8/2002	15,7		0		43
22/5/2002	15,2	3,7	0	83	58	22/6/2002	13,1	8,7	2	99	75	22/7/2002	16,8	6	0	76	53	22/8/2002	14,8	0	0	79	54
23/5/2002	13	7,8	0	93	73	23/6/2002	13,9	9,3	0,2	86	72	23/7/2002	16,7	5,4	0	74	43	23/8/2002	12,2	5,1	0	64	42
24/5/2002	14,9	8,2	0	59	46	24/6/2002	14,8	3	0,2	79	47	24/7/2002	13,8	8,1	0	85	55	24/8/2002	12,3	1,9	0	58	45
25/5/2002	16,4	1,5	0	74	52	25/6/2002	14,4	7,5	0	65	52	25/7/2002	13,4	5,8	0	88	66	25/8/2002	15,9	0,4	0	61	26
26/5/2002	16,3	6,7	0	75	43	26/6/2002	14,3	3,4	0,4	99	52	26/7/2002	15,7	5	0,8	93	54	26/8/2002	19,7	-0,3	0	39	20
27/5/2002	14,1	4,8	0	76	77	27/6/2002	10,4	4,4	0,4	90	77	27/7/2002	21,7	-0,5	0,2	72	36	27/8/2002	20,6	4,9	0	30	17
28/5/2002	14,5	3,4	0,2	76	48	28/6/2002	11,2	2	10	72	63	28/7/2002	10,1	6,2	0,2	93	97	28/8/2002	14,2	9,4	0,6	79	53
29/5/2002	14,7	2	0	86	47	29/6/2002	10,6	0,7	0	68	55	29/7/2002	13,1	0,1	11,2	83	58	29/8/2002	13	2,6	2,4	82	58
30/5/2002	14,5	0,1	0	83	64	30/6/2002	11,6	-1,9	0	72	44	30/7/2002	14,5	0,3	0,2	97	49	30/8/2002	16,9	1,8	0,4	80	51
31/5/2002	13,6	7,5	3,4	99	74							31/7/2002	18	4,6	0	59	41	31/8/2002	17,4	4,6	0,4	71	53
May-02	18,8	7,1	72,2	69,1	47,9	Jun-02	14,4	5,6	51,6	79,7	58,6	Jul-02	14,6	4,7	50,0	80,6	56,1	Aug-02	14,7	3,3	27,6	73,8	49,8
1/9/2002	19,2	5,7	0	93	37	1/10/2002	24,1	3,8	0	25	12	1/11/2002	29,6	8,9	0	46	12	1/12/2002	29,9	10	0	44	11
2/9/2002	24,5	9,8	0	35	41	2/10/2002	23,3	8,4	0	13	11	2/11/2002	26,4	15,3	0	28	36	2/12/2002	23,5	17,3	0	52	47
3/9/2002	17,7	9,8	7	80	50	3/10/2002	15	9,4	1	63	64	3/11/2002	18,5	9,8	1	67	41	3/12/2002	22	8,3	0	58	34
4/9/2002	14,2	9,5	6,6	93	59	4/10/2002	11,8	7,6	2,6	89	90	4/11/2002	18,5	3,2	0,2	55	40	4/12/2002	17,6	10,2	0,6	69	41
5/9/2002	21,1	2,3	0,2	69	27	5/10/2002	14,5	9,2	0,4	87	71	5/11/2002	22,8	2,1	0	57	10	5/12/2002	16,5	11,6	4,4	94	65
6/9/2002	14,1	7,7	2	67	53	6/10/2002	22,2	5,7	0	64	21	6/11/2002	30,9	5,5	0	38	6	6/12/2002	19,5	9,3	5,2	50	37
7/9/2002	15	10,1	0,8	84	89	7/10/2002	20	4,9	0,2	56	53	7/11/2002	33,3	12,6	0	6	2	7/12/2002	26,5	7,9	0	53	11
8/9/2002	16,7	7	1,8	89	57	8/10/2002	13,6	6,4	2,8	55	56	8/11/2002	28,4	9,4	0	48	18	8/12/2002	24,2	8,4	0	29	37
9/9/2002	12,4	9	2,4	72	59	9/10/2002	15,2	1,1	0,8	65	41	9/11/2002	28,6	5,3	0	40	20	9/12/2002	17,8	6,6	0	52	36
10/9/2002	12,5	0,8	0	67	53	10/10/2002	18,1	5,8	0	52	34	10/11/2002	27,2	11,5	0	74	19	10/12/2002	20,2	8,3	0	63	31
11/9/2002	15,2	-0,6	0,2	63	30	11/10/2002	23	6,8	0	51	19	11/11/2002	36,1	13,4	0	31	11	11/12/2002	21,4	6,6	0	66	42
