Optimizing irrigation and response to abiotic stress through development of biochemical, physiological and molecular markers of vine performance.

FINAL REPORT to

GRAPE AND WINE RESEARCH & DEVELOPMENT CORPORATION

Project Number: **CSP 06/03**

Principal Investigator: **Dr Jim Speirs**

Research Organisation: **CSIRO Plant Industry**

Date: **July 2010**

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June 2006 – June 2010

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1. Abstract

In Australian viticulture most vines are periodically exposed to varying degrees of soil water deficit, that is less water than they can potentially use. This is either done deliberately, as in the application of RDI or PRD or as result of undesired reductions in soil water due to water supply restrictions. So that we can optimize the management of deficit irrigation and better respond to water restrictions it is important that we gain a better understanding of the ways that vines respond to water deficits, in particular knowing how roots communicate to the leaves information about soil conditions and whether this communication varies in different rootstocks. By studying the genes that control the synthesis of the chemical substances responsible for regulating water loss and photosynthesis we have shown that during periods of soil water deficit, despite major changes in leaf water status, the primary source of these regulating chemicals is the roots and that rootstocks differ in their ability to supply these chemicals. The work has also begun to unravel the mechanisms that allow leaves to respond very quickly to changes in their environment to improve their efficiency of water use.

2. Executive summary

In order to study the root to leaf hormone signalling pathway, it is crucial to obtain samples that accurately represent the composition of the xylem sap and we tested a number of methods for sampling xylem sap from grapevines so that we could be confident that our methods are the most appropriate. Our studies have centred on the substance abscisic acid (ABA) which is universally present in plant tissues and is primarily responsible for regulating vine water loss through the stomata. Using a leaf pressure bomb to expel petiole sap is the most commonly used method for the routine assessment of xylem-mobile bio-active molecules in field vines. It is important to recognise the factors which may influence their concentration in expressed sap and to gain an understanding of their role in determining vine response to soil water deficits and the environment. The concentration of ABA and its metabolites is higher in petiole sap collected with a leaf pressure chamber than their concentration in sap expelled from the distal half of the same petiole through the application of pneumatic pressure to the roots, suggesting that apoplastic sources contribute to solutes in petiole sap. Even though petiole sap may not quantitatively reflect the sap as it leaves the roots it may be a reasonable representation of the solution that is actually in contact with the stomatal guard cells which ultimately control leaf transpiration. Petiole sap collected at a pressure within 0.2 MPa of the leaf water potential appears to be minimally affected by applied pressure. The concentration of ABA in sap from unstressed vines is not affected at all by the collection pressure but if the vines have been subject to water deficit, the sap collected at a pressure near the water potential has an elevated ABA content but as the pressure is increased the sap becomes progressively diluted so that the ABA content falls. Sap collected from stressed vines at a pressure as close as possible to the leaf water potential will therefore give the best result.

Grapevines typically have a very high leaf ABA content in comparison with many other species but most of this appears not to be available to the stomata. The relationship between xylem sap ABA concentration and stomatal conductance in grapevine is very similar to that in other species with vastly different leaf ABA contents, even under varying conditions of water potential, suggesting that this is a highly conserved relationship and that it is the predominant factor driving plant response to changes in soil water and the environment. Xylem sap ABA concentration always correlated strongly with stomatal conductance but xylem sap ABA also correlated strongly with leaf water potential. This could suggest a direct hydraulic effect on conductance but it is more likely that changes in leaf water potential are influencing apoplastic concentrations of ABA. This does not occur as a result of ABA biosynthetic activity within the range of leaf water

potentials normally encountered on a diurnal basis, but we speculate that this may be through an effect on leaf glucosidase activity liberating ABA from its glucose ester.
Soil water deficit strongly induces the activity of the genes involved in ABA biosynthesis (*ZEP*, *NCED#1* and *NCED #2*). The former is probably involved in the expression of the photoprotecting xanthophyll cycle in leaves whereas the *NCED* genes are induced in roots in response to soil water deficit. Moderate soil water deficit does not induce *NCED* genes in leaves despite changes in leaf water potential. This suggests that the primary response to water deficit occurs in the roots and results in the synthesis of ABA which is available for transport to the leaves via the xylem. More severe water stress resulting in leaf turgor loss may induce *NCED* activity in leaves for *in situ* synthesis.
Our data provide no strong evidence for root-to-shoot signalling compound(s) other than ABA during periods of moderate water deficit and during normal diurnal cycles. However, other conditions, not investigated here, such as severe water deficit or salt stress may induce other mechanisms which may involve other signally compounds.
Despite the documented effect of exogenous application of ABA on grape anthocyanins and sugars we could find no evidence for a link between the increased levels of berry ABA in stressed vines and the composition of those berries. Water deficit did, however, result in significant differences in wine sensory attributes when compared with wines from fully irrigated vines with increases in Dark berry, Dried Fruit, Cooked Fruit characters and reductions in Cough Syrup, Confection and Leaf Stalk characters. Whether there is any link between the production of signalling compounds and the expression of different sensory characters remains to be seen, but if such a link could be established it would open the possibility of targeted manipulation of grape and wine sensory properties.
The primary step in the degradation of ABA results in the formation of phaseic acid via 8'-hydroxy-ABA. The genes responsible for the 8'-hydroxylase enzyme are highly expressed in leaves but less so in roots. This is consistent with a scheme where ABA is synthesised in roots under the control of the *NCED* gene family, is transported to the leaves where it acts to regulate stomatal aperture and is then broken down in the leaves. Major day to day fluctuations in VPD were coincident with changes in the expression of the *ABA 8'-hydroxylase 1* gene, suggesting that this may be part of a regulatory system allowing leaves to respond rapidly to ambient conditions by changing the rate of degradation of ABA at its site of action. This response would have a major impact on the efficiency of water use by favouring high rates of assimilation under conditions or times of the day when there is low transpiration demand and restricting transpiration under hot, dry conditions when there is potential for high water loss.

In the absence of significant changes in leaf water potential, soil water deficit induces large reductions in stomatal conductance in Shiraz scions grafted to Ramsey and Schwarzmann rootstocks and less so in own-rooted Shiraz. The water deficit caused large increases in xylem sap ABA and the expression of *NCED* genes in the roots of Ramsey and Schwarzmann rootstocks but less so in the own-rooted Shiraz. These results suggest that the intensity of expression of genes responsible for ABA synthesis may be an important property defining the characteristics rootstocks are able to confer on their scion. This project has substantially enhanced our knowledge of the basic mechanisms that grapevines have evolved to optimize water use. It has identified points of difference between various rootstock and scions which could be useful in the development of new genotypes or explaining why some rootstock or scions are more or less effective under a range of soil or aerial environments. It also focuses attention on the environment we provide for the roots because it is the roots that are the primary responsive elements when water is restricted. Possibly the most important recommendation from this work is that viticulturists give urgent consideration to increasing the number of commercial vines grafted onto rootstocks displaying greater water use efficiency. This, coupled with breeding programmes also aimed at improved water use efficiency and salt tolerance will probably be of increasing importance in the very near future.

3. Background

During recent years this research group has developed a sound understanding of the ways that grapevines respond to abiotic stress and how these responses can be used to advantage to improve vine water use efficiency. This work has been based largely on traditional plant physiology techniques but more recently we have integrated molecular techniques into the group with the help of Dr Jim Speirs. Central to this understanding has been the idea that roots are able to sense the water status of the soil and transmit this information to the canopy in the form of a chemical signal. There is much evidence that in the canopy this information about soil water status is represented by the plant hormone abscisic acid (ABA) since this is a potent mediator of stomatal function and our working hypothesis has been that it is ABA synthesised in the roots that brings about changes in canopy performance after transport from the roots to the leaves. This idea is supported by the fact that the concentration of ABA in xylem sap changes as vines experience water deficits and also that under more stressful conditions rootstocks that impart better water use efficiency to the canopy have higher xylem ABA concentrations than those rootstocks that are less effective in imparting higher water use efficiency. However, recent work by the group has shown (Soar et al 2006) that the activity of a gene responsible for ABA synthesis (*NCED1*) is much more closely aligned with xylem sap ABA concentration when measured in leaves than in roots (see Figure 1 for an illustration of the ABA biosynthetic pathway and Figure 2 for a representation of the major steps in the breakdown of ABA under the influence of the ABA 8'-hydroxylase enzyme). This is an important observation because it suggests the presence of another chemical message about root water status. It also questions the idea that the contents of the xylem represent the activity of roots since it appears that the ABA content of the xylem changes in its passage through the shoots (Soar et al 2004, Soar 2004). These observations therefore raise some fundamental questions about our concept of how vines function, and in particular how soil moisture status can influence canopy performance. The importance of the canopy genotype has been recognised in GWRDC project CSP05/02 which will categorise major winegrape varieties according to their ability to respond to abiotic stress. The work proposed in the current application will interface strongly with CSP05/02 and will provide significant additional resources to further our understanding of vine stress response and will assist us to make better judgements about irrigation management and rootstock selection. Attention will also be paid to optimizing grape quality through irrigation management. Our current suite of projects is developing new ways to assess fruit quality through the objective measurement of specific chemical compounds and these new techniques will be applied to determine the role of root-derived chemical signals, and therefore the influence of water management and rootstock, in modifying grape quality

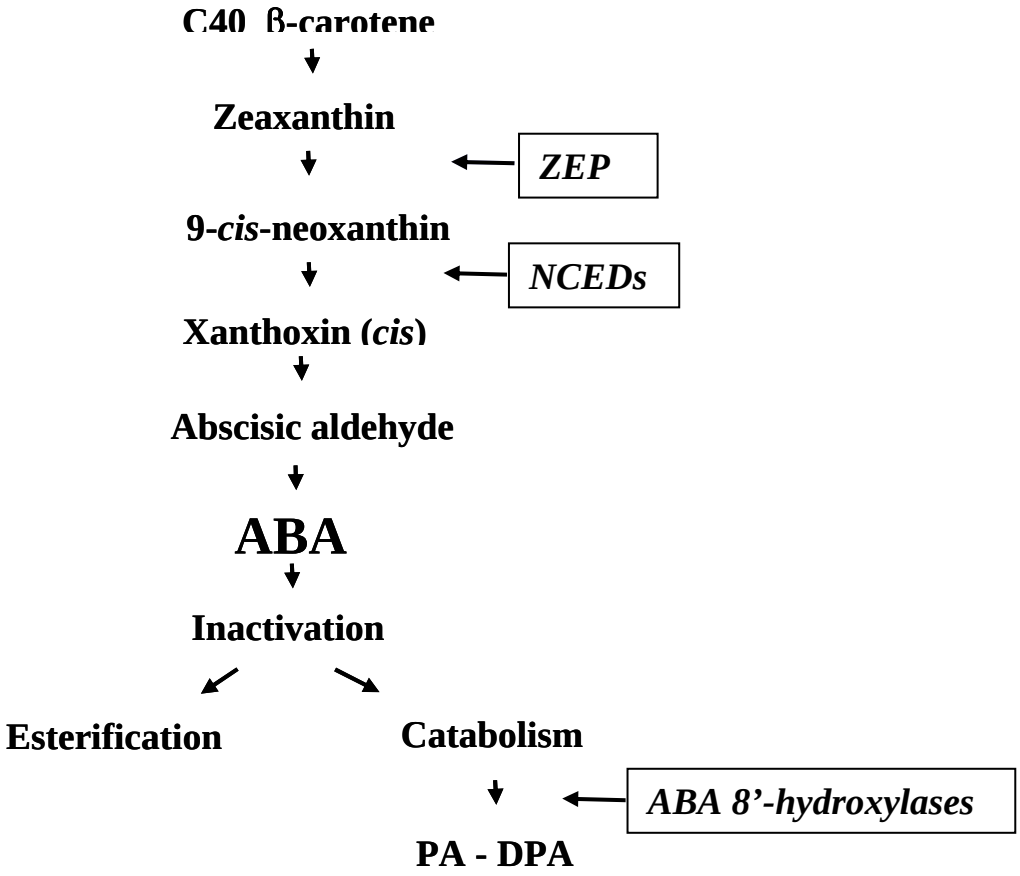


Figure 1. Pathway of synthesis of abscisic acid (ABA) indicating genes of particular interest in the regulation of synthesis (*Zep* and *NCED*) and catabolism (*ABA 8'-hydroxylase).*

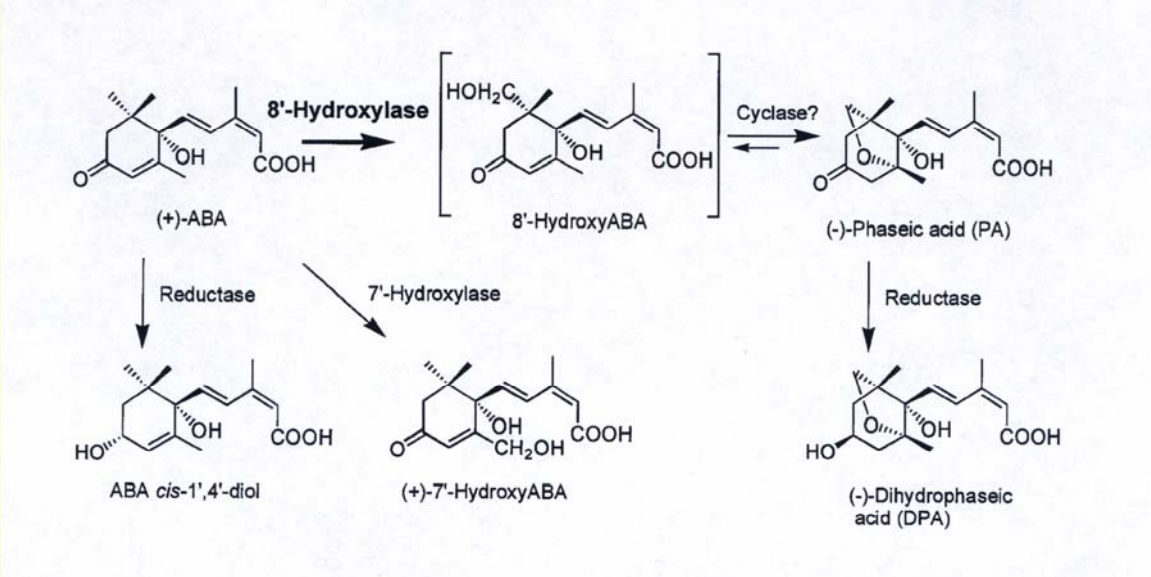


Figure 2. Pathway of metabolism of abscisic acid (ABA) catalyzed by the primary catabolic enzyme (ABA 8'-hydroxylse). Taken from Cutler and Krochko (1999).

Understanding the factors regulating stomatal control over grapevine transpiration is of importance in the endeavour to improve grapevine water use efficiency. It is clear that regulation of stomatal aperture is not simple, involving several interconnecting physiological and biochemical pathways (Düring and Scienza 1975; Düring 1987; Tardieu and Davies 1992; Tardieu and Davies 1993; Tardieu *et al*. 1993; Düring *et al*. 1997). From the biochemical perspective there is good evidence for the involvement of several plant growth regulators and abscisic acid in particular has received significant attention as a signal for stomatal closure (Hartung and Heilmeier 1993; Giraudat 1995; Zhang and Outlaw 2001). However even the ABA signal transduction pathway, ending with stomatal closure, is not simple with the involvement of possible antagonistic hormones such as cytokinins (Düring and Broquedis 1980; Abida *et al*. 1994; Cowan *et al*. 1999) and other "second messenger" compounds, most notably calcium (Leckie *et al*. 1998 and references therein). As with many plant growth regulators ABA has multiple roles in plants and as such it is synthesised ubiquitously. The challenge is to understand where the important sites of synthesis are in terms of control of stomatal aperture in response to changes in environment and to better understand the transport of this hormone to the sites of action. An increase in root ABA concentration in response to drying soil has been implicated as a root derived signal that is transported to shoots, and once there directly influences stomatal aperture though the regulation of ion transport over the guard cell plasmalemma. The evidence for this type of root to shoot communication is strong (Blackman and Davies 1985; Schurr *et al*. 1992; Jackson 1997; Comstock 2002). Furthermore studies using split root grapevines and partial rootzone drying (PRD) have demonstrated that without altering leaf water potential compared to fully irrigated control plants, drying half the root system leads to an increase in the ABA concentration in roots contacting drying soil with a coincident decrease in stomatal conductance in the shoots (Loveys *et al*. 2000). Whilst the importance of root derived ABA is well accepted the suggestion that stomatal aperture is controlled purely by root derived chemical messengers is an oversimplification and there have been several publications that have included hydraulic control in models describing the regulation of stomatal aperture (Tardieu and Davies 1992; Tardieu *et al*. 1993; Tardieu and Davies 1993; Tardieu 1995; Tardieu *et al*. 1996). Hollbrook et al. (2002) also draw attention to the importance of the canopy genotype in determining whole plant response to a water deficit. An area that has received less attention however is whether, from a chemical signalling perspective, grapevine shoots are passive in root to shoot communication or whether they play an active role in modulating or modifying "the message" through ABA metabolism.

The biosynthetic pathway of ABA has been the subject of considerable research in recent years and many of the genes encoding important enzymes in the ABA pathway have been isolated and characterised from a number of plants (reviewed by Liotenberg *et al*. 1999). Two genes or gene families have been identified as rate limiting in the production of stress related ABA and important in the constitutive synthesis of the hormone. One of these genes encodes the zeaxanthin epoxidase (ZEP) enzyme responsible for the conversion of zeaxanthin to violaxanthin. The other gene (family) encodes the 9-*cis*-epoxycarotenoid dioxygenase enzymes (NCED) responsible for cleavage of the epoxy-carotenoids violaxanthin and neoxanthin to form xanthoxin, a precursor of ABA. We have isolated cDNAs encoding the single ZEP enzyme and the two NCED enzymes from *Vitis vinifera* and characterised them in terms of their similarities with other related and functionally characterised genes described in the literature (Soar et al., 2004). These cDNAs can be used as hybridisation probes to examine the activities of their respective genes in vine leaves and other tissues.

We therefore have available to us state-of-the-art physiological and molecular tools with which to study the importance of both roots and shoots in determining vine response to abiotic stress and the particular characteristics imparted by rootstocks and how these may be best managed for optimum water use efficiency and fruit quality.

4. Project aims and performance targets

Summary of project aims:

- Assess the effectiveness of the presence and concentration of metabolites in expressed xylem sap as a measure of root activity
- Determine the relative contribution of roots and shoots to xylem contents
- Develop an understanding of the influence of currently practised deficit irrigation practices (RDI and PRD) on the expression of key genes in the ABA biosynthetic pathway in leaves and roots in a range of scion and rootstock genotypes.
- Assess the evidence for an additional chemical messenger able to communicate soil water status to the leaves
- Use information regarding the presence of chemical messengers to optimize effects of irrigation management on fruit quality
- Produce guidelines for improved irrigation management to optimize efficiency of water use for a range of rootstock and scion genotypes.

Details outputs and performance targets

Outputs and Performance Targets 2006-07

Outputs	Performance Targets
1. Technical Officer appointed	
2.methods of collecting xylem sap compared	Assess sap expressed from petioles and continuously using the root pressure chamber
3.impact of water stress on xylem sap composition determined	Measure sap content of ABA and major ionic species
4.influence of RDI and PRD on key ABA genes determined for a range of scion genotypes	NCED and ZEP Gene expression measured in scions of contrasting water use behavior
5.	

Outputs and Performance Targets 2007-08

Outputs	Performance Targets
1. influence of canopy on xylem sap composition determined	Measure sap content of ABA and major ionic species at a number of points in the canopy
2. influence of RDI and PRD on key ABA genes determined for a range of root genotypes (own rooted)	NCED and ZEP Gene expression measured in scions of contrasting water use behavior
3. . influence of RDI and PRD on key ABA genes determined for a range of rootstock genotypes	Expression of key genes measured in rootstocks with contrasting water use properties
4.influence of PRD and RDI on specific indicators of fruit quality measured	At least three indicator compounds measured in either Cabernet Sauvignon or Riesling
5.	