12/9/2002	19,8	3,9	0	29	18	12/10/2002	26	6,8	0	18	10	12/11/2002	23,9	22,1	0	52	82	12/12/2002	22,6	8,6	0	56	35
13/9/2002	22	5	0	41	29	13/10/2002	16,7	7,5	0,6	59	35	13/11/2002	20,3	6,7	3	52	29	13/12/2002	25,4	7	0	59	27
14/9/2002	26,4	8,9	0	28	14	14/10/2002	17,3	8	0,6	91	58	14/11/2002	21,7	3,7	0	63	35	14/12/2002	32,2	10,4	0	41	9
15/9/2002	24,9	15,9	0	31	25	15/10/2002	25	5	0,2	62	19	15/11/2002	25,8	9,5	0	60	24	15/12/2002	38,7	11,7	0	18	5
16/9/2002	13,9	10,1	5,2	91	60	16/10/2002	26,3	10,4	0	13	31	16/11/2002	32,3	12,8	0	25	7	16/12/2002	40,2	23	0	14	2
17/9/2002	12,9	4,5	6	87	60	17/10/2002	24	11,6	1	75	44	17/11/2002	35,9	14,7	0	7	6	17/12/2002	40,2	22,5	0	24	5
18/9/2002	15,5	0,7	3,4	92	71	18/10/2002	31,7	8	0	25	46	18/11/2002	29,9	11,6	0	47	25	18/12/2002	35	23,7	0	36	11
19/9/2002	14,1	9	3,4	80	62	19/10/2002	18,6	9,2	0	61	41	19/11/2002	27,4	11,9	0	40	35	19/12/2002	38,7	22,4	0	24	11
20/9/2002	21,5	1,5	0	71	37	20/10/2002	19,6	5,1	0	59	37	20/11/2002	21,2	12,3	1,4	71	46	20/12/2002	36	21,5	0	15	10
21/9/2002	24,4	5,1	0	52	33	21/10/2002	24,3	2,6	0	56	25	21/11/2002	26,9	9,2	0	50	19	21/12/2002	40,2	21,2	0	24	8
22/9/2002	18,7	8,4	0	81	52	22/10/2002	26,6	11,4	0	15	52	22/11/2002	26,9	8,1	0	30	17	22/12/2002	26,2	14,6	0	55	26
23/9/2002	24,5	9,4	0	72	22	23/10/2002	15,1	8,2	8	89	58	23/11/2002	35,8	17,2	0	12	10	23/12/2002	24,1	9,5	0	51	22
24/9/2002	17,6	9,5	0	72	36	24/10/2002	20,4	9	1,4	66	37	24/11/2002	28,5	19	0,4	52	61	24/12/2002	25,2	9,1	0	56	26
25/9/2002	16,5	7,7	0,2	60	57	25/10/2002	16,8	4,6	1	76	54	25/11/2002	24,1	16,1	2,8	90	95	25/12/2002	26,6	10,4	0	59	19
26/9/2002	17,5	1,8	1	59	33	26/10/2002	18,3	5,6	0	48	25	26/11/2002	16,2	11,3	11,6	85	54	26/12/2002	29,1	11,8	0	39	9
27/9/2002	18	2,3	0	35	44	27/10/2002	20,6	5,4	0	61	30	27/11/2002	21	11,3	0,2	63	52	27/12/2002	31,5	13,7	0	32	14
28/9/2002	12,3	5,1	5,6	78	77	28/10/2002	24,3	8,1	0	54	17	28/11/2002	28,3	10,4	0	69	25	28/12/2002	36,6	18,4	0	21	7
29/9/2002	14,4	-0,4	1,2	72	71	29/10/2002	31,3	12,3	0	15	6	29/11/2002	24,2	13,2	0	64	34	29/12/2002	33,5	23,7	0	24	24
30/9/2002	17,8	0,2	0	73	35	30/10/2002	19,9	10,9	0,2	72	52	30/11/2002	23,2	10,3	0	58	25	30/12/2002	34,7	20	0	38	90
						31/10/2002	23,5	9,3	0	60	25							31/12/2002	18,2	13,5	22	77	70
Sep-02	17,8	6,0	47,0	67,2	46,4	Oct-02	20,9	7,4	20,8	54,7	37,9	Nov-02	26,5	10,9	20,6	49,3	29,9	Dec-02	28,2	13,5	32,2	44,9	26,5

APPENDIX E: TEMPERATURE, PRECIPITATION AND RELATIVE HUMIDITY AT NURIOOTPA, SA.