Outputs and Performance Targets 2008-09

Outputs	Performance Targets
1.decision on whether there is	Assess all data from this project and from literature

another chemical messenger	
2.existing irrigation trials sampled to test predictive ability of developed methods	sample vines from Langhorne Creek and Wingara sites
3.papers in peer-reviewed and industry journals	Write papers
4.final report	Report written and submitted by due date
5.	

Modified Outputs and Performance Targets 2009-10 agreed to as project variation (GWRDC letter dated December 7th 2009)

Outputs	Performance Targets
1.decision on whether there is another chemical messenger	Assess all data from this project and from literature
2.existing irrigation trials sampled to test predictive ability of developed methods	Use grafted/own-rooted vines at Nuriootpa to assess role of root-derived signals and influence of rootstock *
3. Role of scion genotype assessed	Use reciprocal grafted plants to determine influence of root on scion performance
4.papers in peer-reviewed and industry journals	Write papers
5.final report	Report written and submitted by due date

Materials and Methods

Field Sites and Plant Materials.

Nuriootpa deficit irrigation Trial – Grenache and Shiraz (Fig 1):

Measurements were taken in the 2006/2007 season at the South Australian Research and Development Institute's (SARDI) Nuriootpa field research station. The soil at this site is described as a light pass sandy clay red brown earth (Northcote 1954). The trial consisted of one row each of 15 year old Shiraz (clone 1125) and Grenache (clone 139HT) with like buffer rows to either side. The block orientation was east to west such that most of the canopy was fully sun exposed throughout the measurement period. Readings and samples were taken across six replicate vines at weekly intervals. Pre-bud burst in the previous year, 2006, trenches had been dug close to the measured vines and to adjacent vines on either side to allow access to the roots. The trenches measured 6m x 0.15m x 0.5m and were filled with washed sand and incorporated slow release fertilizer. To encourage root growth, each trench was watered for 1 hour daily with 2 x 4L/h drippers until the commencement of the RDI treatment at the end of December 2006, at which time the control vines were returned to normal irrigation and water was completely withheld from the deficit treated vines until returning to normal irrigation on the 6th March 2007. During the 20 week growing period control vines received 1.3 – 2 ML/hectare delivered via above surface drippers. Sampling was commenced on the 25th January. Shiraz berries were harvested on the 1st March 2007 and the Grenache berries on the 19th March 2007.

Nuriootpa Rootstock Droughting Trial – Shiraz on Commercial Rootstocks (Fig 2):

Measurements were taken in the 2009/2010 season at the South Australian Research and Development Institute's (SARDI) Nuriootpa field research station. The trial consisted of one row each of 15 year old Shiraz (clone 1654) on own roots or grafted on Ramsey or Schwarzmann rootstocks with like buffer rows to either side. The block orientation was east to west such that most of the canopy was fully sun exposed throughout the measurement period. In December 2008, trenches had been dug close to the measured

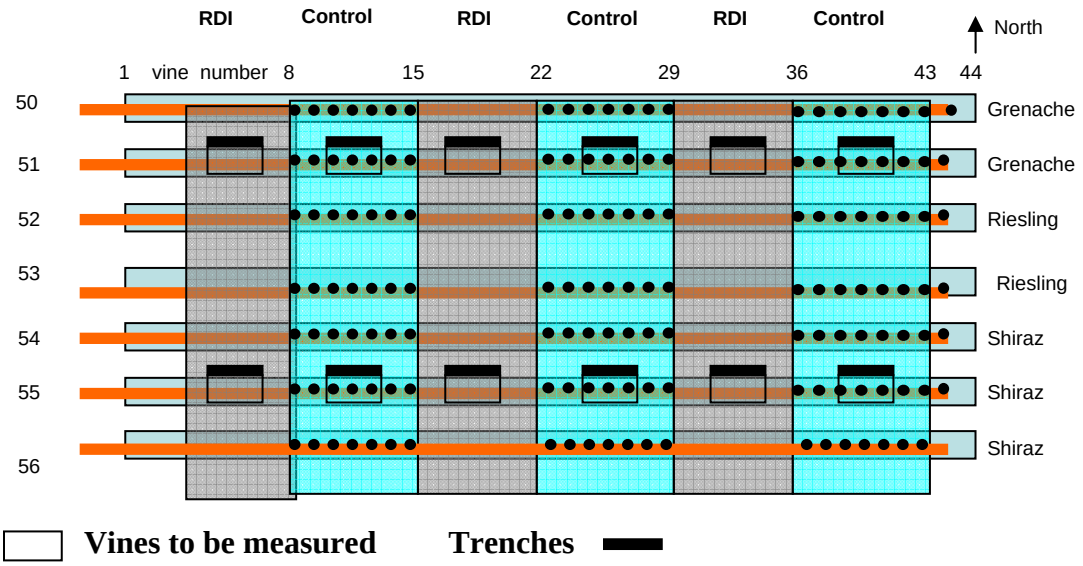


Figure 1. Grenache and Shiraz deficit irrigation Trial – Nuriootpa SARDI Field Research Station

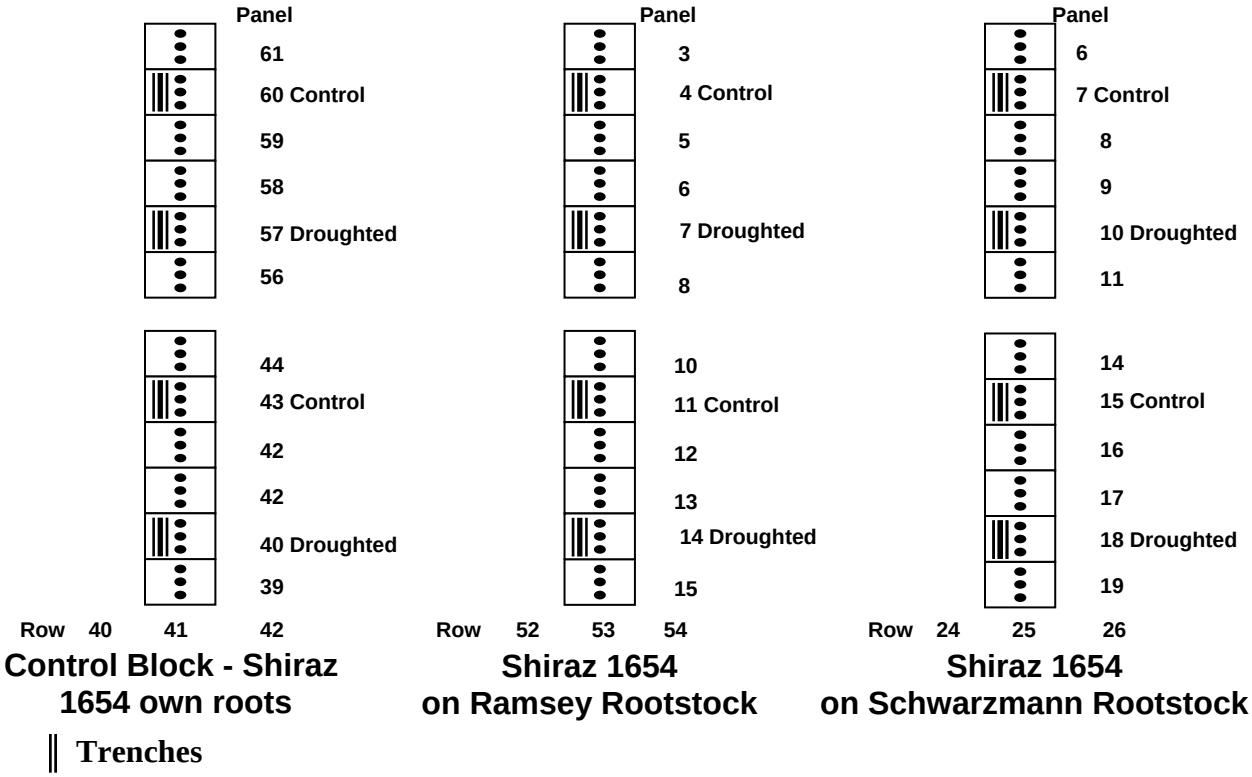


Figure 2. Rootstock Droughting Trial – Nuriootpa SARDI Field Research Station

vines and to adjacent vines on either side to allow access to the roots. The trenches measured 6m x 0.15m x 0.5m and were filled with washed sand incorporating slow release fertilizer. Root systems were allowed to develop over the following season and, to encourage root growth, each vine was watered for 1 hour daily with 2 x 4L/h drippers during Summer months until the commencement of the droughting treatment in February 2010. Droughting was carried out by withholding water from the relevant panels and their two adjacent panels for a period of 6 days between the 11th February and the 17th February when sampling was undertaken and normal irrigation was restored. Stomatal conductance was measured on a total of 15 leaves per trench; leaf water potential was measured with a pressure bomb on 3 leaves per trench. Xylem sap was collected from 3 leaves per trench, 5 leaves were harvested for further anaylses per trench and 1 sample of roots was taken per trench for further analysis. Leaf and root tissues and xylem sap samples were frozen on dry ice and held at -80^{o}C until analysed.

Deficit Irrigation Trial - Oxford Landing (Fig 3) :

12 year old Cabernet Sauvignon vines on Ramsey rootstocks were kindly made available for irrigation trials at the Yalumba, Oxford Landing Vineyard, in the South Australian Riverland. At the end of the 2007 season, 60cm deep trenches were dug alongside selected vines in groups of three panels and these were backfilled with washed sand to allow collection of root samples. In seasons 2007-2008 and 2008-2009 the vines were irrigated from mid September until after berry harvest. Three different irrigation treatments were applied to the vines in groups of five rows, one central (experimental) row and two buffer rows on each side. Irrigation rates were 6.6ML/h, 3.3ML/h and 1.3ML/h where 3.3ML/h was the vineyard standard rate. Measurements of stomatal conductance and leaf water potential of trenched vines were taken at approximately weekly intervals and samples were collected of leaf and root tissue and xylem sap, which were snap frozen under liquid nitrogen and stored for analysis at -80^{o}C, leaf and root tissue and -40^{o}C xylem sap.

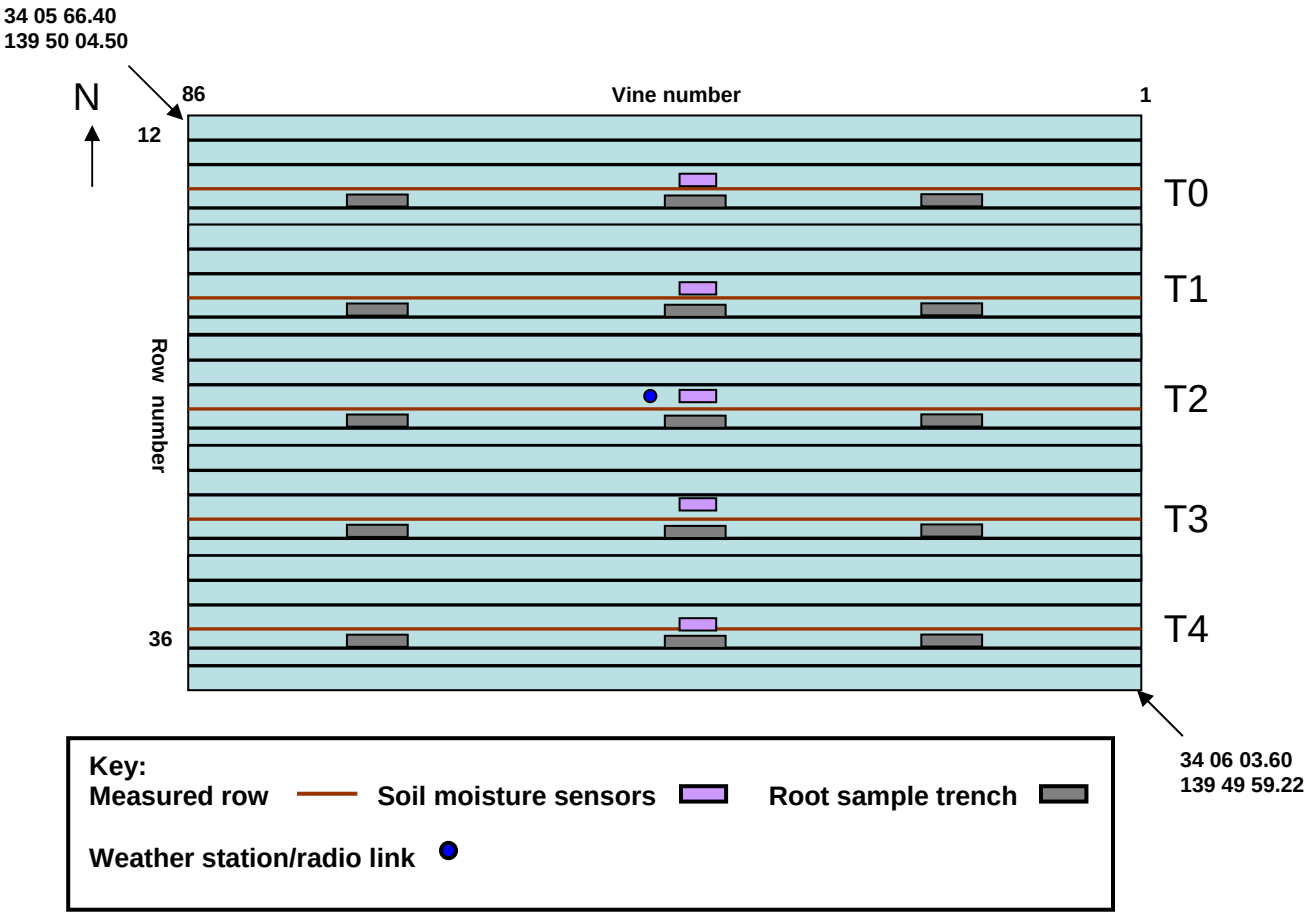


Figure 3. Plan of Oxford Landing Deficit Irrigation Trial Site.

Gas exchange measurements

Stomatal conductance measurements (g_s) were taken from sun exposed fully expanded leaves of all vines being studied using an AP4 porometer (Delta-T Devices Ltd., Cambridge, U.K.) on the northern side of the rows.

Leaf water potential and sap collection for ABA analysis

Leaf water potentials (Ψ_{leaf}) were measured using the method of Scholander *et al*. (1965). Ψ_{leaf} was measured on ten mature leaves per trench. with a 3000 series Plant Water Status Console (Soilmoisture Equipment Corp, Santa Barbera, USA) using industrial grade nitrogen (BOC gases, Australia) to pressurise the chamber. After recording the water potential an overpressure of 100 kPa was applied and exuded sap was collected and transferred to pre-weighed and labeled microcentrifuge tubes before being snap frozen in liquid nitrogen. Samples were stored at –40ºC to await ABA analysis.

Leaf, root harvesting

Leaves used for measuring water potential were snap frozen, ground to a powder under liquid nitrogen and stored at -80^{o}C for future analysis. For access to root tissue, 60cm deep trenches filled with washed coarse sand had been installed adjacent to the experimental vines in the previous season. Approximately 15 g of root tissue was carefully removed from the sand, surface sand and soil was removed carefully by brief washing and the roots dried with paper towels before snap freezing under liquid nitrogen and storage at -80^{o}C for future analysis. Two sets of 10 berries each were collected from vines in each trench. From each bunch, two berries were taken from the top of the bunch, two from the bottom and 6 from the mid regions. berries were frozen under liquid nitrogen and ground frozen before further processing.

Analysis of ABA abundance:

Analysis of ABA abundance in xylem sap, root and leaf tissues was undertaken by liquid chromatography/mass spectrometry (LC MS/MS) using a stable isotope dilution assay.

ABA extraction from leaf and root tissue

Approximately 50-100mg of frozen tissue was weighed into chilled 2mL eppendorf tubes. Aqueous methanol (20%, 500µL) was then added to the tubes, which were vortexed and stored at 4°C overnight. Tubes were then vortexed and centrifuged at 10,000rpm for 3 minutes, the supernatant was decanted and aqueous methanol (20%, 500µl) was added to the residue. Tubes containing the methanol and residue were vortexed and centrifuged at 10,000rpm for 3 minutes, and the two supernatants combined. A solution of deuterated internal standard (400µL, containing D3 - 7', 7', 7'-PA and DPA, D5 – 4, 5, 8', 8', 8'- ABA-GE and D6 - 3', 5', 5', 7', 7', 7'- ABA) was added to the supernatants.

Phenomenex SPE columns (60mg/1mL: 8B-S100-UAK) were equilibrated with 1mL methanol and 1mL nanopure water as per the manufacturers directions. The samples were loaded onto the SPE columns, washed with aqueous methanol (20%, 1mL) and then eluted with aqueous methanol (90%, 1mL). 50µL of the 90% elutant was aliquoted into a 1.5mL eppendorf tube and dried in a vacuum centrifuge. The dried sample was dissolved in 50µL aqueous acetonitrile (10% with 0.05% acetic acid) and centrifuged at 13,000rpm for 5 minutes. 20µL of the sample was aliquoted into a HPLC vial with glass insert which was then analysed by LC-MS/MS (Agilent 6410)

Column temperature was set at 40°C and the column used was a *Phenomenex* C18(2) 75mm *x* 4.5mm *x* 5µm. Solvents used were nanopure water and acetonitrile, both with 0.05% acetic acid. Samples were eluted with a linear 15 minute gradient starting at 10% acetonitrile at time 0 and ending with 90% acetonitrile. Compounds were identified by retention times and MRM. Parent and product ions are shown in Table 1.

Compound	RT (mins)	MRM for native and deuterated ions (m/z)
DPA	7.25-7.75	281/284 → 171/174
ABA-GE	8.25-8.75	425/430 → 263/268
PA	9.0-9.5	279/282 → 139/142
ABA	10.5-11.0	263/269 → 153/159

Table 1. Analytes, retention times and m/z values for parent and product ions of native and deuterated internal standards.

Xylem sap samples were thawed, dried in a vacuum centrifuge and dissolved in 10% acetonitrile containing 0.05% acetic acid before introduction into the HPLC. The analysis was performed as described above.

RNA extractions (Nuriootpa samples)

Leaf tissues were either pooled before grinding under liquid nitrogen or ground separately and aliquots pooled subsequent to grinding. Root tissues were ground under liquid nitrogen prior to aliquoting. For each data point, 1 g of pooled leaf tissue or a 1g aliquot of root tissue was taken for RNA extraction. Total RNA was extracted and purified by the 'Hot Borate" method of Wan and Wilkins (1994). Purified RNA was stored under ethanol at -40^{o}C.

RNA extractions (Oxford Landing samples)

Leaf tissues were pooled in sets of five leaves per sample point before grinding under liquid nitrogen. Root tissues were ground under liquid nitrogen. Ground leaf and root tissues were stored at -80^{o}C prior to use. For RNE from leaf tissues, 50mg of ground (pooled) tissue was extracted and DNased on column using the Spectrum Plant Total RNA Kit (Sigma). For RNA from root tissues, RNA was first extracted from 1g of ground (pooled) tissue by the 'Hot Borate" method of Wan and Wilkins (1994) and was subsequently further purified and DNase treated on column using the Spectrum Plant Total RNA Kit (Sigma).

RNA treatment with DNase (Nuriootpa samples).

10µg aliquots of RNA were dried down and resuspended in 44µL of RNase free water. The RNA was treated with Turbo DNase (Ambion) according to the product instructions and was reprecipitated after treatment by the addition of 1/10th volume of 3M NaOAc pH 5.2 and 2.5 volumes of ethanol and storage overnight at -40^{o}C. After centrifugation and washing with 250µL of 70% ethanol followed by drying, the RNA was redissolved in 10µL of RNase free water and 2µL were taken for quantitation using a NanoDrop

spectrophotometer (Thermo Fisher Scientific). 5µg of the DNased RNA was taken for cDNA synthesis.

cDNA synthesis:

First strand cDNA was synthesized from DNased total RNA from leaf and root tissues from each data point using either i) 5µg of RNA per reaction in a SuperScript™ III RNase H- Reverse Transcriptase reaction with RNAseOUT™ (Invitrogen, Carlsbad CA, USA) and either oligo $dT_{(18)}$ or the B26 primer (Frohman et al., 1988) for 3'-RACE, or ii) 1µg of RNA per reaction and using the reverse transcription component of the Phusion RT PCR Kit (Finnzymes) and the oligo $dT_{(18)}$ primer as per kit instructions.

Real Time PCR analysis of mRNAs:

Primer pairs for Real Time PCR of the various gene probes are shown in Table 2. The cDNA templates were diluted 1:10 with H_2O to provide sufficient template for experiments and to increase specificity of the reaction. Template cDNA (5 µL) was placed in each PCR reaction in a final volume of 15 µL containing 266 nM of each primer and 1 x Absolute™ QPCR SYBR® Green Buffer (ABgene®, Surrey, UK). Thermocycling conditions were as follows: an initial enzyme activation of 15 min at 95°C, followed by 30-35 cycles of denaturation for 30 s at 94°C, annealing for 30 s at 58°C, and extension for 30 s at 72°C, followed by a melt gradient starting from 50°C and heating to 96°C at a rate of 0.2°C s^{-1}. The PCR reactions were carried out in a Rotor-Gene™2000 Real Time PCR instrument (Corbett Research, NSW, Australia). The fluorescence of reactions was measured with an excitation wavelength of 470 nm and a detection wavelength of 510 nm at the end of each extension step and at each 1°C increment of the melt profile.

The specificity of the PCR product generated for each set of primers was tested in three ways: i) by the melt gradient in which fluorescence decreases at a single discrete temperature indicating separation of the two strands of a single DNA species, ii) by agarose gel electrophoresis showing a single PCR product of the correct size and iii) by obtaining the correct DNA sequence of the gene from a PCR product extracted from an

Gene	5’ primer	3’ primer	Gene Bank Accession	Frag Size	Dist 3’-end
Vvnced1	5’ TTTGTGCACGACGAGAAGAC 3’	5’ TCTGCAATCTGACACCAAGC3’	AY337613	210	279
Vvnced2	5’ CACCCTGGATTTGGAAAATG 3’	5’GCTGAGCTCCAATTCTACCG3’	AY337614	169	158
VvZep	5’ GGCTTCATTTCGAGTCAAGG 3’	5’ TGATGCTTTCTGCTTGGATG 3’	AY337615	208	126
VvABA8’OH-1	5’ ATGGACTTCCAGCCAGATTG 3’	5’ GGACATCTCTCCAACCCAGA 3’		180	88
VvABA8’OH-2	5’ATTTGGAAATGGGGTCCACT 3’	5’ ATTGTGATGGGCAAACCATT 3’		168	485
VvABA8’OH-2	5’ CTCTCAGTGCCACCAATCAA 3’	5’ TCAGCTTGCAATCTTTCTGG 3’		168	233
VvABA8’OH-3	5’ GTGGGATCCAAGACAAATGG 3’	5’ CCGGAATGAACACAAACTGA 3’		203	188
VvUbiquitin	5’GGTGGTATTATTGAGCCATCCTT3’	5’AACCTCCAATCCAGTCATCTACT3’	CF406001	181	321
VvEF1α	5’TGTTAGGGACATGCGTCAAA3’	5’CCCCACAAGTGAGGCTAGAG3’	TC38276	232	456
VvEF1α (2)	5’ TTGAGGTCCCTTGGATGTTT 3’	5’ CCGCCTGCTGAAATCTGTAT 3’	TC38276	158	146

Table 2. Details of Primer pairs for Real Time PCR

agarose gel. All cDNA samples to be compared for gene expression levels were analysed in a single batch for each primer pair and each set of analyses were conducted in triplicate.

Normalisation of Real Time results

Comparative abundances of the mRNAs (cDNAs) encoding the six gene products of interest were calculated as follows. The efficiencies of primer amplification of each of the three target cDNAs , and of the stable internal standards *Ubiquitin* cDNA were tested by the method of Muller et al., 2002, and appropriate adjustments made. The abundances of the target cDNAs relative to the *Ubiquitin* cDNA were then calculated and means and standard errors from three separate determinations for each target cDNA were calculated.

Abundances of the target cDNAs relative to a second stable internal standard *EF1α* cDNA were also calculated. These closely reflected the abundances relative to the *Ubiquitin* cDNA.

The sizes of the fragments used for calculation of cDNA abundances were similar and no corrections were made on the basis of their sizes. Expression values for each of the 6 genes examined were considered to be approximately relative to each other.

Isolation and characterisation of *Vitis vinifera* ABA 8'-hydroxylase genes.

The cDNA template for the initial PCR amplification of 8'-hydroxylase fragments was derived from RNA from ABA-treated Chardonnay culture cells. All subsequent sequencing work used cDNA derived from Shiraz leaves dried to 85% fresh weight as described in Soar et al, (2004).

Three potential 8'-hydroxylase fragments were obtained using redundant PCR primers based on conserved amino acid sequences of 8'-hydroxylases. The redundant primers were constructed on the basis of conserved amino acid sequences of a number of ABA 8'-hydroxylases obtained from the NCBI data base : Arabidopsis 1, 2, 3 and 4 (AB122149, NM_128466, AB122150, NM_112814), potato A1 and A2 (DQ206630,

DQ206631), barley A1 and A2 (DQ145931, DQ145932), and rice A5 (DQ887714). Using these primers three fragments of *V.vinifera* DNA of approximately 300bp were obtained with close similarity to each other and to the specific conserved regions of the published sequences. 3'-RACE (Frohman et al., 1988) was carried out using non-redundant primers based on the derived sequences of the *V.vinifera* fragments and the B25 primer (Frohman et al., 1988). 5'-ends of the three putative *V.vinifera* genes were obtained by designing primers based on homologous sequences in grape genomic contigs.

The open reading frames of the three genes were initially amplified from cDNA by PCR using pfu-Ultra polymerase (Invitrogen) and their sequences verified after cloning into pCR11-Blunt-TOPO (Invitrogen). The amplicons were digested from the TOPO plasmids and were ligated into the pYeDP60 vector and transformed into *S. cerevisiae* strain WAT11 (Pompon et al., 1996) by the method of Geitz et al., (1992). Transformed colonies were selected by growing for 48 h on SG1 plates (Pompon et al., 1996) and the presence of the plasmid was tested by PCR amplification of the respective amplicons. Positive lines were grown overnight in SG1 medium at 28^{o}C and stored in 30% glycerol at -80^{o}C until required. Functional analysis of the constructs was tested by the method of Yang and Zeevaart (2006) with slight modification. Transformed cells were grown to stationary phase in 2mL of SG1 medium and were pelleted by centrifugation at 500g for 5 min. The pelleted cells were resuspended in 3mL of SL1 induction medium (Pompon et al., 1996) at pH 5.4 and the cells were incubated for 8 h before the addition of 3µL of 10mM ABA in ethanol. After a further 18 h incubation, the cells were disrupted by sonication for 5 min, the pH was adjusted to 3.0 with 0.1 M HCl and the mixture was extracted 2 x with 3mL ethyl acetate. The organic extract was dried over sodium sulphate crystals, centifuged at 450g for 60 secs, room temperature and dried under a nitrogen stream. The dried sample was dissolved in 3 mL 20% methanol + 0.2% acetic acid and loaded onto a pre-conditioned Sep-Pak C18 cartridge (Phenomenex strata-X 33um Polymeric Reversed Phase 500mg/6mL). After centrifugation at 450g for 60 seconds, room temperature, the cartridge was washed with 3mL 20% methanol, with the wash discarded. The cartridge was further washed with 100% methanol, and the wash collected and retained. The sample was allowed to dry over night, in the dark, to a

volume of approximately 1mL. From this, 20 uL was taken and was added to 15uL LCMS solution (10% acetonitrile, 0.05% acteic acid in nanopure water) and 25uL ABA standard (ABA D6 100ng/mL, PA D3 140ng/mL, ABAGE D5 88ng/mL, DPA D3 11.4ng/mL from NRC-CNRC, in MeCN) and centrifuged for two minutes in a microfuge. 4ul aliquots of the mix were analysed by LCMS/MS. Separation was on a Phenomenex C18(2) 75mm x 4.5mm x 5µm column set at 40^{o}C. Samples were eluted with a linear 15min gradient starting at 10% acetonitrile, 0.05% acetic acid and ending at 90% acetonitrile, 0.05% acetic acid. Compounds were identified by retention times and MRM as detailed in Table 1.

Each hydroxylase construct generated a distinct peak of phaseic acid with the principle identifying ion at m/z 139, confirmed by the qualifying ion at m/z 205. Conversion of ABA into phaseic acid by the constructs is shown as a percentage of the residual ABA in Figure 4. Sequences of the three hydroxylase genes will be submitted to the NCBI data base when the work is published.

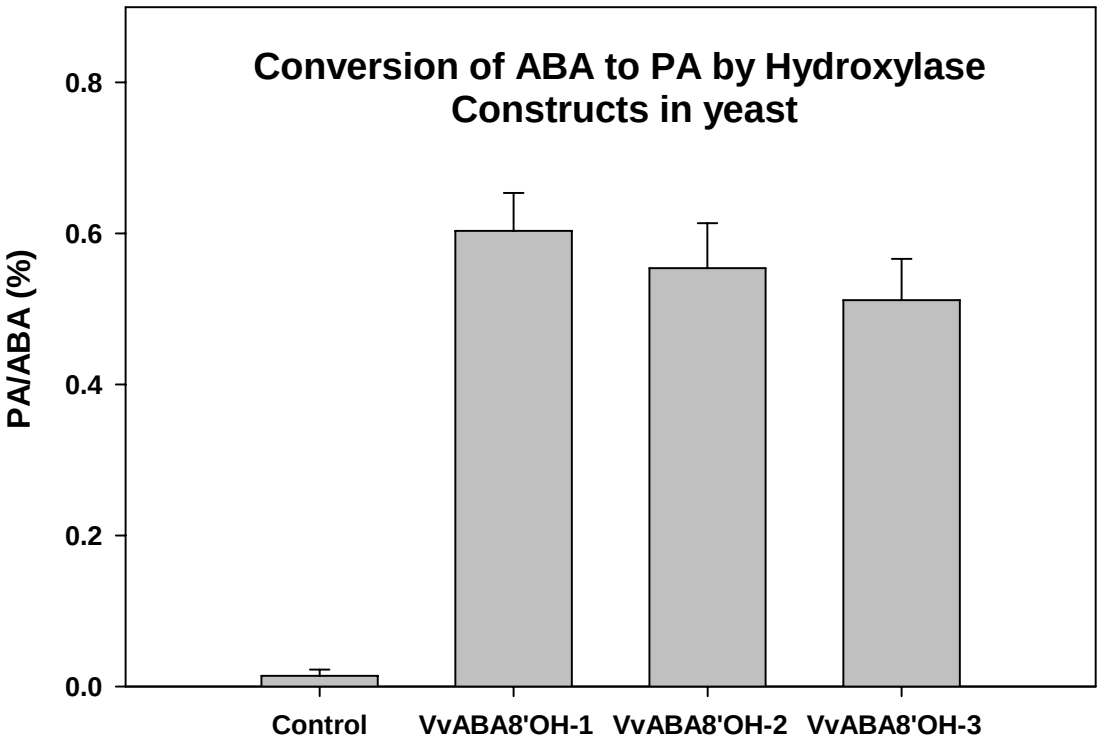


Figure 4. Percentage conversion of ABA into PA in culture by *Vitis* hydroxylases synthesised from *Vitis* 8'-hydroxylase genes expressed in yeast cells according to the procedure of Pompon et al., 1996.

Chapter 1. An assessment of different methods for sampling xylem sap.

Introduction

Roots exposed to drying soil synthesize abscisic acid (ABA), some of which may then be transported through the xylem to reduce stomatal conductance in the leaves (Dodd et al., 2008; Goodger et al., 2005; Jia and Zhang, 1999). In order to study the root to leaf hormone signalling pathway, it is crucial to obtain samples that accurately represent the xylem sap. The problems likely to be encountered in obtaining meaningful samples of xylem sap have been outlined by (Schurr, 1998). Schurr points out that the xylem is often under negative pressure and so once the integrity of the xylem vessels have been breached air will enter the conduit rather than the contents leaking out, thus requiring some form of pressurization to extract a sample of sap for analysis. This is certainly true for grapevines. With the exception of a short period of time in spring before budburst when the xylem is under positive pressure, stem and leaf water potentials are nearly always negative, even at pre-dawn when it is assumed the water potential is in equilibrium with soil water potential.

A number of different methods are available to obtain plant xylem sap. Leaf pressurisation, as pioneered by Scholander *et al* (1964) as a means of measuring leaf water potential, remains one of the fastest and easiest means to collect sap from plants (Cochard et al., 2001). The technique involves removing a leaf and sealing it in a chamber with the petiole protruding through a seal (Fig 2). The pressure within the chamber is then slowly increased until it balances the tension in the xylem that existed before the leaf was removed. This is a measure of the xylem water potential. Sap can be observed at the cut surface of the petiole at this pressure and can be collected via further small increases in chamber pressure (Scholander et al., 1964). Sampling is relatively non-destructive at the whole vine level since only individual leaves are removed, and is really the only technique that can conveniently be conducted on plants grown in the field. However, the technique suffers from several potential problems. Fluid from the contents of damaged cells at the cut surface of the petiole may be collected along with the sap sample, thus rendering the sap unrepresentative of xylem sap. Additionally, raising the overpressure too high above the balancing pressure can force water and solutes from intercellular or intracellular sources in the leaf into the sap sample, thus rendering the sample unrepresentative of the undisturbed xylem contents (Jachetta et al., 1986). Despite these potential problems

the technique is still widely used as a means of sampling sap due to its simplicity and ease of use in field measurements and the lack of alternatives. An extension of this technique involves the excision of a whole vine cane and sampling the sap contained in the xylem either by pressurization in a chamber similar to that used for leaf sampling or by removing the sap from the xylem conduits under vacuum or by centrifugation. This is not a practical technique for routine field use but can be useful for confirming other techniques.

Another technique involves sealing the entire root system of plants grown in specialised pots in a chamber with the cane protruding through a seal (Passioura, 1980) (Fig 1). Pressurisation of roots and soil within the chamber occurs in a similar fashion to the leaf pressure chamber. Sap can be collected by detopping the shoot or by excising a leaf when sap is forced through the stump or the petiole by the pressure applied to the roots (Goodger *et al.*, 2005). Sealing the entire root system of a plant within the chamber gives the advantage of enabling sap collection over longer time periods (up to several days, if required) which in turn allows collection of larger sap volumes. Control of sap flow enables collection at a rate approximating the transpiration rate of an unpressurised plant, giving further confidence that the sample is representative of i*n vivo* xylem fluid. . Sap collection via a root pressure chamber is potentially the best technique to obtain sap representative of the xylem (Goodger et al., 2005; Jokhan et al., 1999). However, due to the specialised equipment and specifically grown plants necessary to conduct measurements, this technique cannot be utilised to collect sap from plants grown in the field.

We aim to compare the concentrations of abscisic acid and several related metabolites in xylem sap collected using a range of these techniques. In addition we will assess the concentration of major inorganic ions in the sap collected using these techniques. Potassium and calcium in particular have well defined roles in stomatal opening and closing (Allen et al., 1998; Macrobbie, 1990; Macrobbie, 1992) and when compared with ABA may be subject to different mechanisms controlling their concentration in xylem. For example, the pH of the xylem and apoplast may significantly influence the movement of a weak acid like ABA for reasons discussed elsewhere in this report (Davies et al., 2002). This will provide us with information on the validity of the sap sampling techniques we have adopted to better understand the role of root-sourced chemical signals in determining vine canopy response to environment and irrigation management.

Materials and Methods Specific to this Chapter.

Root and Leaf Pressure Chamber Comparison

Shiraz and Grenache vines (own roots) were grown individually in pots designed to fit in a Passioura-type pressure chamber in a glasshouse, vines were watered to field capacity twice daily. Four vines of each cultivar were randomly selected for use in this experiment.

A pot was placed in the pressure chamber and sealed inside, with the cane protruding through the chamber lid (Fig 1). A leaf near the base of the cane was excised approximately halfway along the petiole using a razor blade. This leaf was then placed into a Scholander-type leaf pressure chamber (Fig 2), chamber pressure was increased using nitrogen until sap was visible at the cut surface of the petiole. An over-pressure of less than 0.2 MPa was applied and sap collected into a 1.5mL Eppendorf tube. To collect sap from the root pressure bomb, the pressure within the chamber was slowly increased until xylem sap was visible at the cut surface of the petiole. The initial droplet of sap was discarded and an additional overpressure of 0.05MPa was applied to the chamber and sap was collected for approximately five minutes. Sap was collected from the petiole into a 2mL Eppendorf tube. Two further leaves at the mid and upper levels of the cane were excised and sap was collected in the same manner as above via the leaf and root pressure chambers. Sap collected via the two techniques was snap frozen in liquid nitrogen and then stored at -40°C before analysis.

Sap samples collected with the root pressure chamber were analysed for ABA and metabolites, major anions and cations, nitrate and nitrite. An aliquot (30µL) was taken and analysed via LC-MS/MS for abscisic acid and metabolites. A further aliquot (250µL) was taken and freeze dried before being sent to CSIRO-PI Black Mountain for ICP-MS ion analysis. The mass of remaining sap was recorded, freeze dried then sent to CSIRO-PI Black Mountain for Flow Injection Analysis (FIA) of nitrate and nitrite. Leaf chamber sap samples were too small in volume for ion analysis, so the entire sample was used in LC-MS/MS analysis.

Figure 1. Grapevine inserted in a root pressure chamber for sap collection.

Figure 2. Scholander-type pressure chamber used for routine measurement of leaf water potential and collection of petiole sap.

Vacuum pump extraction of sap

Four randomly selected, glasshouse grown Shiraz plants were used in this experiment. Canes were removed from the vines and 6 internode sections were connected to a vacuum pump with Tygon tubing. A sample collection device was inserted between the cane and the vacuum source which allowed sap to be collected in a 2mL Eppendorf tube. A vacuum of approximately 200mm Hg was applied. Sap was freeze dried and sent to CSIRO-PI Black Mountain for ICP-MS ion analysis.

Cut surface contamination of sap.

Six Grenache vines were selected at random for use in this experiment. The vines were grown in a glasshouse and watered to field capacity twice daily. Water stress was imposed on half of the selected vines for 9 days through the removal of their watering lines. Water stress was verified through daily monitoring of stomatal conductance with an AP-4 porometer. On the 9th day leaf water potential was determined on six randomly selected leaves for each plant using a leaf pressure chamber; xylem sap was collected in the standard manner with an over pressure of no more than 0.2MPa for half of the leaves (see methods above). For the other half, the first droplet exuded from the petiole was wiped away and discarded. Pressure was increased to 0.2MPa beyond the balancing pressure and the exuded sap was collected into 0.5mL Eppendorf tubes, snap frozen in liquid nitrogen and then stored at -40°C until analysis by LC-MS/MS.

Variations in overpressure

Merlot and Shiraz vines (six of each) were selected at random for use in this experiment. The vines were grown in a glasshouse and watered to field capacity twice daily. Water stress was imposed on half of the selected vines for 9 days through the removal of their watering lines. Water stress was verified through the monitoring of stomatal conductance with an AP-4 porometer. On the 9th day leaf water potential was determined on six randomly selected leaves for each plant using a leaf pressure chamber (see methods above). Pressure was then increased in steps of 0.1 MPa and sap collected at each increment until no more sap could be collected. Sap was collected into 0.5ml Eppendorf tubes and snap frozen in liquid nitrogen, then stored at -40°C before analysis for ABA and metabolites by LC-MS/MS.