APPENDIX E4: (2003): Max or Min °C = mean ambient temperature; Rain = precipitation (mm); RH am/pm = relative humidity at 9 am/3 pm

Date	Max°C	Min°C	Rain	RH am	RH pm	Date	Max°C	Min°C	Rain	RH am	RH pm	Date	Max°C	Min°C	Rain	RH am	RH pm	Date	Max°C	Min°C	Rain	RH am	RH pm
1/1/2003	17	10.2	4.2	86	53	1/2/2003	25.1	7.1	0	74	36	1/3/2003	18.3	10	0.4	83	41	1/4/2003	26.5	10.5	0	41	22
2/1/2003	23	8.6	0.2	79	49	2/2/2003	28.5	12.2	0	55	25	2/3/2003	19	8.5	0	78	43	2/4/2003	23.7	15.5	0.4	68	49
3/1/2003	25.2	11	0	87	36	3/2/2003	30.6	11.2	0	30	15	3/3/2003	20.4	10.5	0	68	45	3/4/2003	19	11.4	0	86	31
4/1/2003	26.7	10.1	0	62	12	4/2/2003	30.5	23.8	0	19	13	4/3/2003	22	12.3	0	71	50	4/4/2003	19.4	7	0	56	25
5/1/2003	33.5	13.9	0	40	11	5/2/2003	29.2	20.1	0.6	67	44	5/3/2003	23.9	10.5	0	73	40	5/4/2003	25	2.9	0	40	17
6/1/2003	37.2	12.8	0	17	4	6/2/2003	33	12.5	0	66	25	6/3/2003	26.4	12.3	0	50	22	6/4/2003	28.4	5.8	0	30	21
7/1/2003	38.3	16.2	0	20	12	7/2/2003	29.2	11.6	0	55	31	7/3/2003	29	9.8	0	46	10	7/4/2003	23.3	11.4	0	22	32
8/1/2003	23.7	9	0	48	23	8/2/2003	27.6	11.7	0	66	23	8/3/2003	22.2	11.4	0	66	27	8/4/2003	16.8	10	2.2	85	73
9/1/2003	25.1	6.6	0	52	24	9/2/2003	31.6	12.4	0	54	14	9/3/2003	23.1	9	0	64	32	9/4/2003	19.6	10.1	0	57	44
10/1/2003	29.1	12.3	0	56	21	10/2/2003	28.1	13.3	0	64	22	10/3/2003	24.4	10.5	0	77	36	10/4/2003	16.3	12.1	0	41	81
11/1/2003	33.4	18	0	30	13	11/2/2003	31.4	13.3	0	54	19	11/3/2003	27.9	13.9	0	68	33	11/4/2003	21.2	9.1	3.2	88	51
12/1/2003	36.9	20.8	0	19	8	12/2/2003	28.3	18.2	0	31	51	12/3/2003	31.2	13.2	0	59	22	12/4/2003	24	5.8	0	74	43
13/1/2003	31.8	25.6	0	10	42	13/2/2003	31.4	15.9	0	28	12	13/3/2003	29.7	12	0	61	22	13/4/2003	20	11.9	7.6	65	32
14/1/2003	23.6	9.7	0	54	28	14/2/2003	33.6	16.7	0	34	18	14/3/2003	22.4	10.9	0	63	33	14/4/2003	16.7	11.6	1.8	89	60
15/1/2003	26.3	10.7	0	51	22	15/2/2003	33.1	21.1	0	21	21	15/3/2003	21.1	10.3	0	71	50	15/4/2003	18.1	12.6	2.4	96	53
16/1/2003	31	14.2	0	47	19	16/2/2003	28.1	19.3	0.4	73	46	16/3/2003	27.2	9.7	0	74	29	16/4/2003	16.3	6.5	0.8	65	35
17/1/2003	38.6	19.8	0	21	3	17/2/2003	26	13.7	4	76	34	17/3/2003	32.8	8.6	0	52	15	17/4/2003	18	7.4	0	64	34
18/1/2003	34.3	11.3	0	27	15	18/2/2003	26.8	14	0	48	16	18/3/2003	35.2	12.3	0	42	12	18/4/2003	20.9	7	0	44	27
19/1/2003	39.9	19.2	0	24	10	19/2/2003	19.1	14.5	0	39	80	19/3/2003	17.8	12.5	2	96	46	19/4/2003	23.2	4.1	0	47	26
20/1/2003	35.6	24.1	0.2	64	23	20/2/2003	20	15.4	21.9	96	97	20/3/2003	18.3	7	0.6	76	55	20/4/2003	24.3	5.9	0	47	26
21/1/2003	28.8	15.1	0	50	27	21/2/2003	21.3	16.1	40	99	89	21/3/2003	17.3	10.6	0	75	42	21/4/2003	24.9	7.8	0	35	22