Sap analysis by LC-MS/MS

Sap samples were thawed and weighed, 30 µL of internal standard mix was added to the sap. The internal standard mix contained deuterium-labelled analogues of abscisic acid (ABA), phaseic acid (PA), dihydrophaseic acid (DPA) and the glucose ester of ABA (ABAGE). Specifically, these were (-) 7', 7', 7' D3- PA ,(-) 7', 7', 7' D3 DPA, (+) 4, 5, 8', 8', 8'- D5 ABA-GE and (-) 3', 5', 5', 7', 7', 7' D6 ABA, all at a concentration of 100ng/mL. Samples were centrifuged at 13,000 rpm for 5 minutes, 20µL of the sap/internal standard mix was then aliquoted into a HPLC vial with glass insert and analysed by LC-MS/MS (Agilent 6410 QQQ LC-MS/MS with Agilent 1200 series HPLC). Column temperature was set at 40°C, the column used was a *Phenomenex* C18(2) 75mm *x* 4.5mm *x* 5µm. Solvents used were nanopure water and acetonitrile, both with 0.05% acetic acid. Samples were eluted with a linear 15 minute gradient starting at 10% acetonitrile at time 0 and ending with 90% acetonitrile. Compounds were identified by retention times and mass through multiple reaction monitoring (MRM).

Compound	**RT (mins)**	**MRM parent and product ions for native/deuterated compounds (m/z)**
DPA	7.25-7.75	281/284 → 171/174
ABA-GE	8.25-8.75	425/430 → 263/268
PA	9.0-9.5	279/282 → 139/142
ABA	10.5-11.0	263/269 → 153/159

Data analysis.

Statistical analysis was conducted using *Instat* by GraphPad, version 3.0. 2-tailed *t* tests or one way ANOVA were used to analyse the concentrations of metabolites in sap. Differences were deemed to be statistically significant if they returned a *P* value of less than 0.05.

Results

Sap sampling methods

The concentration of ABA in sap collected with the leaf pressure chamber was higher than in sap collected with the root pressure chamber (figure 3a) and as reported by Soar et al (2004) the concentration in the leaf chamber samples tended to increase as sap was sampled from nodes closer to the shoot apex. This did not appear to be the case with the root chamber samples. On average, the concentration of ABA in both leaf and root chamber samples was higher from Shiraz than from Grenache. The concentration of the ABA metabolites PA, DPA and ABAGE were barely detectable in the root chamber samples (Fig 3 b, c and d) but much higher in the leaf chamber samples and as for the ABA analyses there was a tendency for the concentration of metabolites to increase with higher node position. Ion analysis of the root chamber samples showed that potassium was the dominant cation in both Shiraz and Grenache (Fig 4). Relative amounts of NO_3/NO_2 were significantly higher in Grenache. There was no effect of node position on the concentration of any of the ions. In another experiment the root pressure chamber was used to collected sap from Shiraz and Merlot vines. Ion concentrations were essentially the same as in the previous experiment and there were no differences between the cultivars (Fig 5). There was also no difference between the concentration of various ions from the Shiraz and Merlot sap compared with sap collected from the internodes of Shiraz using a vacuum chamber (Fig 6).

Contamination of the sap sample with cell contents at the petiole cut surface is a possibility. To test this, sap was collected using the leaf pressure chamber and the initial droplet of sap expressed from the petiole was discarded. The concentrations of ABA and its metabolites in these samples were then compared with sap samples collected using the leaf pressure chamber with our standard technique (*i.e.* not discarding the first droplet). The stomatal conductance on the wet and dry Grenache vines was respectively 963.25 ± 80.99mmol.$m^{-2}s^{-1}$ and 104.13±16.30mmol.$m^{-2}s^{-1}$ (mean plus/minus standard error, n=16) on the day the sap samples were collected, showing that the stress treatment had been effective. .There was no significant difference between any of the samples within any metabolite class in either well watered or water stressed Grenache vines (Fig 8). The concentration of ABA, PA and DPA were all significantly increased in the sap collected from stressed vines.

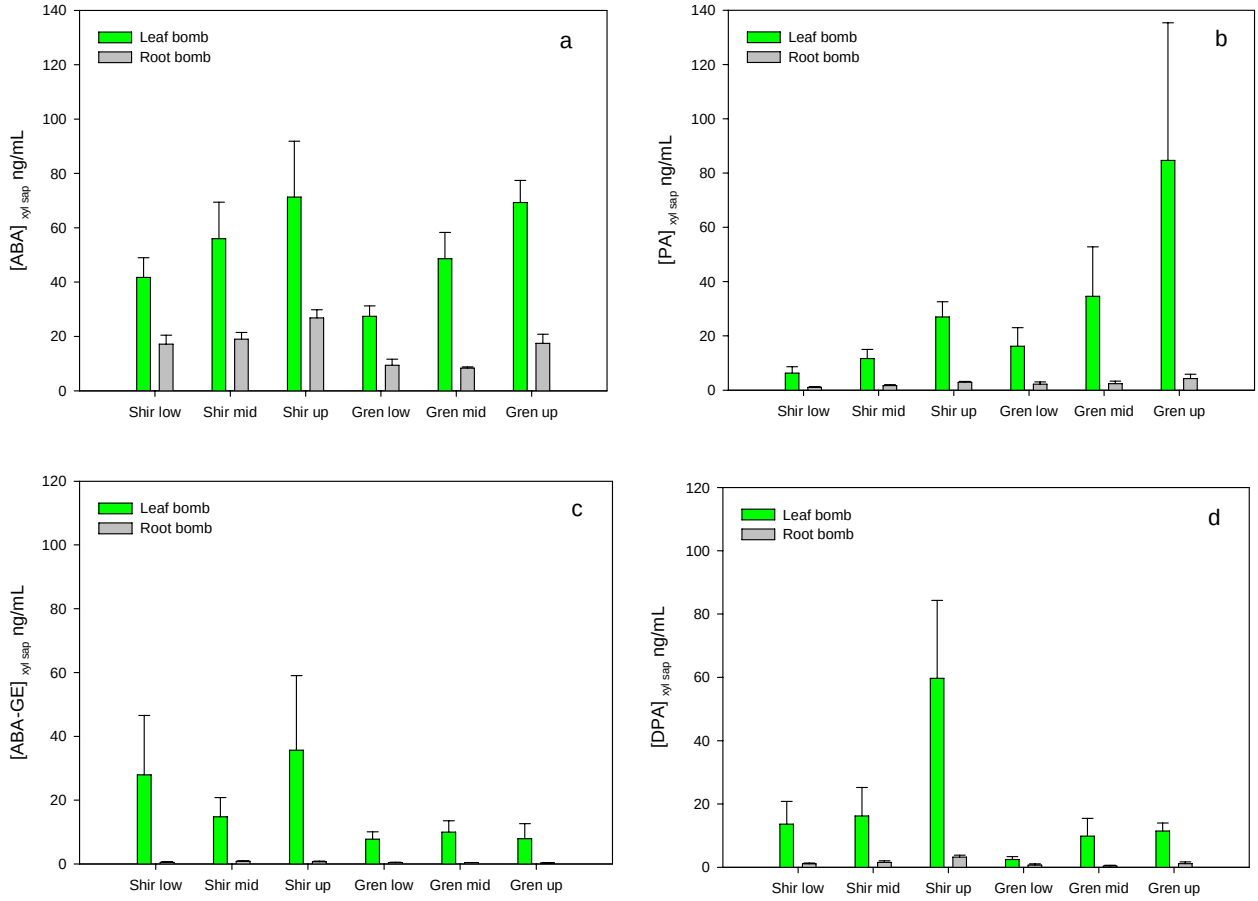


Figure 3: **Mean metabolite concentrations +1 standard error (n=4) for xylem sap samples collected with a leaf pressure chamber (leaf bomb) or root pressure chamber (root bomb).** A) ABA, abscisic acid. B) PA, phaseic acid. C) ABA-GE, abscisic acid glucose ester. D) DPA, dihydrophaseic acid. The cultivars were Shiraz (shir) and Grenache (gren) and sap was collected at 3 points along the cane low (low), middle (mid) and upper (up).

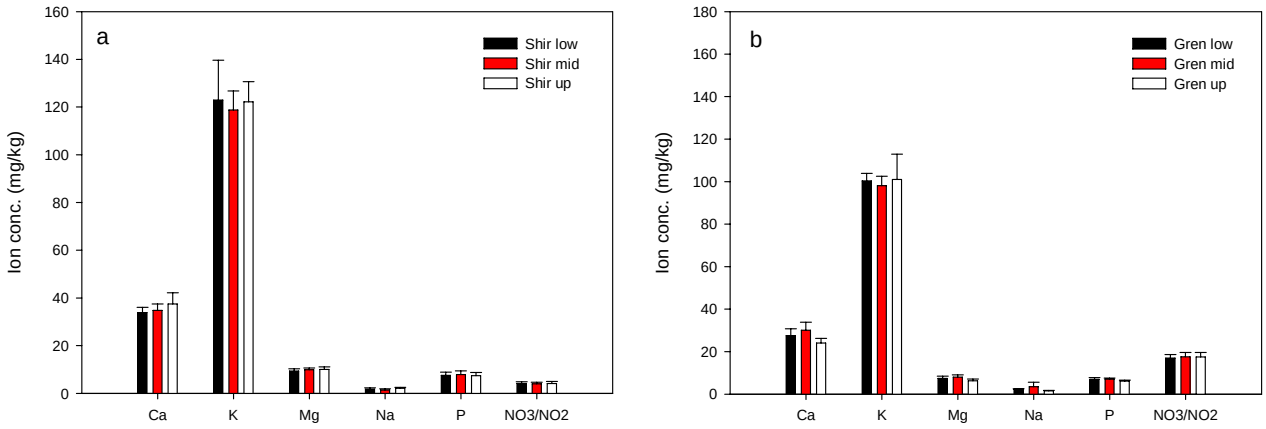


Figure 4: Mean ion concentration + 1 standard error (n=4) in xylem sap collected with the root pressure chamber from 3 positions on a) Shiraz and b) Grenache cultivars. Sap was sampled at 3 different positions along the cane; low (low), middle (mid), upper (up). Ions analysed were calcium (Ca), potassium (K), magnesium (Mg), sodium (Na), phosphorous (P) and nitrate/nitrite (NO_3/NO_2).

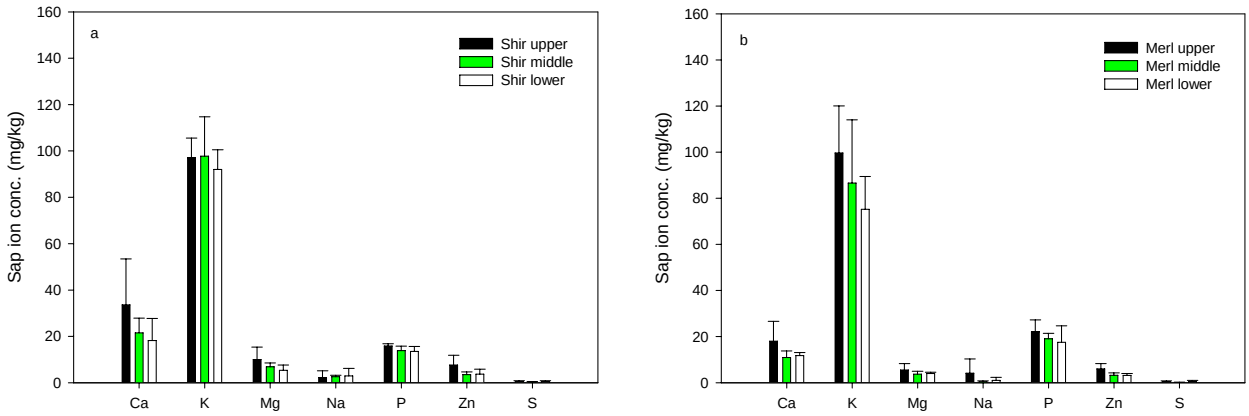


Figure 5: Mean concentration of ions in xylem sap exuded using the root pressure chamber +1 standard error (n=9) for a) Shiraz and b) Merlot at three heights of the cane; upper, middle and lower. Ions analysed were calcium (Ca), potassium (K), magnesium (Mg), sodium (Na), phosphorous (P), zinc (Zn) and sulphur (S).

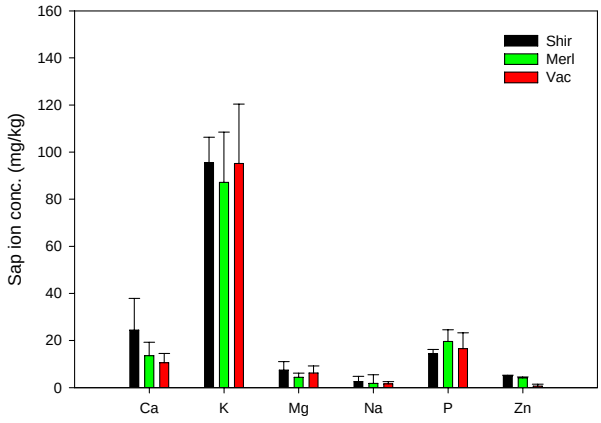


Figure 6: Mean concentration of ions +1 standard error collected at 3 node positions using the root pressure chamber for Shiraz (Shir) and Merlot (Merl) (n=9) and for Shiraz vines using a vacuum chamber (Vac) to extract sap from canes (n=4). Ions analysed were calcium (Ca), potassium (K), magnesium (Mg), sodium (Na), phosphorous (P) and zinc (Zn).

By contrast, the concentration of ABAGE in the sap was not affected by the stress treatment.

When the leaf pressure chamber is used to measure leaf water potential and collect xylem sap we have adopted the technique of applying only a minimum over-pressure. This is due to the assumption that higher over-pressures are likely to create artefacts due to modification of the sap analyte concentration by dilution or addition from cellular compartments other than the xylem or apoplast. To test whether this is reasonable assumption we collected sap from well watered and water stressed Shiraz and Merlot leaves over a range of over-pressures. The collection pressure had no effect on the sap ABA concentration of well watered Merlot or Shiraz vines (Fig 8, open circles). However, differences were evident in the water stressed vines. Samples collected at the prevailing leaf water potential (lowest collection pressure) had significantly elevated concentrations of ABA (Fig 8 closed circles). As the collection pressure was increased the ABA concentration fell and in Merlot approached that of the well watered vines at the highest collection pressure. Shiraz showed the same trend but the ABA concentration remained elevated, even at the highest collection pressure.

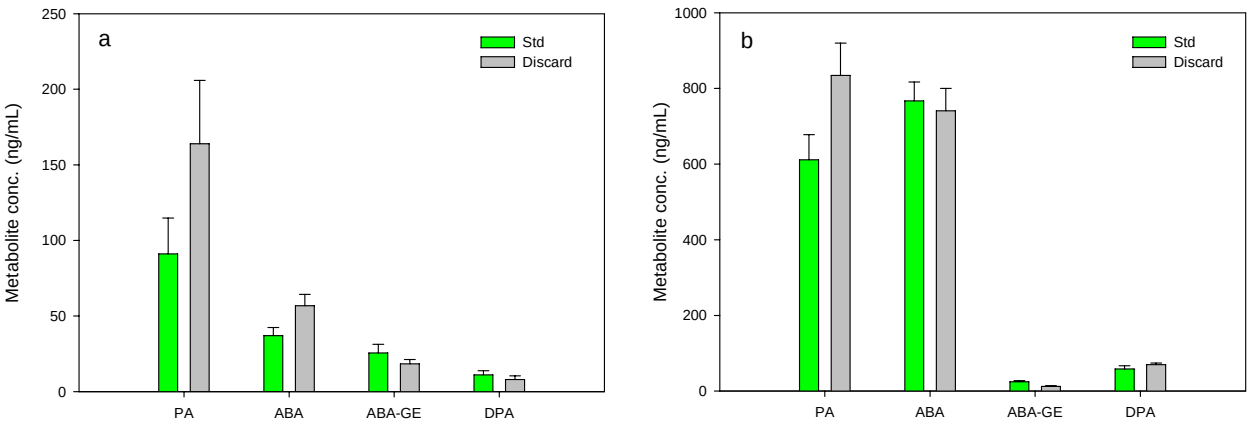


Figure 7: Mean concentration of xylem sap metabolites, phaseic acid (PA), abscisic acid (ABA), abscisic acid-glucose ester (ABA-GE), dihydrophaseic acid (DPA), +1 standard error (n=9) in a) well watered and b) water stressed Grenache vines. Sap was collected by leaf pressure chamber in the standard manner (std) or by discarding the initial droplet of expressed sap (Discard). There was no significant difference in metabolites collected with either technique (2 tailed *t* test, *P*>0.05 for std vs discard).

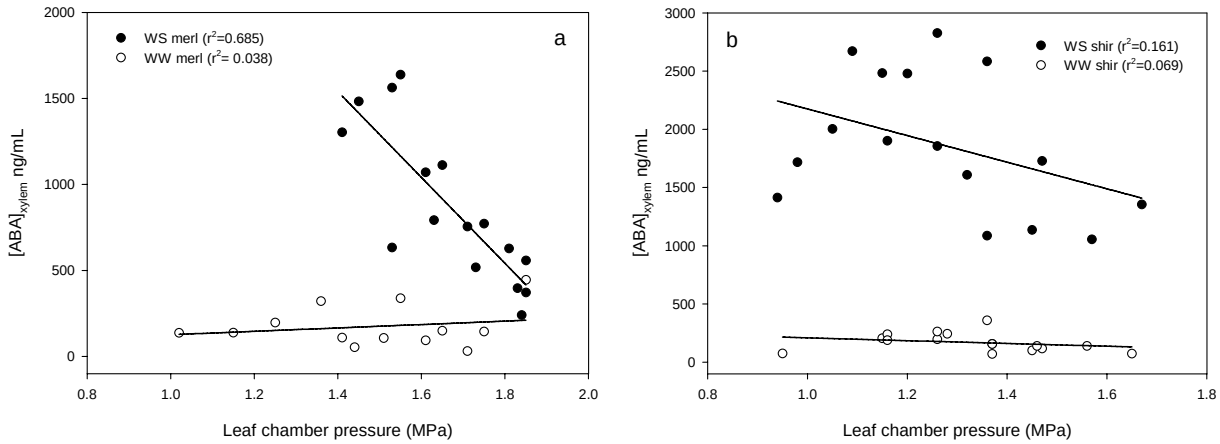


Figure 8: Effect of collection pressure on xylem sap abscisic acid (ABA) in a) water stressed (WS) and well-watered (WW) Merlot (merl) and b) water stressed (WS) and well-watered (WW) Shiraz (Shir). R-squared values are 0.685 and 0.038 for water-stressed and well-watered merlot respectively, 0.161 and 0.069 for water-stressed and well-watered Shiraz respectively. The only statistically significant regression was in water-stressed merlot (one-way ANOVA, $p<0.05$), all others were not statistically significant (one-way ANOVA, $p>0.05$).

Metabolomic analysis of xylem sap from water stressed Shiraz vines

Introduction

During water stress a wide range of metabolites and ions change in concentration within xylem sap (Alvarez et al 2008; Schachtman and Goodger 2008). Recent developments in analytical techniques enable rapid identification of a wide range of organic compounds within xylem sap. One such technique is time-of-flight (TOF) mass spectrometry, which enables the identification of compounds by accurate mass determination. This technique was used to analyse the compounds within the xylem sap of water stressed and well watered grapevine. This was a collaborative venture with the Australian Wine Research Institute and we thank Jeremy Hack and Meagan Mercurio for performing the analyses.