22/1/2003	29	12.3	0	60	23	22/2/2003	23.2	16.3	0.4	94	73	22/3/2003	18.9	7.4	0.2	96	52	22/4/2003	26.3	7.7	0	48	23
23/1/2003	33	13	0	37	6	23/2/2003	27.6	14.5	0			23/3/2003	19.6	8.6	0	82	42	23/4/2003	26.4	8.6	0	44	29
24/1/2003	40	19.9	0	33	7	24/2/2003	31.4	16.3	0	69	44	24/3/2003	25.5	6.5	0	74	30	24/4/2003	26	10	0	26	26
25/1/2003	44.1	18.1	0	19	2	25/2/2003	29.3	17	0	74	52	25/3/2003	25.4	5.3	0	70	22	25/4/2003	23.1	7.1	0	63	26
26/1/2003	29	20.4	0	60	41	26/2/2003	30.6	17.3	0	57	43	26/3/2003	26.5	11.9	0	65	35	26/4/2003	24.4	9.8	0	44	28
27/1/2003	28.7	12.9	0	53	16	27/2/2003	26.3	14.3	0	67	39	27/3/2003	24.7	13.1	0	71	35	27/4/2003	25.7	6.4	0	50	18
28/1/2003	30.3	15.2	0	36	16	28/2/2003	18.6	12.8	0.6	72	49	28/3/2003	22.2	10.2	0	75	38	28/4/2003	21.8	9.3	0	49	58
29/1/2003	40	19.2	0	15	11							29/3/2003	23.8	10	0	70	37	29/4/2003	19.2	3.9	0	78	55
30/1/2003	20.8	15.2	1.2	96	36							30/3/2003	22	10.7	0	71	35	30/4/2003	18.1	4.6	0	87	90
31/1/2003	23.1	7.3	0.4	53	31							31/3/2003	23.4	11.3	0	56	37						
Jan-03	30.9	14.6	6.2	45.4	20.8	Feb-03	28.4	15.1	67.9	58.6	38.2	Mar-03	23.9	10.3	3.2	68.5	34.5	Apr-03	21.9	8.5	18.4	57.0	38.6
1/5/2003	15.8	7.3	7.6	72	52	1/6/2003	18.7	8.5	0	71	38	1/7/2003	13.1	6.5	5.8	100	73	1/8/2003	13.4	-0.8	0.2	72	56
2/5/2003	17.1	4.8	0	66	51	2/6/2003	17.8	11.4	0	88	44	2/7/2003	13.4	3.9	2	99	79	2/8/2003	16.3	-0.2	0	51	43
3/5/2003	17.3	11.8	0	65	65	3/6/2003	16.5	9.1	0	88	57	3/7/2003	14.5	2.9	0.6	96	72	3/8/2003	15.9	3.4	0	47	30
4/5/2003	16	8	0	70	34	4/6/2003	16.1	6.4	0.4	57	61	4/7/2003	14.5	7.2	0.8	97	65	4/8/2003	17.5	3.8	0	45	37
5/5/2003	20.2	3.7	0	37	17	5/6/2003	14.1	6.8	14	92	66	5/7/2003	16.7	5.9	0	53	39	5/8/2003	11.1	7.6	4	90	88
6/5/2003	21.4	8.1	0	38	29	6/6/2003	14.5	9.6	0.8	65	95	6/7/2003	16.4	2.3	0	58	41	6/8/2003	13.5	6.9	1	96	64
7/5/2003	23.6	9.6	0	44	29	7/6/2003	13.4	6.3	9	86	59	7/7/2003	15.9	9.6	0	53	50	7/8/2003	14	4.2	0.2	81	63
8/5/2003	22.6	3.4	0	55	26	8/6/2003	12.5	8.3	0	74	58	8/7/2003	13.6	4.7	0	95	91	8/8/2003	14.1	1	0.2	87	63
9/5/2003	24.8	6.3	0	72	20	9/6/2003	12.9	2.6	0	85	59	9/7/2003	13.5	3.1	5.2	100	88	9/8/2003	10.8	6.2	3.6	85	57
10/5/2003	16.5	12.2	0.4	76	89	10/6/2003	14.5	0.8	0	78	45	10/7/2003	15.9	4.3	0.2	83	51	10/8/2003	8	0.9	0	66	75
11/5/2003	17	12.4	5.2	79	71	11/6/2003	15.9	0.1	0	82	44	11/7/2003	18.3	3.5	0	57	37	11/8/2003		3.1	13.8	92	74
12/5/2003	15.7	10.3	0.4	88	83	12/6/2003	13.1	3.7	0.2	72	72	12/7/2003	12.9	7.1	8	78	65	12/8/2003	16.1	5	0.2	77	68
13/5/2003	18.4	12	1.8	85	61	13/6/2003	13.5	6.9	3	83	78	13/7/2003	13.7	5.6	0.4	100	73	13/8/2003	12.2	8.7	7.2	98	94
14/5/2003	16	10.5	2.8	95	67	14/6/2003	12.5	7.6	2	95	82	14/7/2003	16	2.6	0	70	42	14/8/2003	11.5	3.2	6	78	61
15/5/2003	17.2	6.2	0	99	68	15/6/2003	12.5	8	1	97	91	15/7/2003	17.4	6	0	66	87	15/8/2003	10.5	5.3	0	66	51
16/5/2003	17.2	7.2	0.4	94	72	16/6/2003	13.7	8.2	1	99	70	16/7/2003	11.7	7.9	0	89	78	16/8/2003	12.7	0.5	0.2	65	54
17/5/2003	20	10.2	0	85	52	17/6/2003	12.8	4.8	0.4	99	79	17/7/2003	12.1	3.1	0	81	58	17/8/2003	14.9	1.3	0	62	59
18/5/2003	18.1	12.4	3.4	80	59	18/6/2003	12.4	6.9	0	82	88	18/7/2003	12.3	3.7	0	84	56	18/8/2003	10.7	2.4	1.6	80	52
19/5/2003	14.9	12	3	84	69	19/6/2003	11.1	7.3	2	85	75	19/7/2003	11.2	3.2	0	81	65	19/8/2003	11.5	2.1	0	82	52
20/5/2003	16.2	10.4	15.6	96	83	20/6/2003	11.5	6.7	0	96	72	20/7/2003	13.5	0.3	0	81	57	20/8/2003		4.8	0	57	67
21/5/2003	13.8	8.6	3.4	68	51	21/6/2003		6.7	1	93	87	21/7/2003	17.2	3.8	0	66	41	21/8/2003	14.6	5.5	18.6	76	62
22/5/2003	15.6	6.7	0	67	59	22/6/2003	13.5		2		61	22/7/2003	14.4	8.1	0.6	80	48	22/8/2003	16.1	6.8	2	79	50
23/5/2003	15.6	9.4	1.6	89	80	23/6/2003	11.8	-0.6	0	95	61	23/7/2003	10.4	6.9	0	75	63	23/8/2003	11.3	8	12.2	96	90
24/5/2003	16.8	7.1	2.4	94	89	24/6/2003	13.5	3.9	0	80	54	24/7/2003	11.2	5.4	10.8	88	85	24/8/2003	12.3	7.1	21	91	95
25/5/2003	15.4	6.6	10.4	80	64	25/6/2003	12.4	3.2	0	83	58	25/7/2003	10.4	5.9	13.2	90	67	25/8/2003	12.7	8.2	4.6	94	56
26/5/2003	12.8	8.7	0.2	64	53	26/6/2003	15.9	4.1	0	62	44	26/7/2003	10.3	0.1	2.4	95	60	26/8/2003	11.7	2.6	0.6	86	63
27/5/2003	13.9	2.6	0	87	64	27/6/2003	14.8	9.1	11	92	91	27/7/2003	14.7	1.5	0	60	46	27/8/2003	13	4.9	1	96	77
28/5/2003	15.4	1.7	0.2	87	51	28/6/2003	14.8	8.5	11	94	89	28/7/2003	15.4	7.1	0	44	80	28/8/2003	15.1	3.9	0.6	87	47
29/5/2003	18	1.9	0.2	69	44	29/6/2003	13.9	10.2	13	97	74	29/7/2003	11.3	1.9	5.6	95	56	29/8/2003	16.6	5.8	0	48	35
30/5/2003	18.6	3.3	0	75	38	30/6/2003	11.8	9.3	0.6	97	92	30/7/2003	10.7	-0.9	0.6	97	62	30/8/2003	10.9	4.7	5.8	83	56
31/5/2003	19.1	7	0	59	57							31/7/2003	11.5	-1.3	0	88	62	31/8/2003	12.3	6.3	1.8	93	59
May-03	17.5	7.8	59.0	74.8	56.4	Jun-03	13.9	6.4	72.4	84.4	68.1	Jul-03	13.7	4.3	56.2	80.6	61.8	Aug-03	13.1	4.3	106.4	77.0	61.0
1/9/2003	11.7	1.1	2	81	71	1/10/2003	14.4	6.5	6.8	95	93	1/11/2003	14.6	7.6	2.6	83	67	1/12/2003	23	13.4	0	65	41
2/9/2003	12.1	7.3	1.4	97	82	2/10/2003	14.1	9	13.8	87	62	2/11/2003	18	5.9	2	77	42	2/12/2003	29.2	12.9	0	51	30
3/9/2003	15.5	6.7	0.2	74	60	3/10/2003	13.4	7	0.2	70	77	3/11/2003	21.4	3.8	0.2	61	31	3/12/2003	30.8	12.8	0	46	24
4/9/2003	18.1	3.3	0	64	33	4/10/2003	14.6	6.4	0	80	50	4/11/2003	27.3	8.6	0	34	12	4/12/2003	20.5	17.1	0	90	79
5/9/2003	16	7.4	0	81	59	5/10/2003	17.3	3.6	0.2	66	46	5/11/2003	26.4	10.7	0	36	16	5/12/2003	19.6	10.9	2.4	76	39
6/9/2003	14.3	8.9	1	91	63	6/10/2003	16.7	6.9	0.4	84	58	6/11/2003	25.4	13.9	0	41	30	6/12/2003	23.7	8.2	0	55	51