Methods specific to this section

Xylem sap was collected using a Passioura type pressure chamber from two Shiraz vines grown in a glasshouse. Water stress was imposed upon one of the vines by removing its watering line, whilst the other was watered to field capacity twice daily. To collect sap, the root system of a vine was sealed in the pressure chamber with the cane protruding through the lid. A leaf was excised near the base of the cane and pressure was increased inside the chamber until sap was visible at the cut surface of the petiole. Pressure was increased by 0.05MPa and sap was expressed from the petiole, the initial droplet of sap was discarded and sap was then collected into a 2mL Eppendorf tube for approximately 5 minutes. The same collection method was repeated for 2 other leaves in the middle and near the top of the cane. The tubes were then snap frozen in liquid nitrogen and then stored at -40°C.

Sap was thawed and an aliquot was subject to metabolomic analysis with a *Bruker* micro TOF-Q mass spectrometer. Mass range was 50-1000m/z and samples were analysed in both positive and negative modes. Data were transformed into a bucket table with buckets of 0.1m/z (counts greater than 200) and this was analysed by principle components analysis (PCA) for each mode (positive and negative). PCA analysis was performed using Camo's *Unscrambler* (v9.5). The metabolites were identified in the metabolomic database *MassBank* (www.massbank.jp) using accurate masses. However, many of the masses identified by the TOF-Q could not be found in the database.

Results and Discussion.

The PCA identified 4 masses, 115.05, 133.05, 149.05 and 341.15m/z, which accounted for 72% of the variance of the negative scan results. In positive mode 12 ions accounted for 55%

of the variance of the PCA. However, no compounds could be identified from the MassBank database for any of the accurate masses found.

The problem with this technique is two fold. The lack of accurate masses in the metabolite database hinders conclusions, as we cannot be sure about the identification of ions as putative signalling compounds. The main problem is that the PCA is unable to elucidate cause and effect, as it identifies ions that change in concentration during water stress, but are not necessarily involved in signalling. One example of this is mannitol, which is an osmoticum that increases in xylem sap during water stress, but does not cause stomatal closure when fed to epidermal leaf strips (Patonnier *et al*., 1999). It may nevertheless be part of a general stress response, even if not a signalling molecule as such.

We were unable to identify further chemical messengers through metabolomic analysis of water stressed xylem sap. The technique is potentially very useful, but would require much more development. Abscisic acid is known to be a vitally important signalling molecule and thus our current efforts focus on measuring its response to water stress. Abscisic acid was not identified in these samples but we know from all our other work that it increases in sap as a result of stress. It is likely that other potential signalling compounds will similarly be present at low concentration in sap and may be masked by the presence of the more abundant compounds. Alvarez et al (2008) adopted a similar metabolomic approach but used a HPLC/ms technique to look for changes in xylem sap of water stressed maize plants. The inclusion of a chromatography step may increase the chances of identifying minor compounds and they found 31 compounds that changed during drought stress including ABA and PA and several cytokinins including the aromatic compound 6-benzyl amino purine (BAP). Interestingly, in response to stress, the cytokinins zeatin and zeatin riboside decreased in abundance whereas BAP increased. It has been suggested (Stoll et al 2000) that it is the ratio of cytokinin to ABA that is important in determining the effect of transpired xylem sap on stomatal conductance.

This metabolomic approach thus shows great promise as a means of identifying new compounds involved in stress response, but it will require significant fine tuning and probably incorporation of a liquid chromatography step to allow meaningful analysis of minor sap components.

General Discussion

ABA and ABA metabolite concentration was higher in the sap collected via the leaf pressure chamber compared with sap collected using the root pressure chamber. There could be a number of reasons for this. The concentration of solutes in xylem sap

collected from detopped plants with a root pressure chamber is very dependent on the pressure applied to the root system and therefore the sap flow rate (Munns, 1985; Schurr, 1998). Solute concentration will not equate to the *in vivo* condition unless the sap flow rate is closely matched to plant transpiration rate. While we attempted to make collection rates approximate transpiration by first measuring transpiration gravimetrically, mismatch of the rates would be a probable source of error, with higher collection rates resulting in sap dilution. However, there was close correspondence between ion concentration measured in samples collected with the root pressure chamber and by vacuum extraction (Fig 6). The latter technique would not be subject to this flow rate error, suggesting that our collection rates did approximate *in vivo* transpiration rate. These were two different experiments, however so we cannot eliminate the possibility of a dilution error in the first experiment. A more likely explanation for the disparity may be that the leaf pressure technique is actually sampling more than one source of solutes. The exuded petiole droplet may be composed of a mixture of true xylem contents (as represented by the root pressure and vacuum techniques) plus a contribution from the leaf apoplastic fluid (Jachetta *et al.*, 1986). Elsewhere in this report we have shown strong negative correlations between sap ABA concentration (sampled by the leaf pressure bomb technique) and stomatal conductance, suggesting a cause-and-effect relationship. Sap sampled with the leaf pressure chamber may therefore be more representative of what the stomatal guard cells are seeing than the xylem sap extracted from the canes, nearer to its source – the roots. Concentrations of ABA in the leaf apoplast may be subject to influence of pH shifts and metabolic modification not present in the xylem. Nevertheless, the source of some of the ABA present in the leaf pressure sap is likely to be the roots, given the strong effects of soil moisture deficit on root NCED expression that we have observed and documented elsewhere in this report. The contribution of the leaf apoplast to sap ABA and metabolite content appears to vary with node position since ABA and its metabolites increase in concentration in nodes closer to the apex, whereas the concentration of inorganic ions remains independent of node position. Scrutiny of the data in Fig 3 suggests further mechanisms that might be impacting on the concentrations of ABA and its metabolites in Shiraz and Grenache. Ratios of ABA to its primary acidic metabolite PA change with node position in Shiraz (Fig 3a). The proportion of PA increases in samples taken nearer to the apex. This is true for both leaf pressure chamber and root pressure chamber sampling techniques, although the

relative amount of PA is always greater in the leaf chamber samples. This suggests that ABA degradation is occurring as the sap passes up the stem or that there is active exchange of ABA and its metabolites with surrounding tissues. Interestingly, the data also show differences between Shiraz and Grenache, there being more PA and less DPA relative to ABA in Grenache. This suggests that aspects of ABA transport and metabolism may be involved in the expression of the more isohydric response shown by Grenache.

Sap collected using the leaf pressure chamber may be contaminated with the intracellular fluid of cells damaged during leaf excision (Jokhan *et al.*, 1999). However, we found that discarding the first drop of sap produced by leaf bomb pressurisation did not cause a significant change in ABA or metabolite concentration compared with sap samples which included the first drop. Thus we can be confident in using the whole of the exuded sap volume for analysis, provided the chamber pressure in excess of the water potential is minimised. This is important as another potential cause of sap contamination associated with the leaf pressure chamber is excessive overpressure, defined here as pressure of >0.2MPa from the balance pressure (Jachetta *et al.*, 1986). However, we found that an over-pressure of as much as 0.8MPa caused no change in the ABA concentration in sap of well watered Merlot and Shiraz vines. By contrast, the ABA content of sap from water stressed vines was reduced as the over-pressure was increased, thus confirming the idea (Jachetta *et al.*, 1986) that ABA collected using the leaf pressure chamber is derived from 2 sources: the roots via the xylem and the leaf apoplast. In non stressed plants the source is predominantly from the former whereas in stressed vines the latter plays a greater role in determining ABA content. The activity of leaf NCED is not greatly affected by the changes in leaf water potential encountered during mild water deficits or as a result of diurnal changes (described elsewhere in this report) but bulk leaf ABA does show some fluctuation, suggesting that a source of this apoplastic ABA, other than *in situ* synthesis, may be responsible. It is possible that hydrolysis of ABA glucose ester as described by Dietz et al (2000) may represent this source. However, substantial work would be required to test this idea.

Conclusions.

Using a leaf pressure bomb to expel petiole sap is the only option available for the routine assessment of xylem-mobile bio-active molecules in field vines. It is

important to recognise the factors which may influence their concentration in expressed sap and to gain an understanding of their role in determining vine response to soil water deficits and the environment. The concentration of ABA and its metabolites is higher in petiole sap collected with a leaf pressure chamber than their concentration in sap expelled from the distal half of the same petiole through the application of pneumatic pressure to the roots, suggesting that apoplastic sources contribute to solutes in petiole sap. Petiole sap may therefore be a reasonable representation of the solution in contact with the stomatal guard cells which ultimately control leaf transpiration and response to root-sourced chemical signals. Petiole sap collected at a pressure within 0.2 MPa of the leaf water potential appears to be minimally affected by applied pressure. The concentration of ABA in sap from unstressed vines is not affected at all by the collection pressure but if the vines have been subject to water deficit, the sap collected at a pressure near the water potential has an elevated ABA content but as the pressure is increased the sap becomes progressively diluted so that the ABA content falls.

Chapter 2. The influence of irrigation strategies on the production of leaf and root sourced chemical signals and their importance in determining vine performance under a range of environmental conditions

2.1 The Influence of Deficit Irrigation on ABA synthesis and distribution in leaf and root tissues of two self rooted grape varieties with contrasting water use properties.

Introduction.

The regulation of stomatal aperture is of considerable importance in that it is the plant's predominant short term mechanism for regulating water loss through transpiration, although xylem cavitation is sometimes argued to be of importance under drought conditions (Schultz, 2003). In the longer term, modification of canopy area through leaf shedding may also become important. Different plant species and different cultivars within species can respond in differing ways to reductions in soil water. These differences have been classified on a physiological basis as isohydric, which conserve water and anisohydric, which do not (Stocker 1956; Tardieu and Simonneau, 1998). While grapevine (*Vitis vinifera*) has been generically classified as 'drought avoiding' (Smart and Combe, 1983), or near-isohydric (Schultz, 2003), there is a wide disparity in the responses within *Vitis vinifera*. For example, two cultivars, Grenache and Shiraz (Syrah), have been demonstrated to have near-isohydric and anisohydric responses respectively to soil drying when grown under field conditions (Schultz, 2003) and to changes in VPD under field conditions and when grown in pots under well watered conditions (Soar et al., 2006). The mechanisms used by plants to sense changes in their soil and aerial environment remain largely unknown but they are expressed in the form of chemical messages that transmit information to the responsive elements, in this case the stomata and there is much evidence that the dominant chemical messenger is abscisic acid (ABA).

The plant hormone ABA is associated with the regulation of many developmental and physiological processes in plants including responses to a number of stresses (Zeevaart

and Creelman, 1988). It has been well established that reductions in soil water availability result in increasing levels of ABA in the xylem and apoplastic sap which, in turn are associated with decreasing stomatal conductances (g_s) (Loveys 1984, Davies and Zhang, 1991; Correia and Pereira ,1994; Tardieu et al., 1996; Jia and Zhang, 1999). However ABA may not be the only factor involved in stomatal regulation and there are good arguments proposing hydraulic and/or chemical signals other than ABA in the same role (Christmann et al., 2007; Rodrigues et al., 2008; Neumann, 2008; Schachman and Goodger, 2008; Wilkinson et al, 2007). It is also unclear whether signaling from the roots initiates the stomatal response or whether the key signal or signals originate in the leaf, for example as a function of leaf water potential (Ψ_l) (Rodrigues et al., 2008), *de novo* synthesis of ABA in the leaf (Taylor et al., 2000; Thompson et al., 2000; Tan et al., 1997; Liotenberg et al., 1999; Qin and Zeevaart, 1999; Soar et al, 2004, 2006) as distinct from synthesis in the root , redistribution of sequestered leaf ABA in response to changes in pH (Stoll et al, 2000; Rodrigues et al., 2008), changes in ABA transport in response to changes in xylem sap pH (Else et al., 2006; Sauter and Hartung, 2002), or re-activation of esterified ABA (ABA-GE) by increased glucosidase activity (Lee et al., 2006).

There is good evidence linking increases in ABA abundance in water stressed tissues with the activity of one or more of the ABA biosynthetic genes, in particular the gene or genes encoding the 9-*cis*-epoxycarotenoid dioxygenase (NCED) enzyme (Tan et al., 1997; Burbidge et al., 1997, 1999; Qin and Zeevaart, 1999, Liotenberg et al., 1999; Soar et al, 2004). Concurrent with increased levels of ABA in stressed tissues are increased levels of its catabolic products, primarily phaseic acid (PA) (Zeevaart, 1980; Pierce and Rasche, 1981; Babiano, 1995; Gergs et al., 1993; Uknes and Ho, 1984: Jia et al., 1996) which has been shown to be regulated by the cytochrome P450 enzyme 8'-hydroxylase (c.f. the review by Cutler and Krochko, 1999) and recently the genes encoding the 8'-hydroxylases have been identified and characterized in a number of plants (Kushiro et al., 2004; Saito et al., 2004; Millar et al., 2006; Saika et al, 2007; Yang and Choi, 2006; Yang and Zeevaart, 2006). Using molecular probes to determine the activities of the genes associated with ABA synthesis and catabolism in grapevine, we set out to examine three aspects of its function in field grown grapevine under control and water deficit

conditions. Firstly, we wished to establish the primary source of ABA found in the xylem sap and how droughting affected the source and the abundance of the ABA in the sap. Secondly, we asked whether, under our experimental conditions, we would observe physiological and molecular differences between Grenache and Shiraz vines consistent with their near-isohydric and anisohydric classifications according to Schultz (2003). Thirdly, we wished to determine whether there was any evidence for differences in ABA catabolism associated with the near-isohydric and anisohydric classifications associated with the two cultivars. These experiments have been designed to address objectives 1), 2), 3) and 4) of this project.

Materials and Methods.

Details of materials and methods common to all the experiments presented in this report are included in the Materials and Methods. A probability level of 0.05 or less is considered as significant unless otherwise stated. Information specific to these particular experiments is as follows:

Plant material

Measurements were taken in the 2006/2007 season at the South Australian Research and Development Institute's (SARDI) Nuriootpa field research station. The soil at this site is described as a light pass sandy clay red brown earth (Northcote 1954). The trial consisted of one row each of 15 year old Shiraz (clone 1125) and Grenache (clone 139HT) with like barrier rows to either side. The block orientation was east to west such that most of the canopy was fully sun exposed throughout the measurement period. Readings and samples were taken across six replicate vines at weekly intervals. Pre-bud burst in the previous year, 2006, trenches had been dug close to these vines and to adjacent vines on either side to allow access to the roots. The trenches measured 6m x 0.15m x 0.5m and were filled with washed sand. This was necessary because the soil at this site becomes too hard to allow root sampling as it dries. To encourage root growth in the trenches, each trench was watered for 1 hour daily with 2 x 4L/h drippers until the commencement of the deficit irrigation treatment at the end of December 2006, at which time the control

vines were returned to normal irrigation and water was completely withheld from the deficit treated vines until returning to normal irrigation on the 6th March 2007. During the 20 week growing period control vines received 1.3 – 2 ML/hectare delivered via above surface drippers. Sampling was commenced on the 25th January. Shiraz berries were harvested on the 1st March 2007 and the Grenache berries on the 19th March 2007.

Results:

Mid morning stomatal conductances of both Grenache and Shiraz control vines were comparatively high early in the year and then dropped to a relative stable level of around 120 mmol m^{-2} s^{-1} for Grenache and 100 mmol m^{-2} s^{-1} for Shiraz (Fig 1a). Withdrawal of irrigation from the vines in mid December resulted in both cases in a significant lowering in stomatal conductance (g_s) to approximately 25 mmol m^{-2} s^{-1} returning to control levels only after irrigation was re-applied in early March (Fig 1a). Leaf water potential (Ψ_l) of control vines from both cultivars, measured at the same time as stomatal conductance, slowly declined from about -0.9 MPa in January to between -1.2 and -1.25 MPa in mid February rising again from late February to between -1.0 and -0.8 MPa at the end of March. There were no consistent differences between Grenache and Shiraz. Leaf water potentials in the deficit vines were significantly lower than in the control vines but had similar profiles of decline and recovery except that the deficit Shiraz recovered more rapidly after re-irrigation than did the deficit treated Grenache (Fig1b).

With the exception of the initial values (25th January) there were no significant 'variety' effects on the pattern of change with time in g_s for either control or deficit treated vines in this experiment. In contrast, there was a significant variety effect in the pattern of change with time in Ψ_l between the cultivars in both the control vines and the deficit treated vines with the greatest difference being associated with the more rapid recovery of the Shiraz vines upon re-watering.

Xylem sap was collected from leaf petioles at the same time as g_s and Ψ_l were being

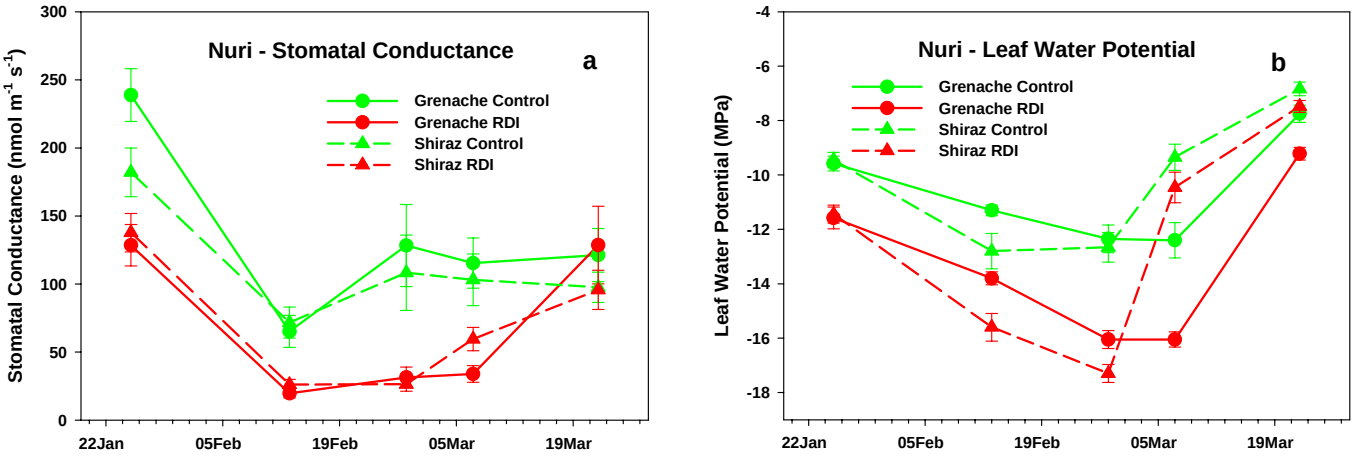


Figure 1. Stomatal conductance (a) and leaf water potentials (b) of Grenache and Shiraz vines under normal irrigation and regulated deficit irrigation (RDI). Water was withheld from the RDI vines between the end of December 2006 and the 6th March 2007. S.E^{s}. are indicated by bars (n = 3 x 15 for g_s and 3 x 5 for Ψ_l).

measured. Levels of ABA in the xylem sap (Fig 2a and b), rose slightly in both sets of control vines from approximately 60 ng mL^{-1} in January peaking at approximately, 100ng mL^{-1} in late February early March and dropping again in late March. In the deficit treated Grenache, xylem sap ABA levels rose from around 80 ng mL^{-1} in January , peaking at 280 ng mL^{-1} in late February before falling again. Deficit treated Shiraz vines showed a similar rise in xylem sap ABA levels rising from 100 ng mL^{-1} in January to a peak of 305 ng mL^{-1} in late February before falling again after rewatering
Within each cultivar there was a significant effect of droughting on the sap ABA levels with only the late January and late March levels in Grenache being similar and the early and late March levels in Shiraz being similar. There was a significant variety effect on the pattern of change with time in sap ABA levels in both control vines and deficit treated vines however this resulted from differences after rewatering on the 6th March 2007 when Shiraz had a greater reduction in the levels in both deficit and control vines compared to Grenache and this was consistent with the concurrent measurements of stomatal conductance.