7/9/2003	15.8	7	0.2	85	52	7/10/2003	16.9	4.1	0.2	93	56	7/11/2003	24.6	8.8	0	61	32	7/12/2003	30.9	8.4	0	46	14
8/9/2003	18.6	7.9	0	63	46	8/10/2003	14.2	8.9	2.6	75	73	8/11/2003	25.7	8.8	0	66	29	8/12/2003	32.1	12.5	0	43	15
9/9/2003	18.3	9.1	0	63	63	9/10/2003	13.3	1.3	0.2	58	45	9/11/2003	25	9.1	0	72	34	9/12/2003	38.5	17.4	0	25	10
10/9/2003	17.6	4.5	0	89	50	10/10/2003	13	5	0	62	63	10/11/2003	27.5	10.6	0	62	32	10/12/2003	23.4	13.2	0.4	79	35
11/9/2003	14.3	7	0.2	89	82	11/10/2003	14.6	3.6	0	58	39	11/11/2003	26.8	11.9	0	58	31	11/12/2003	23	12.5	0.4	89	37
12/9/2003	15.3	6.9	14.6	81	59	12/10/2003	16.6	4.1	0	63	42	12/11/2003	25.6	10.8	0	85	18	12/12/2003	21.1	12.3	0	62	46
13/9/2003	16.1	5.5	0.8	33	86	13/10/2003	21.2	3.2	0	37	22	13/11/2003	29.6	11.7	0	45	11	13/12/2003	22.5	12.5	0	74	42
14/9/2003	10.8	5.3	16.2	77	76	14/10/2003	26	4.6	0	33	13	14/11/2003	35.7	10.8	0	11	8	14/12/2003	31.3	11.5	0	62	15
15/9/2003	12.7	6	2.4	83	92	15/10/2003	17.4	12.1	0	82	50	15/11/2003	38.5	12.3	0	21	4	15/12/2003	35.3	12.6	0	23	7
16/9/2003	13.1	9	9	95	89	16/10/2003	18.4	6.1	0	71	48	16/11/2003	24.2	11.6	0	63	18	16/12/2003	39.8	17.9	0	16	7
17/9/2003	13.8	9.6	8.4	95	83	17/10/2003	24.6	8.8	0	51	22	17/11/2003	28.9	13	0	32	13	17/12/2003	29.7	25.4	0	32	28
18/9/2003	15.9	9.7	2.8	94	64	18/10/2003	30.8	9.5	0	27	19	18/11/2003	32.6	15.2	11.2	86	32	18/12/2003	32.7	18.4	0	52	32
19/9/2003	15.6	7.7	0.4	86	59	19/10/2003	15.7	7.7	0.4	47	56	19/11/2003	36.9	19	0	44	24	19/12/2003	35	20.6	0	44	70
20/9/2003	23.2	6.6	0	63	34	20/10/2003	19.3	8.8	0	73	49	20/11/2003	27.3	16.2	2	96	29	20/12/2003	29.3	16.7	8.2	80	50
21/9/2003	22.3	11.7	0.4	76	46	21/10/2003	15.1	10.4	0	70	77	21/11/2003	23	13	0	57	36	21/12/2003	26.1	20.9	13.4	88	81
22/9/2003	27.6	7.6	0	41	59	22/10/2003	17.9	5.6	5.8	74	40	22/11/2003	17.2	9.3	0	59	45	22/12/2003	20	12.5	8.2	89	61
23/9/2003	24.6	5	0	54	33	23/10/2003	16.6	10.6	1.4	71	45	23/11/2003	18.8	8.6	0	69	39	23/12/2003	25.1	9.7	0	64	33
24/9/2003	18.5	8	0.2	69	34	24/10/2003	18.1	2.7	0	59	41	24/11/2003	22.6	8.5	0	63	32	24/12/2003	32.3	10	0	50	11
25/9/2003	17.1	10.1	0	73	91	25/10/2003	15.8	6.6	0	59	51	25/11/2003	26.2	10.8	0	53	22	25/12/2003	30.4	11	0	19	22
26/9/2003	14.4	7.1	2.4	90	55	26/10/2003	16.3	5.5	0	70	42	26/11/2003	28.4	13.2	0	35	14	26/12/2003	26.3	12.5	0	68	33
27/9/2003	13.7	3.7	4.6	58	51	27/10/2003	18.1	5.8	0	55	33	27/11/2003	32.3	16.8	0	21	11	27/12/2003	25.6	10.2	0	47	23
28/9/2003	19.2	2.4	0	43	32	28/10/2003	20.4	9.9	0	47	77	28/11/2003	30.8	18.2	0	14	11	28/12/2003	32.1	13.1	0	39	22
29/9/2003	12.8	3.6	0	66	50	29/10/2003	15.5	7.5	3.4	76	39	29/11/2003	30.3	21.3	0	22	21	29/12/2003	36.2	18.4	0	13	32