Leaf and root tissues were collected throughout the course of the trial and analysed for ABA levels (Fig 2c and d). In Grenache, ABA levels in both leaf and root tissues rose during the course of the trial with the levels reaching a maximum in the leaves in mid February and in the roots in late February. Unexpectedly the ABA levels in the leaves of the control Grenache vines were consistently higher than in the deficit treated vines however these differences were not significant. In contrast in the roots the higher levels of ABA found in the deficit treated vines in comparison with the control vines were significant prior to the 7th March but not subsequently. In Shiraz, a constant low level of ABA was present in the leaves of the control plants with a significantly higher level in the deficit treated vines (Fig 2d) prior to the 7th March. the concentration of ABA in Shiraz leaves was significantly lower than in Grenache leaves for control treatments. Levels of ABA in the root tissues of the control vines were highest through mid to late February before falling again (Fig 2e and f). Levels in the roots of the deficit treated vines peaked significantly in late February before dropping in response to rewatering

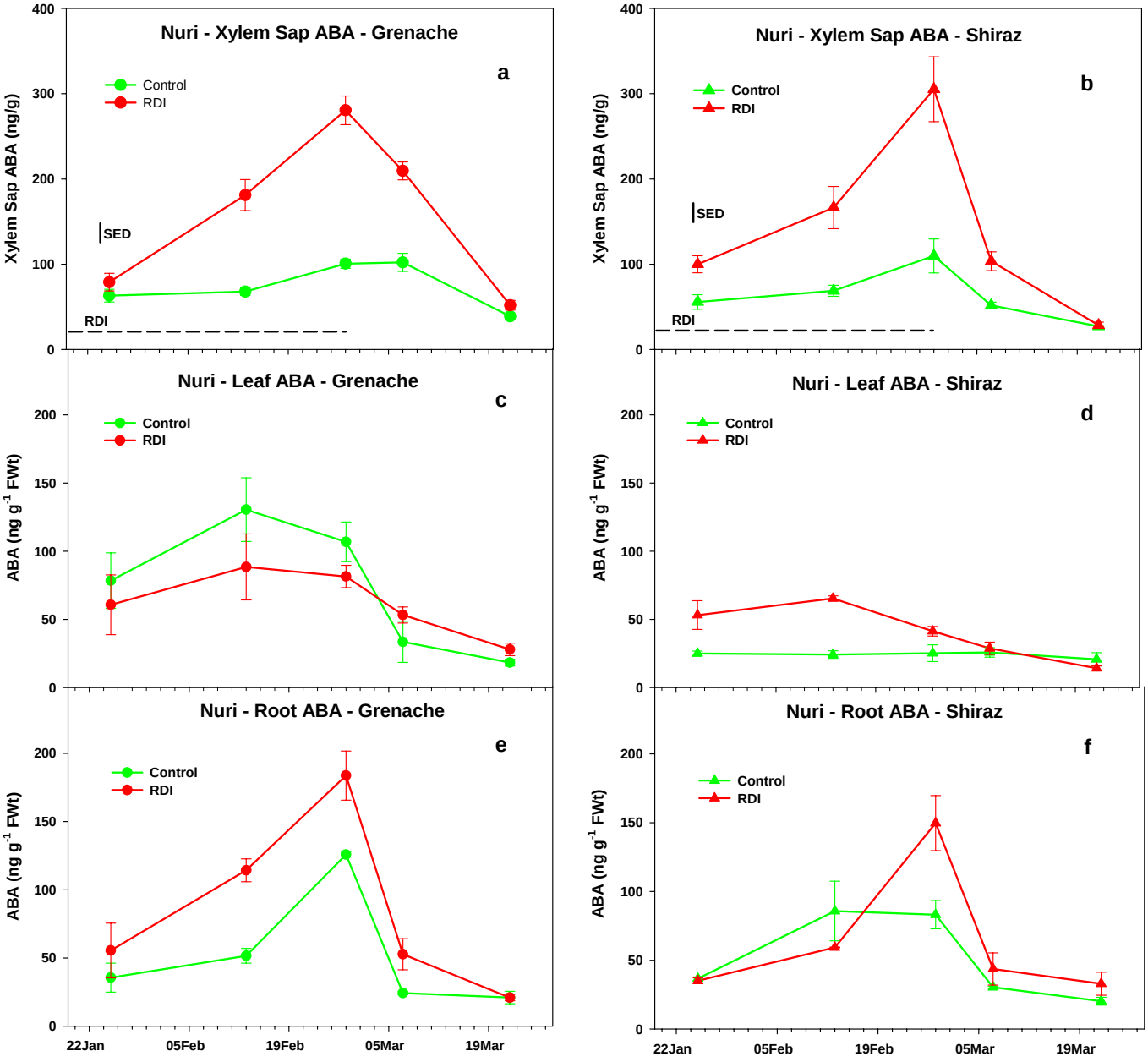


Figure 2 ABA abundances in the xylem sap (a and b), leaf (c and d) and root tissues (e and f) of Grenache and Shiraz vines under normal irrigation and RDI.
S.E. bars are shown (n = 3 x 3 for xylem sap and 3 x (5 leaves pooled) for leaf tissue and 3 x 1 for root tissue. ABA abundances were determined by LC MS/MS as detailed in Materials and Methods.

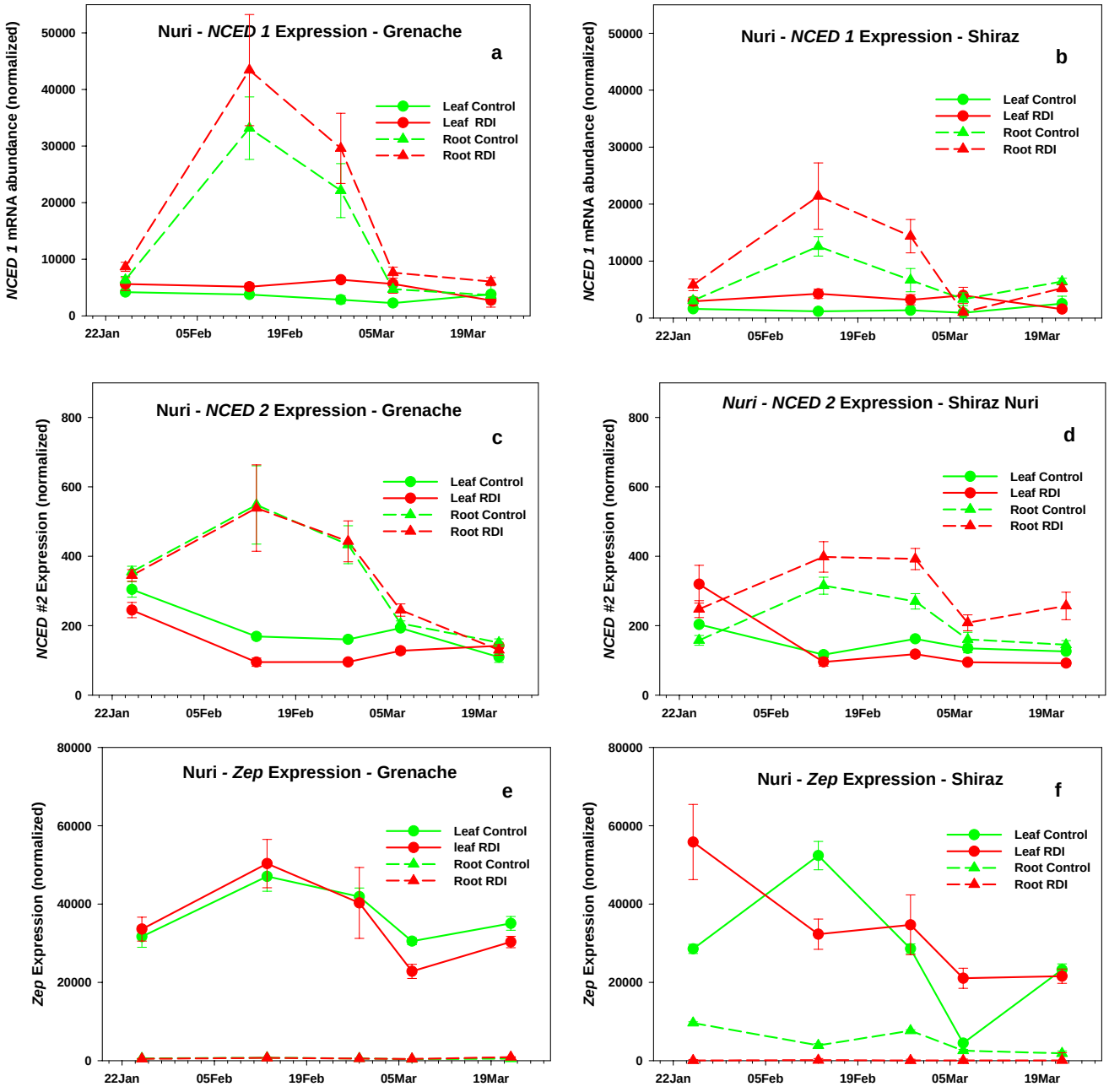


Figure 3 Expression levels of the *NCED 1* (a and b), *NCED 2* (c and d) and *Zep* (e and f) genes in leaf and root tissues of Grenache and Shiraz vines under normal irrigation and RDI.

S.E^{s}. are indicated (n = 3 in all cases). Expression levels were determined by real time PCR (qPCR) as described in Materials and Methods. Results have been normalised against the expression of two internal standards *Vitis Ubiquitin* and *Vitis EF1*. and the values for each gene are relative to the values for the other genes.

The activities of genes associated with the regulation of synthesis of ABA in the various tissues were determined by Real Time PCR. The expression of the *NCED 1* gene (9-*cis*-epoxycarotenoid dioxygenase gene1) was low in deficit and control leaves of both Grenache and Shiraz (Fig 3a and b). In contrast, in root tissues of both cultivars, levels of expression of the gene were strongly enhanced during mid to late February in both deficit and control plants which was consistent with the enhanced ABA concentrations measured in the roots. Expression of the *NCED 2* gene (9-*cis*-epoxycarotenoid dioxygenase gene 2) was an order of magnitude lower than that of the *NCED 1* gene in all tissues, with the expression in root tissues being higher than in the leaf tissues (Fig 3c and d) and elevated, like the expression of the *NCED 1* gene, during mid to late February. Expression of the zeaxanthin epoxidase gene (*Zep*) was low in root tissues in both cultivars throughout the trial but elevated in all leaf tissues particularly during mid to late February in Grenache and in early season in Shiraz, dropping towards the end of the season (Fig 3e and f).

We examined the expression of the three ABA 8'-hydroxylase genes of *Vitis vinifera, VvABA8'OH-1 (Hyd 1), VvABA8'OH-2 (Hyd 2)* and *VvABA8'OH-3(Hyd 3)* (see Materials and Methods), in order to determine if variations in their expression were linked with changing levels of ABA during or, more particularly, towards the end of the growing season. Of the three genes *Hyd 1* was the most highly expressed although at levels almost two orders of magnitude lower than the levels of expression of the *NCED 1* gene (Fig 4a-d). In both Grenache and Shiraz cultivars expression of *Hyd 1* was higher in leaf tissues than in root tissues and remained relatively constant throughout the season. Expression of the *Hyd 2* gene was lower than that of the *VvABA8'OH-1* gene with some perturbations of expression being evident in root tissues of Grenache late in the season and in Shiraz in mid February. Expression of the third ABA 8'hydroxylase gene, *Hyd 3* , was essentially undetectable throughout the season in either cultivar (data not shown)

Water deficit caused a significant increase in berry ABA in both Shiraz and Grenache at veraison (Fig 5a and b). From then until harvest berry ABA concentration fell in both control and deficit berries. The water deficit had no significant effect on the rate of berry sugar and anthocyanin accumulation (Fig 5a-d).

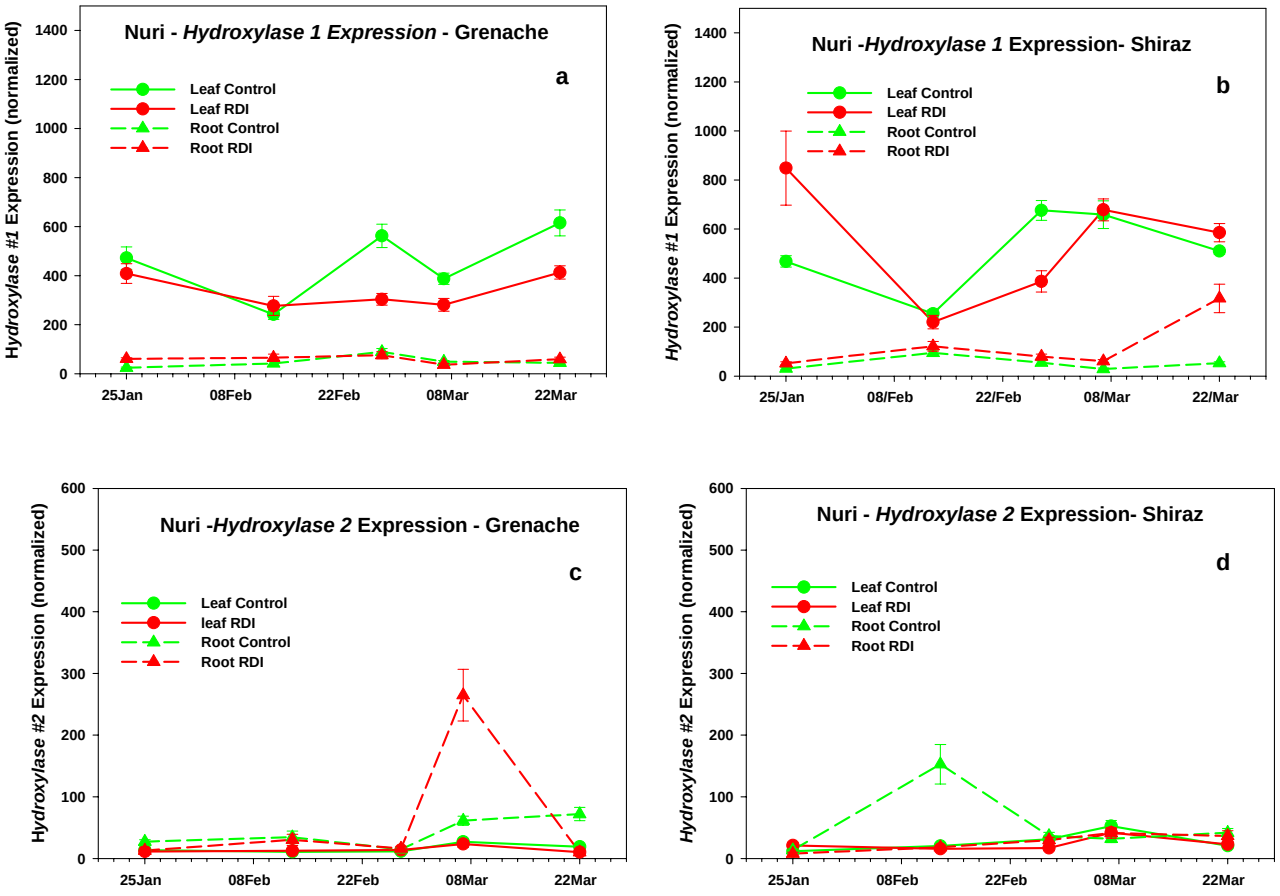


Figure 4 Expression levels of the *Hyd 1* (a and b) and *Hyd 2* (c and d) genes in leaf and root tissues of Grenache and Shiraz vines under normal irrigation and RDI. Experimental details are as in the legend to Fig 3.

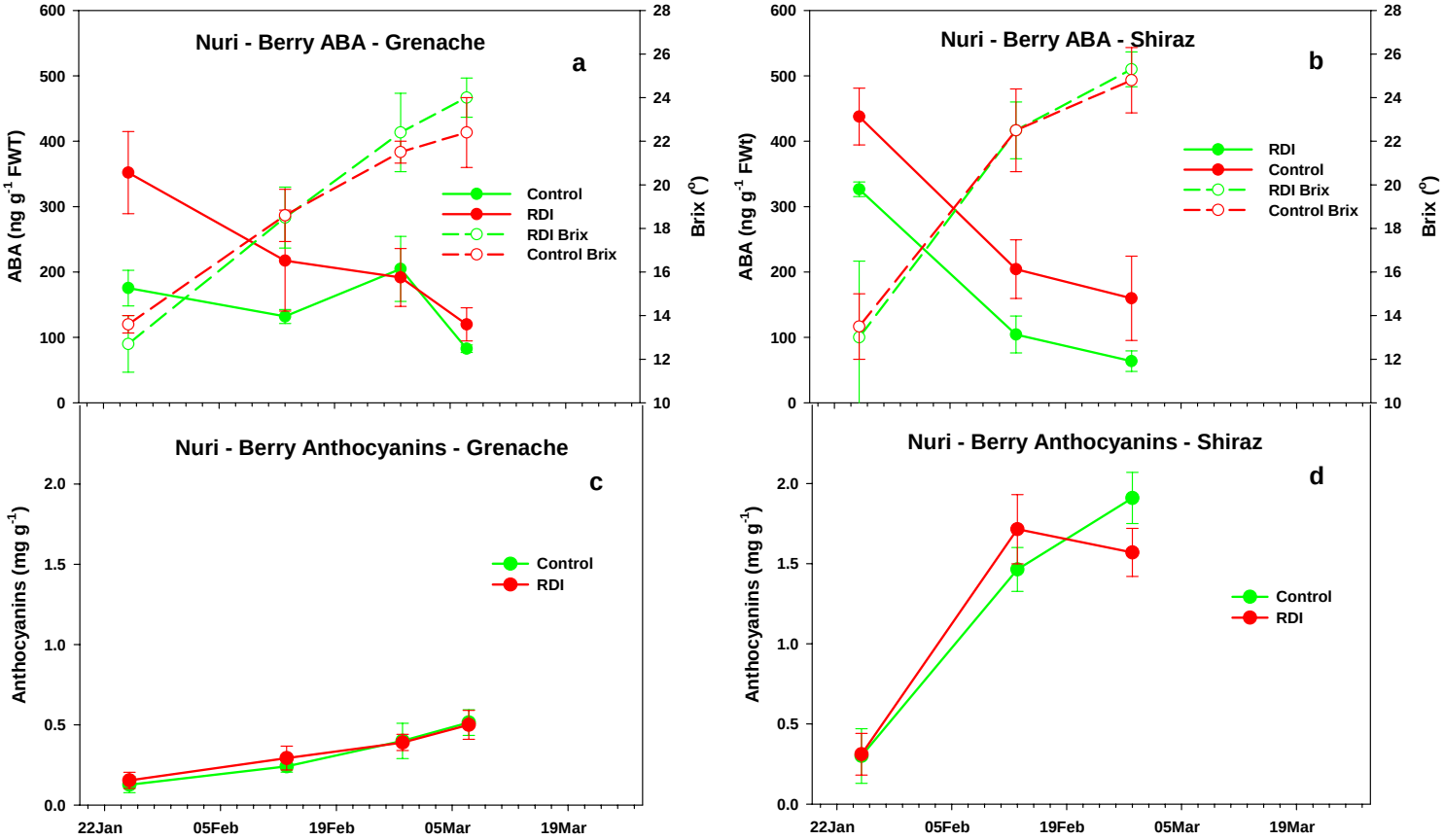


Figure 5 ABA abundances and Brix levels (a and b) and anthocyanin levels (c and d) in the berries of Grenache and Shiraz vines under normal irrigation and RDI.
S.E^{s}. are indicated (n = 3 x 2). Analysis was as described in Materials and Methods.

Discussion:

Stomatal conductance of the control Grenache and Shiraz vines fluctuated across the season but the profiles of the fluctuations were closely similar. Droughting (water deficit) resulted in a significant decrease in conductance in both cultivars but again the comparative profiles were closely similar. This was unexpected in view of the suggested differences in the water use strategies of the two cultivars (Schultz, 2003; Soar et al, 2006). Leaf water potential of both cultivars also fluctuated across the season and both were significantly lowered by droughting unlike the observations of Schultz (2003) where a stress related lowering of Ψ_l was only evident in the stressed Shiraz (Syrah) vines. Unlike the g_s, the profiles of change in Ψ_l in both the control and deficit vines were statistically different between cultivars with the differences being most pronounced in the droughted vines after the reapplication of water when the Shiraz vines recovered more rapidly than the Grenache. This distinction between Grenache and Shiraz may be related to differences in hydraulic architecture identified by Schultz (2003). Schultz showed that during the imposition of water stress Grenache showed considerably higher rates of xylem cavitation than Shiraz which presumably would take longer to repair once a favourable water status was restored.

The levels of ABA in the xylem sap of the two cultivars were enhanced some 3 fold by droughting which is consistent with a correlation between xylem ABA and the observed reductions in stomatal conductance. Again the patterns of change in levels across the season did not appear to differ between cultivars except after re-watering of the two sets of stressed vines when Shiraz recovered more quickly. An analysis of ABA abundance in leaves and roots of the vines (Fig 2c-f) did not give a clear indication of the most likely source of the xylem ABA although the maximum levels of ABA in both cultivars were in the roots of the deficit treated vines and coincided with the maximum levels in the xylem sap. Sap ABA generally correlated more strongly with root ABA than with leaf ABA, suggesting that the roots were the more likely source of xylem ABA. Regression analysis of combined Grenache and Shiraz data revealed that xylem sap ABA was not

significantly correlated with leaf ABA (r^2=0.09, p=0.19) whereas xylem sap ABA correlated strongly with root ABA (r^2=0.62, p<0.0001).

In order to investigate in more detail the origin(s) of the xylem ABA, we looked at the activities of the primary genes associated with regulation of ABA synthesis in root and leaf tissues. Activities of both *NCED 1* and *NCED 2* were several fold higher in the root tissues of both cultivars under both control and stress conditions. The activities in the roots increased and decreased across the season in a similar way, although slightly ahead of the increase and decrease in xylem, leaf and root ABA abundance while no such changes were evident in the expression of the genes in the leaf tissues. This strongly points to the roots as the source of the xylem ABA . The lag that was observed between maximum gene expression and maximum ABA levels may have resulted from the 7 day interval between sampling of the tissues, in other words, the real maxima could have occurred between the two sampling dates.

Expression of the zeaxanthin epoxidase gene (*Zep*) was high in the leaves in both cultivars but barely detectable in the roots. The Zep enzyme is responsible for the epoxidation of zeaxanthin to violaxanthin (Marin et al., 1996) both of which, together with antheraxanthin, are components of the xanthophyll cycle which is believed to play an important role in photoprotection in the chloroplasts (Demmig-Adams and Adams 1992; Chaves et al., 2003). High expression of the *Zep* gene in the leaves is therefore consistant with the role of the Zep enzyme in photoprotection. However its low level of expression in the roots raises the question of whether it could be rate limiting in these tissues with respect to ABA synthesis as suggested previously (Soar et al., 2006). The pool of carotenoid precursors that are converted by Zep on the way to ABA (reviewed by Liotenberg et al. 1999) has been reported to be small in the root and potentially rate limiting in itself (Thompson et al. 2000; Borel et al., 2001). If the roots are the primary source of the xylem sap ABA as is suggested by the high expression of the *NCED* genes in the roots and, to a certain extent, the relative abundances of ABA in the root and leaf tissues, it is tempting to speculate that an intermediate precursor of ABA, for example 9-*cis*-neoxanthin, is being synthesised in the leaves and transported to the roots for

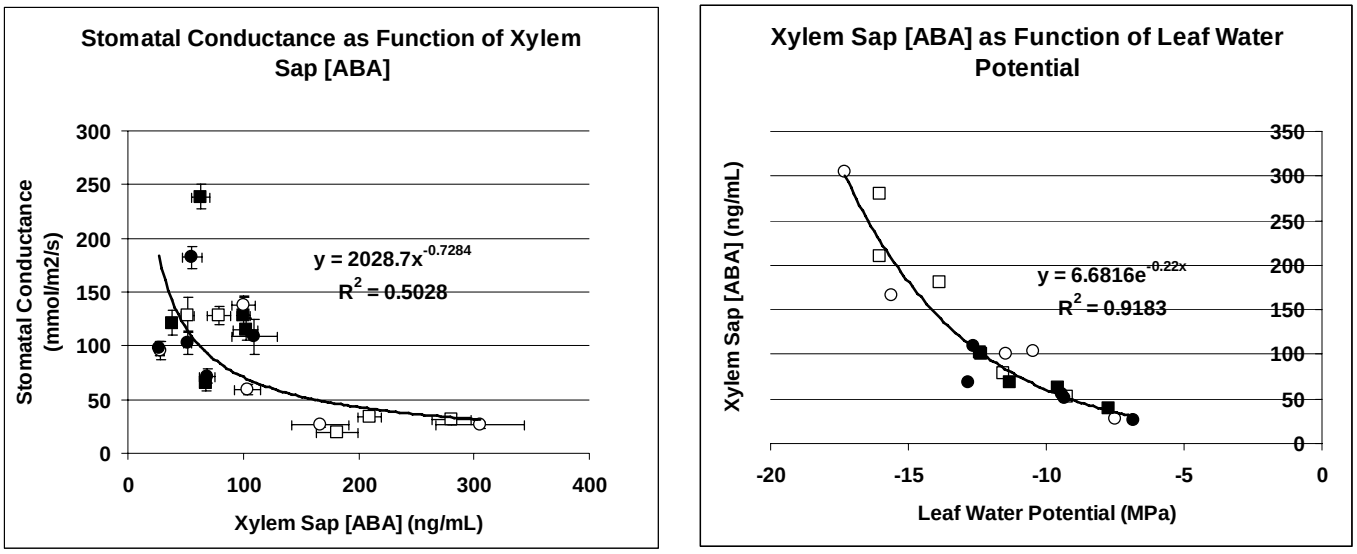
conversion to xanthoxin and on to ABA. However this seems unlikely as both the Zep and NCED enzymes are located in the chloroplast or plastid and such a pathway would involve the bidirectional transport of the 9-*cis*-neoxanthin across the plastid membrane. The simplest explanation is that the reported small pool of ABA precursors in the root tissues is the result of rapid turnover and subsequent transport of the synthesised ABA out of the roots.

In order to determine whether differential catabolism was playing a role in the regulation of ABA accumulation or decline, we looked at the expression of the three genes encoding the primary ABA-catabolic enzyme (ABA 8'-hydroxylase) in grapevine. To facilitate this we first isolated and characterised the three *Vitis vinifera* genes encoding the 8'-hydroxylases, *VvABA8'OH-1 (Hyd 1), VvABA8'OH-2 (Hyd 2)* and *VvABA8'OH-3 (Hyd 3)* and confirmed their identities by expression in yeast cell culture (see Materials and Methods). In the grape tissues, *Hyd 1* was the most highly expressed with *Hyd 2* at least two orders magnitude lower. The only grape tissue in which significant levels of mRNA from the *Hyd 3* gene were detected was dry grape seed (data not presented here). Expression levels of all three genes were very low relative to the expression of the *NCED 1* gene. Expression of *Hyd 1* in leaf tissue was approximately two orders of magnitude lower than the maximum expression of the *NCED 1* gene in roots, while its expression was barely detectable in the roots. Some fluctuation in expression of *Hyd 1* was evident particularly in the Shiraz leaves and expression in both Shiraz and Grenache tended to increase later in the season when ABA levels were declining in response to rewatering. Similar expression profiles were seen with *Hyd 2* although at even lower levels. As with *Hyd 1*, there was some fluctuation in expression levels with one apparent increase in expression in deficit treated Grenache roots late in the season but although the increase was consistent in multiple leaves from three separate vines, it would be difficult to allocate a significance to the single point. An alternative explanation for the observed changes in expression of the hydroxylase genes involves a direct short term response to environmental variables and data to support this idea are presented in a subsequent section of this report. The relatively long time intervals between sampling in the Nuriootpa experiments may not have allowed us to see short-term changes in gene

expression or their consequences. Expression of *Hyd 3* was undetectable in root or leaf tissue throughout the season and likewise, has not been detected in berry or seed tissues during development or germination respectively (data not presented here).

Our results differ from those of Schultz (2003) in that the Shiraz and Grenache vines behaved in a very similar manner to each other both under normal irrigation and in their responses to deficit irrigation. This difference may have resulted from differences in irrigation or environment (soil type, humidity, average temperature), but our data have proved to be consistent as the 2006-2007 season data was essentially replicated in the 2007-2008 season (data not included here). While it is noticable that the leaf water potentials of our vines, both irrigated and stressed, are quite low and suggest a baseline of water stress under both irrigation strategies, they are similar to those of Schultz (2003) and therefore do not explain why our vines did not demonstrate anisohydric and near-isohydric behaviours described by Schultz (2003). It is of interest to note that significant differences can be seen in ABA levels particularly in the stressed roots of both cultivars and in the expression of the *NCED* genes in both cultivars while the ABA levels in the xylem sap are essentially identical. As differences in the supposed sources of the sap ABA would be expected to result in differences in the sap ABA levels themselves, this suggests some unidentified regulatory mechanism is functioning, but it does not appear to be at the level of ABA catabolism, at least not at the level of expression of the genes encoding the ABA-catabolic enzymes.

We looked for correlations between Ψ_l, g_s and xylem sap ABA to investigate the proposed hydraulic component of stomatal regulation (Lovisolo et al, 2002; Schultz 2003; Liu et al, 2003). There appeared to be a good correlation between xylem sap ABA and stomatal conductance up to an ABA concentration of approximately 100ng mL^{-1} above which the stomates were essentially closed and further increases in ABA had little effect (Fig 6a). Such a relationship appeared in other experiments carried out in this project and has also been described for other plant species (Tardieu et al 1996). Leaf water potential compared with xylem sap ABA (Fig 6b) showed a very strong correlation ($r^2 = 0.9183$) between the pooled data sets suggesting, again, that under our experimental



F**ig 6. Correlations.** Graphs show relationships between Stomatal Conductance and Xylem Sap [ABA] (a), and between Leaf Water Potential and Xyle Sap [ABA] (b). Symbols are Grenache control ■, Grenache RDI □, Shiraz control ●, Shiraz RDI ○.

conditions there was no significant difference between the cultivars. All of these comparisons would indicate a connection between the three parameters, Ψ_l, xylem sap ABA and g_s but do not support either xylem sap ABA on its own, or leaf water potential on its own, as being solely responsible for stomatal regulation.

2.2 ABA synthesis and catabolism in *Vitis vinifera* under varied levels of irrigation. The relationship between leaf water potential and xylem sap ABA and the source of the xylem sap ABA.

Introduction.

Given the evidence above that it is the activity of ABA biosynthesis genes that are driving vine response to water deficit, we have investigated this in more detail by applying a range of irrigation treatments to Cabernet Sauvignon vines.

Our main objectives were to determine the effects of varied irrigation on the physiology of the vines, to establish the primary source of the ABA found in the xylem sap and how reduced irrigation affected the source and the abundance of the ABA in the sap. In addition, we wished to determine whether there was any evidence of specific induction of the genes encoding the ABA catabolism enzyme 8'-hydroxylase associated with rapid response(s) to changes in irrigation and/or environment. As part of our collaboration with the GWRDC-funded "Vines to Wines" project (CSP05/04), wines made from the same vines used in the experiments detailed below were subject to detailed sensory analysis as it was of interest to see if a link could be established between physiological response to water deficit and the expression of compounds giving rise to the sensory attributes of the grapes. These experiments address project objectives 1), 2), 3) and 5).

Materials and Methods.

Details of materials and methods common to all the experiments presented in this report are included in the Materials and Methods. A probability level of 0.05 or less is considered as significant unless otherwise stated. Information specific to these particular experiments is as follows:

Plant material

Cabernet Sauvignon vines on Ramsey rootstocks were kindly made available for irrigation trials at the Yalumba, Oxford Landing Vineyard, in the South Australian Riverland. At the end of the 2007 season, 60cm deep trenches were dug alongside selected vines in groups of three panels and these were backfilled with washed sand to facilitate collection of root samples. In seasons 2007-2008 and 2008-2009 the vines were irrigated from mid September until after berry harvest in late February. Three different irrigation treatments were applied to the vines in groups of five rows, one central (experimental) row and two barrier rows on each side. Irrigation rates were 6.6ML/h, 3.3ML/h and 1.3ML/h where 3.3ML/h was the vineyard standard rate. Measurements of stomatal conductance and leaf water potential of trenched vines were taken at approximately weekly intervals and samples were collected of leaf and root tissue and xylem sap, which were snap frozen under liquid nitrogen and stored for analysis at -80^{o}C for leaf and root tissue and -40^{o}C for xylem sap.

Results:

Sampling of the Cabernet Sauvignon irrigation trial was undertaken on a weekly basis over two growing seasons, 2007-08 and 2008-09. As results from both seasons are closely similar we have presented here only those from the 2008-09 season except where data from the previous season conflicts with or emphasises the 2008-09 seasons findings.

Irrigation was applied at the prescribed levels three to four times times per week. We measured soil moisture using gypsum blocks (Watermark) at depths of 10, 20, 40, 80 cm and found that only the treatment receiving the least water did not influence soil moisture below 40cm but that the other 2 treatments were both influenced by irrigation down to

80cm. Soil moisture potentials, averaged over the upper 40cms, ranged between -6.9 to -33.5 kPa in the T_0 treatment, -6.9 to -46.1 kPa in the T_1 treatment and -12.5 to -73.4 kPa in the T_3 treatment. Ambient temperatures fluctuated between 18^o and 38^{o}C max., 4^o and 17^o min. in mid November 2008 increasing to 26^o and 46^o max., 6^o and 25^o min. between late January to mid February 2009.

Leaf water potentials (Ψ) reflected the differential levels of irrigation throughout the season (Fig 1b), with Ψ-T_0 the highest, Ψ-T_1 intermediate and Ψ-T_3 the lowest. Over the complete season Ψ-T_0 was significantly higher than Ψ-T_1 and Ψ-T_3 which were not significantly different to each other except in late December and late February. The stomatal conductance (G_s) of the vines in each irrigation treatment also reflected the differences in irrigation strategies (Fig 1a), differing significantly from each other at all dates throughout the season.

Levels of ABA in the leaves of the T_0 vines did not change significantly across the season except for a brief rise on the 21st January (Fig 2a). However there were significant increases in leaf ABA in the T_1 and T_3 vines subsequent to the 30th Dec 08 peaking in late January early February '09. Across most of the season levels in the T_1 and T_3 leaves were comparable. Levels of ABA in the xylem sap varied significantly in all three treatments across the season with a significant decline in abundance mid December '08 to early January '09 in all three and a significant rise in both the T_1 and T_3 treatments on the 17th February '09. Levels of xylem sap ABA in the T_1 vines were significantly higher than in the T_0 vines for most of the season except on the 20th November '08 and the 30th December '08. Levels in the T_3 vines were higher than in the T_1 vines for most of the early season up to mid January and higher than in the T_0 vines across the whole season. ABA levels in the roots of all three treatments changed significantly across the season, with increased levels evident from mid January '09 onwards and the increases in the T_1 and T_3 roots being significantly greater than in the T_0 roots.

Immediate products of ABA breakdown, phaseic acid (PA) and dihydrophaseic acid (DPA) were present in varying amounts in all of the tissues examined (Fig 2d-f and g-i).

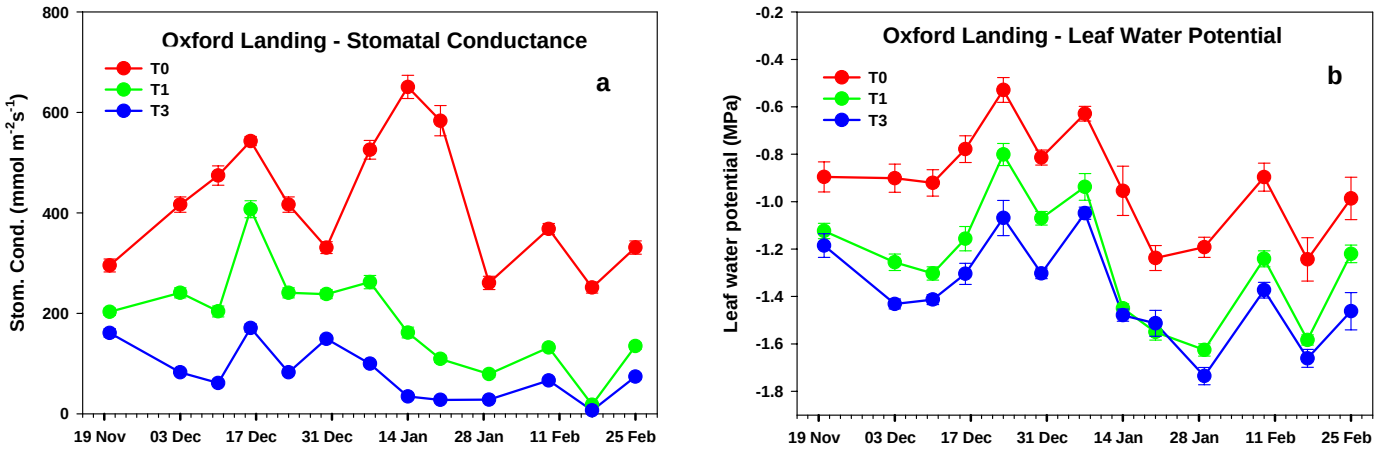


Figure 1 Stomatal conductance (a) and leaf water potentials (b) of Cabernet Sauvignon vines on Ramsey rootstocks under three irrigation schedules.
Irrigation was at the rates of 6.6ML/h (T_0) , 3.3ML/h (T_1) and 1.3ML/h (T_3) where 3.3ML/h was the vineyard standard rate. S.E^s. are indicated by bars (n = 3 x 15 for g_s and 3 x 5 for Ψ_l).

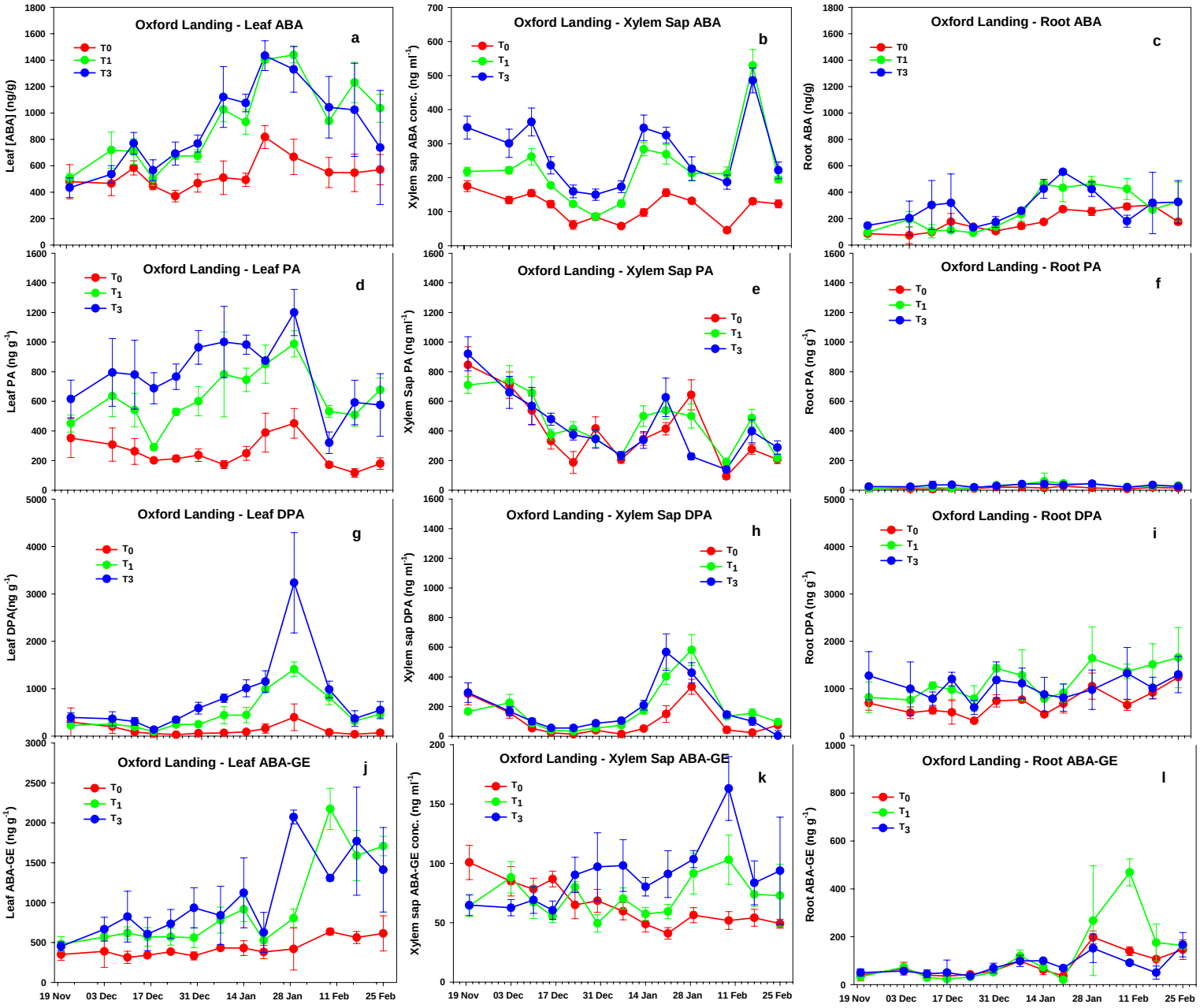


Figure 2 ABA abundances in the leaf (a,d,g and j) xylem sap (b,e,h and k)) and root tissues (c,f,I and l) of Cabernet Sauvignon vines on Ramsey rootstocks under different irrigation schedules.