30/9/2003	13.2	3.7	0	56	27	30/10/2003	20.9	2.5	0	59	26	30/11/2003	23.4	14.6	0.4	71	51	30/12/2003	32.4	22.1	1.2	26	47
						31/10/2003	14.2	7.1	7.4	87	51							31/12/2003	34.4	15.4	0	30	21
Sep-03	16.4	6.6	67.2	74.3	59.4	Oct-03	17.5	6.5	42.8	65.1	48.5	Nov-03	26.5	11.8	18.4	53.3	26.5	Dec-03	28.8	14.3	34.2	52.4	34.1

APPENDIX F: STOMATAL CONDUCTANCE OF POTTED CABERNET SAUVIGNON IN THE 2003 VINTAGE

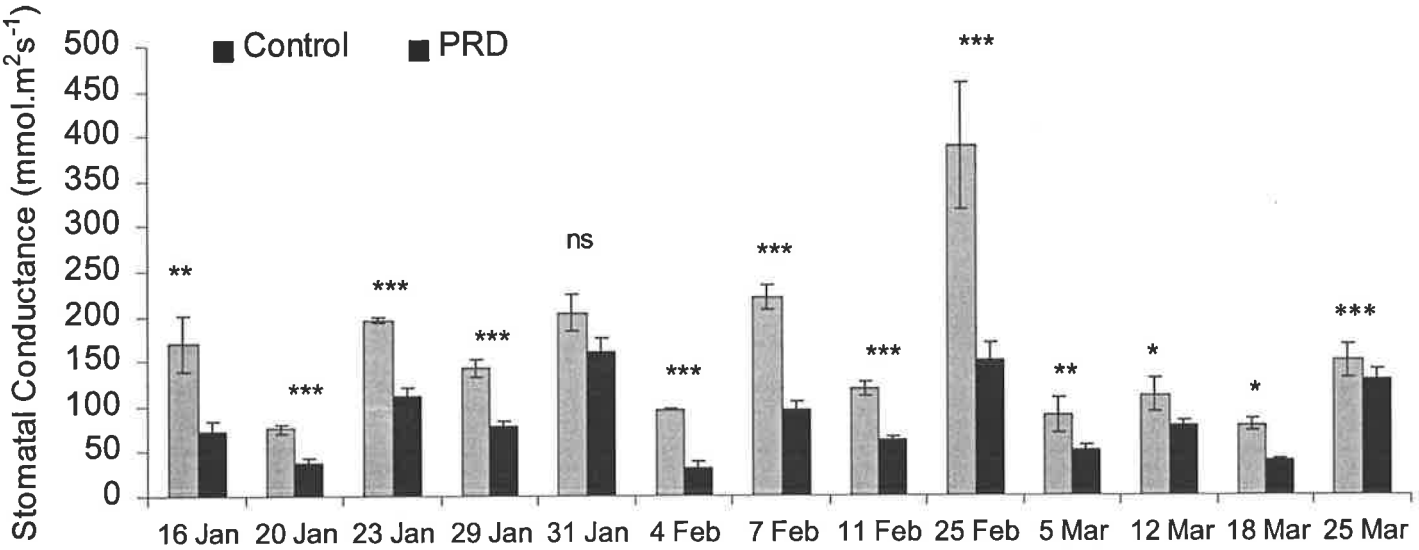

Figure F: Effect of PRD on stomatal conductance of leaves of potted Cabernet Sauvignon over the 2002/2003 season. Data were courtesy of G. du Toit, Adelaide University (T-test; n=11; *=P<0.05; **=P<0.01;***=P<0.001).

APPENDIX G: FRUIT COMPOSITION OF SHIRAZ CLONES

Table G: Fruit composition of four Shiraz clones grown at Nuriootpa in 2000. There were no treatments imposed on the vines, which were pruned and irrigated according to standard practice for the region (M. McCarthy pers comm. 2004).

	Berry weight (g)	Harvest TSS (°Brix)	Anthocyanin (mg/g)	Phenolics (A_{280}/g)
BVRC12	1.2	24.7	1.85	1.53
121S	1	26.5	2.5	1.5
PT15N	1.23	25.6	1.63	1.31
1125S	1.1	26.5	1.85	1.49

APPENDIX H: SHOOT AND VINE LEAF AREA MEASUREMENTS

Leaf area was determined at veraison and harvest in only one season (2000-2001) in the Nuriootpa Shiraz experiment (Section 2.1.1.1, Chapter 2). Leaf area per shoot was determined non-destructively. A sample of six main shoots of differing shoot lengths was selected at random from a buffer vine on each test plot. For each leaf, the length (cm) of the main lobe was measured from the petiolar point to tip of lobe. This was also determined for leaves from lateral shoots of each main shoot sample. Leaf area (A) of the Shiraz leaves was calculated from the length of the main lobe (L) from an equation derived by Dry (1997) for mature Shiraz leaves: $A = 0.798(L)^2 + 3.63(L)$. The sum of individual leaf areas was pooled to give a total leaf area per main shoot. Average shoot length for each of the treatments was measured from six representative shoots per plot at veraison and harvest. The total number of shoots was counted after leaf fall, prior to winter pruning. For each pruning level and irrigation treatment within the Shiraz experiment, the relationship between shoot length and leaf area was calculated graphically using a polynomial regression, and average leaf area per shoot was estimated from the derived equation using the calculated average shoot length per vine. Leaf area per vine was then estimated by multiplying average shoot length per vine and average shoot number per vine.

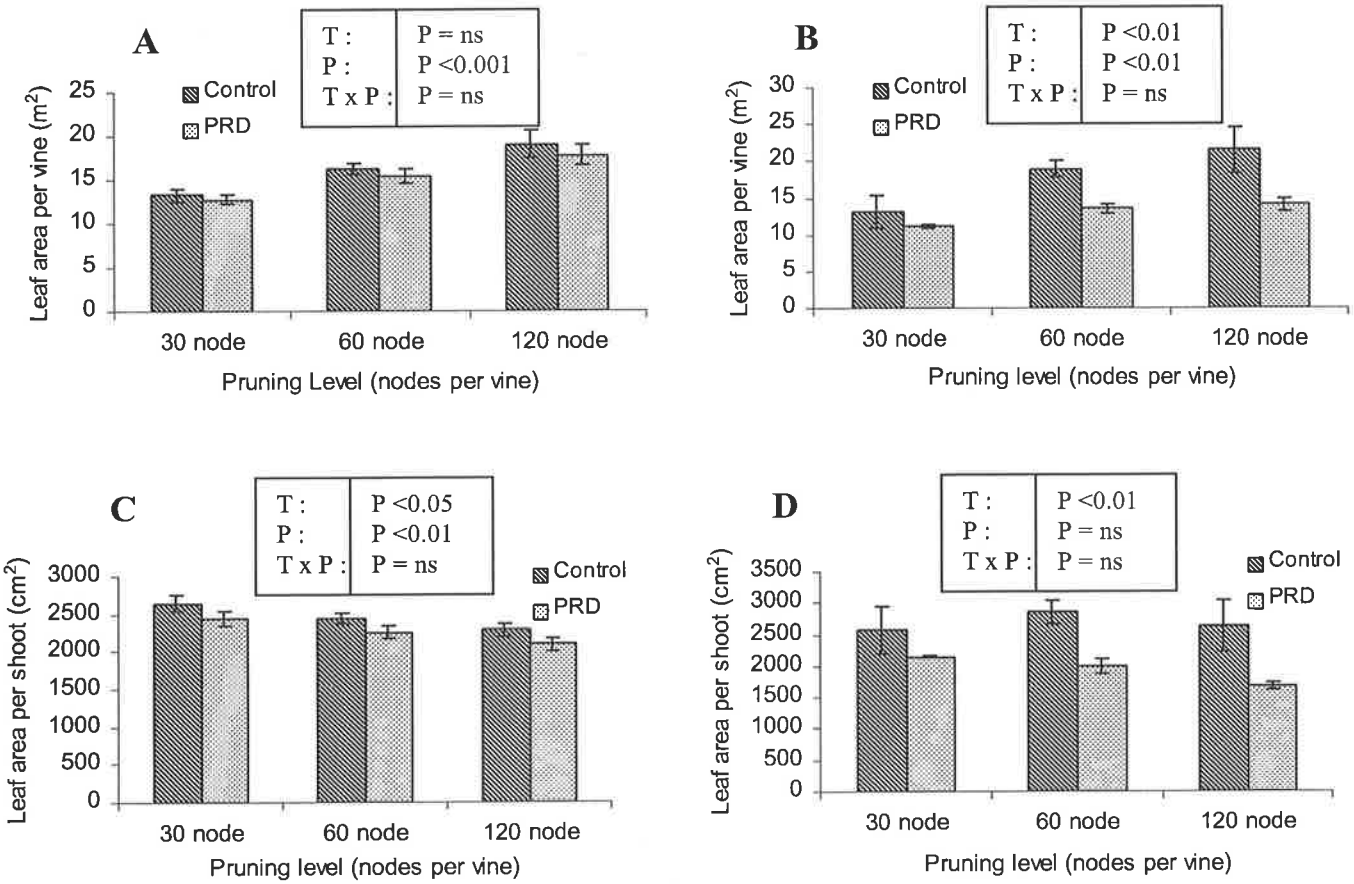

Figure H: Effect of node number per vine and PRD on leaf area in Shiraz grown at Nuriootpa in 2001, where PRD received half the irrigation water of the control. **A**. Leaf area per vine at veraison; **B**. Leaf area per vine at harvest; **C**. Leaf area per shoot at veraison; **D**. Leaf area per shoot at harvest (ANOVA, n=30, T = irrigation treatment; P = pruning level; T x P = interactive effect; ns = not significant).