Irrigation rates are detailed in the legend to Fig 1. S.E. bars are shown (n = 3 x (5 leaves pooled) for leaf tissue, 3 x 3 for xylem sap and 3 x 1 for root tissue). ABA abundances were determined by LC MS/MS as detailed in Materials and Methods.

In the leaf there was a relatively constant level of PA in the T_0 vines across the season with a small non significant peak in mid to late January. In the T_1 and T_3 vines the levels of PA increased significantly from the 29th Jan to the 9th Feb and dropped again before rising towards the end of the season. For the majority of the season PA levels in the T_3 leaves were significantly higher than in the T_1 leaves which, in turn, were significantly higher than in the T_0 leaves. PA levels in the xylem sap reflected to an extent the ABA levels in the sap. There was a steady significant decline in abundance in the sap of all three treatments prior to the 14th January '09 with significant peaks in abundance in all three between the 14th January and the 29th January PA abundance in the sap of the T_3 treatment was significantly higher that in the T_0 treatment on the 29th January as was the abundance in the T_1 treatment on the 9th and 17th February. PA was barely detectable in the roots of all three treatments across the season.

DPA levels were relatively constant in all three treatments across the season with the exception of peaks in all three on the 29th January, which were significant in the T_1 and T_3 treatments but not in the T_0 treatment. Across most of the season the levels of DPA in the T_1 and T_3 leaves were significantly higher than in the T_0 leaves. The DPA abundances in the xylem sap of the three treatments declined significantly early in the season then remained relatively stable with a significant increase in abundance peaking in all three between the 21st and 28th January at which time the T_1 and T_3 abundances were significantly higher that in the T_3 sap. Root DPA levels increased significantly in the later part of the season in all three treatments with levels in the T_1 and T_3 roots higher than in the T_0 roots for the majority of the season.

The abundance of the glucose ester of ABA, ABA-GE in the leaf tissues of all three treatments increased significantly across the season with the abundance in the T_0 treatment increasing the least (Fig 2j-l). Across most of the season the levels in the T_1 and T_3 leaves were similar to each other and significantly higher than in the T_0 treatment. Large increases in the abundances in the T_1 and T_3 treatments were evident but not coordinated towards the end of the season with the increase in the T_3 leaves preceding that in the T_1 leaves. ABA-GE abundances in the xylem sap of all three treatments were

an order of magnitude lower than in the leaves. The abundance in the T_0 sap dropped significantly across the season while the abundances in the T_1 and T_3 saps remained relatively constant until peaking significantly on the 9th February.

ABA-GE abundances in the roots of all three treatments were also very low compared with the levels in the leaf tissues. A significant increase in levels in all three treatments was evident towards the end of the season culminating in peaks of abundance between 9th and 17th February with the abundance in the T_1 roots becoming significantly higher than in both the T_0 and T_3 roots.

The activities of the genes associated with the regulation of synthesis and degradation of ABA in the leaf and root tissues were examined from first berry colour, 30th December '08, until the end of the season, 25th February '09. Gene activities were determined by qPCR and have been presented in the figures as raw copy number data, which approximately relates the expression levels of each gene to those of the other genes
The expression of the *NCED 1* gene (9-*cis*-epoxycarotenoid dioxygenase gene1) was low in leaves from all three treatments and relatively constant (Fig 3a). It was similarly low in roots from all three treatments prior to the 14th of January after which it increased markedly in the T_3 vines, to an intermediate extent in the T_1 vines and to a lesser extent in the T_0 vines (Fig 3b). Expression of the *NCED 2* gene in the leaves was an order of magnitude lower in all three treatments and relatively constant (Fig 3c). Its expression in roots mirrored the expression of the *NCED 1* gene but, as in the leaves, was an order of magnitude lower (Fig3d). We also examined the expression of the gene encoding zeaxanthin epoxidase (the *Zep* gene) although it is of greater importance in the xanthophyll cycle which confers photoprotection to photosynthetic tissues (Demmig-Adams and Adams 1992; Chaves et al., 2003). Expression of the *Zep* gene in the T_0 leaves peaked over the second half of January and dropped off later in the season (Fig 3e). Its expression in the T_1 and T_3 leaves tended to drop during January and recovered towards the end of the season. Expression in the roots was at least two orders of magnitude lower than in the leaves and it showed no discernable trend with time or with treatment (Fig 3f).

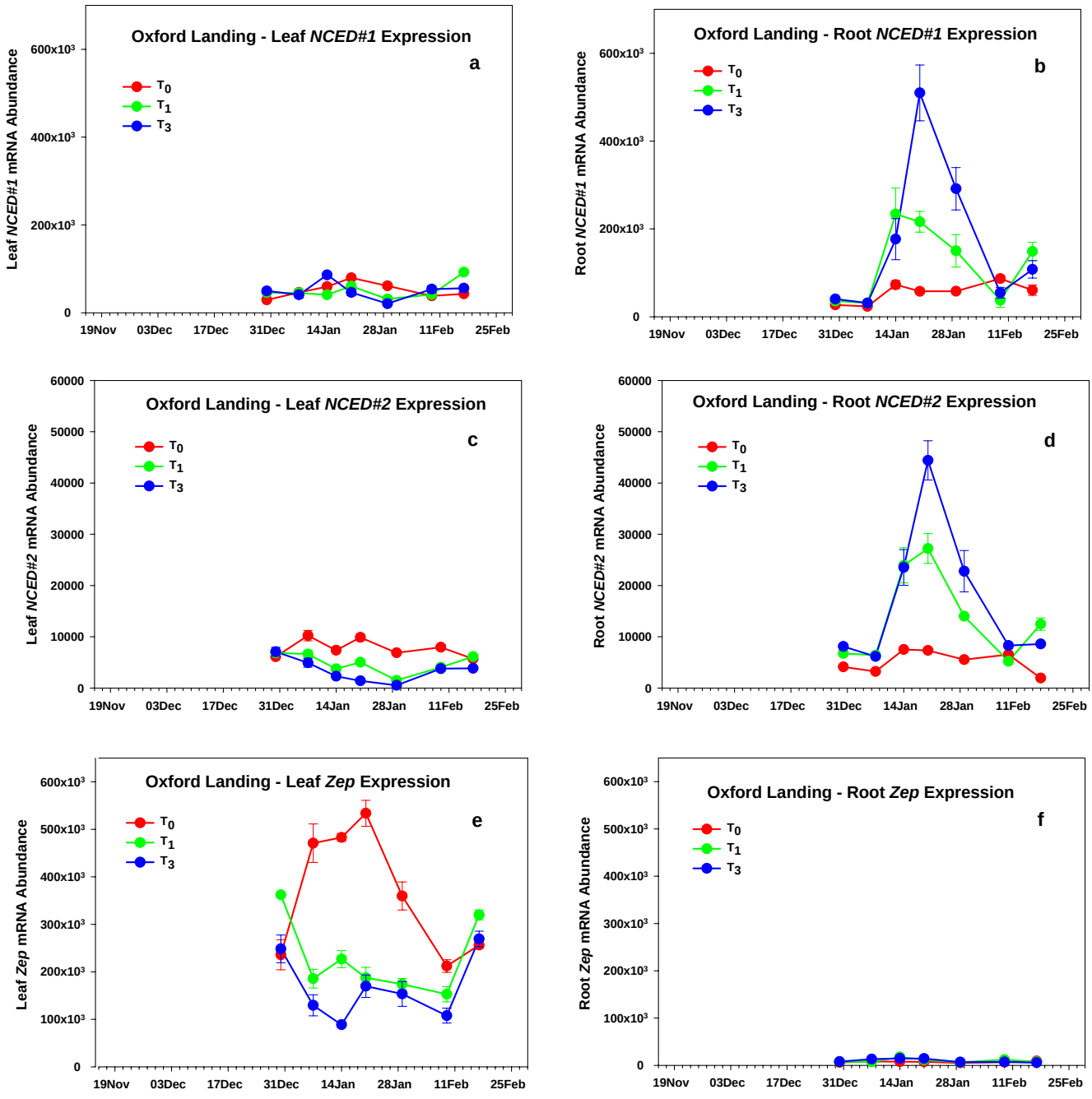


Figure 3 Expression levels of the *NCED 1* (a and b), *NCED 2* (c and d) and *Zep* (e and f) genes in leaf and root tissues of Cabernet Sauvignon vines on Ramsey rootstocks under different irrigation schedules.
S.E^{s}. are indicated (n = 3 in all cases). Expression levels were determined by real time PCR (qPCR) as described in Materials and Methods. Results have been normalised against the expression of two internal standards *Vitis Ubiquitin* and *Vitis EF1* and the values for each gene are relative to the values for the other genes.

In order to examine the breakdown of ABA in the leaf and root tissues, we measured the expression of the gene(s) encoding the ABA 8'-hydroxylase enzyme(s) which catalyze the primary (regulatory) catabolic step. As described in the preceeding section we first isolated and characterised the three *Vitis vinifera* genes encoding the 8'-hydroxylases, *VvABA8'OH-1 (Hyd 1), VvABA8'OH-2 (Hyd 2)* and *VvABA8'OH-3 (Hyd 3)* and confirmed their identities by expression in yeast cell culture (see Materials and Methods). In the grape tissues, *Hyd 1* was the most highly expressed with *Hyd 2* at least two orders magnitude lower. The only grape tissue in which significant levels of mRNA from the *Hyd 3* gene were detected was dry grape seed (data not presented here).

Two peaks of expression of the *Hyd 11* gene were evident in the leaf tissues of all three treatments (Fig 4a). Both peaks, the first on the 7th January and the second on the 9th February, coincide with sudden drops in VPD suggesting an interrelationship. We have investigated this possible regulation of ABA metabolism by an environmental variable in more detail in a later section of this report. Expression of the *Hyd 1* gene in root tissue was three orders of magnitude lower than in leaf tissue, however increased expression of the gene was evident in the T_1 and particularly the T_3 treatments between the 14th and 21st January. Expression of the *Hyd 2* gene in both leaf and root was low relative to that of the *Hyd 1* gene in the leaf and did not appear to fluctuate to any extent.
Wine sensory attributes were first assessed by simple difference tests which established that both T0 and T3 wines differed significantly from the control (T1) wines. A detailed sensory evaluation showed that T0 wines differed significantly ($P<0.05$) from T1 and T3 wines in dark berry, Dried Fruit, Menthol, Cough Syrup, Earthy and Leather aromas but that T1 and T3 wines did not differ significantly in these attributes. Similarly, taste attributes Fresh Berry and Cough Syrup differed significantly between T0 and the other 2 wines. A Brine/Salty attribute was significantly higher in the T3 wines than in the T0 and T1 wines.

Discussion:

The differing irrigation strategies had the expected influence on the g_s and Ψ_l of the

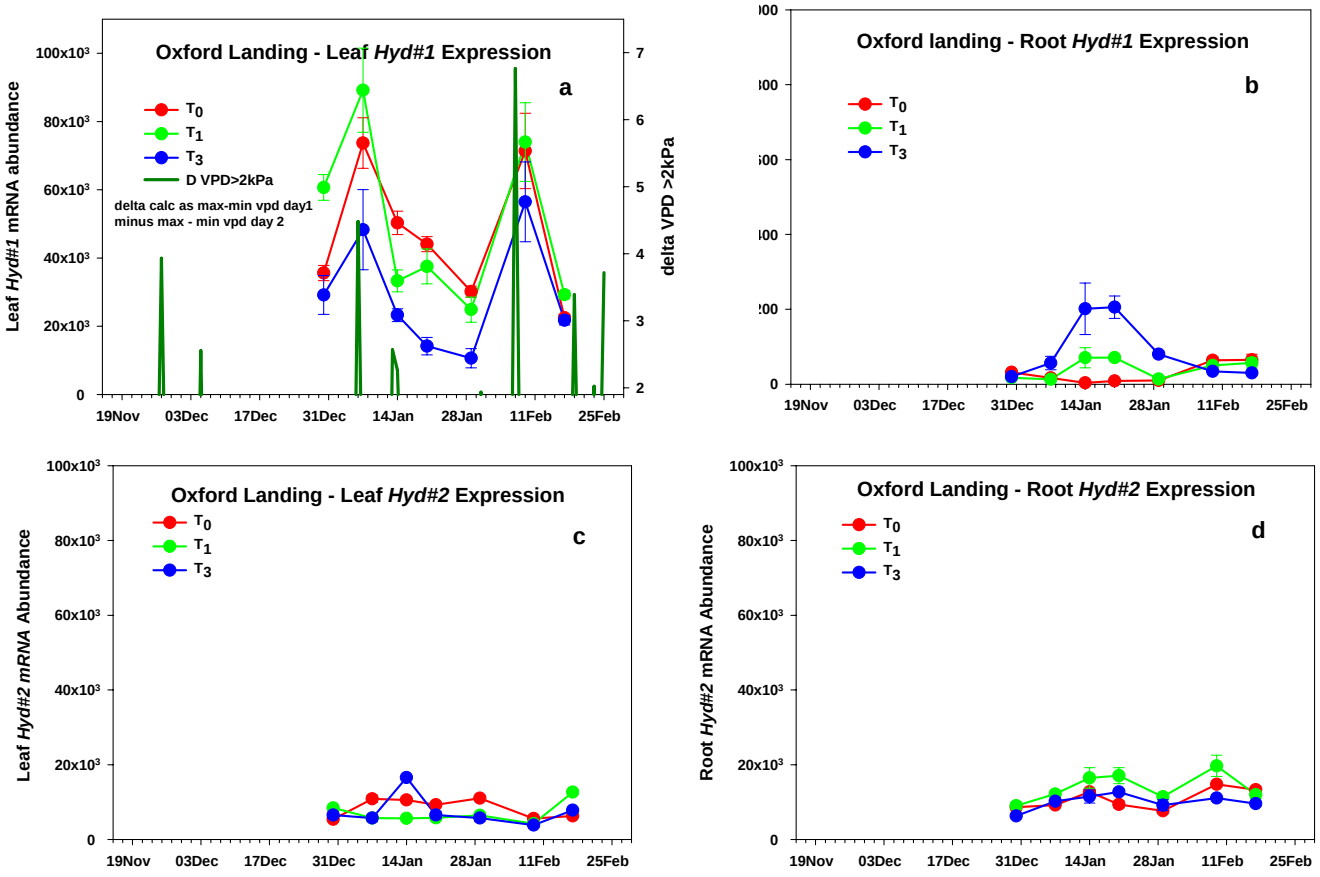


Figure 4 Expression levels of the *Hyd 1* (a and b) and *Hyd 2* (c and d) genes in leaf and root tissues of Cabernet Sauvignon vines on Ramsey rootstocks under different irrigation schedules.

Experimental details are as in the legend to Fig 3. Included in graph (a) showing expression of the *Hyd 1* gene in leaf is an indication of periods of rapid change in VPD (delta VPD) calculated as the $VPD_{max} - VPD_{min}$ on day one minus $VPD_{max} - VPD_{min}$ on the subsequent day.

vines in both the 2007-08 and the 2008-09 seasons, with the highest level of irrigation (T_0) resulting in vines with the highest g_s and Ψ throughout and those with the lowest irrigation level (T_3), the lowest g_s and Ψ. Pooling data from all three irrigation treatments and both growing seasons, there was a correlation between g_s and Ψ_l which was best fitted by an exponential function $g_s = a^{b\Psi}$. Similar exponential correlations have been described between g_{max} and pre dawn Ψ for stressed and unstressed Grenache and Shiraz, (Schultz 2003). Individual correlations for our data from each season differed slightly from each other but were sufficiently similar to give an $r^2 = 0.632$ when combined (Fig 5) suggesting a continuity of sampling across the two seasons.

In a previous section of this chapter, dealing with the results from the Nuriootpa trials, we looked for correlations between g_s or Ψ_l with [ABA] in leaf, root or xylem sap of Grenache and Shiraz vines with and without water stressing. While there was a good correlation between g_s and xylem sap [ABA] up to an [ABA] of approximately 100ng mL^{-1}, the best correlation that we found in that experiment was between Ψ and xylem sap [ABA] with an $r^2 = 0.8168$ between the pooled data from both stressed and unstressed Grenache and Shiraz. In the current experiment, data from all three irrigation treatments and across both seasons did not show a particularly strong correlation of g_s or Ψ_l with [ABA] in any of the locations, the best being a logarithmic correlation between g_s and leaf [ABA] with an $r^2 = 0.518$. Treating the data from each season separately gave better correlations in the '07-'08 season, the best being between g_s and root [ABA] with an $r^2 = 0.7244$ and between Ψ_l and root [ABA] with an $r^2 = 0.6225$. However correlations in the '08-'09 season were all lower and, across both seasons individually and pooled, there was little difference between the r^2 values of a logarithmic fit as proposed by Zhang and Outlaw (2001) and a linear fit.

Good correlations have been demonstrated in *Vicia faba* L. between g_s and leaf [ABA], between g_s and leaf apoplast [ABA] (Zhang and Outlaw, 2001a), and between g_s and guard cell [ABA] (Zhang and Outlaw, 2001b) while others have shown correlations of g_s with xylem sap [ABA] in maize and *Commelina communis* (Tardieu and Davies, 1992;

Tardieu et al., 1992; Tardieu, Zhang and Gowing, 1993; Davies, Tardieu and Trejo, 1994).

As the primary intention of this trial was to examine the effects of differential irrigation treatments on the physiology of the vines and the levels of ABA synthesis in the leaf and root tissues, we looked for a link between ABA synthesis (as indicated by the expression of the *NCED* genes associated with ABA synthesis) and irrigation. During the period we investigated, the expression of both *NCED* genes was significantly higher in the semi-stressed root tissues than in the leaf, despite the ABA levels being higher in the leaf. During the same period, the levels of expression were inversely related to the levels of irrigation supplied to the vines. Similar relationships were noted in the previous season and are consistent with an induction of ABA synthesis in the roots in response to reduced soil water availability which is central to our understanding of vine response to deficit irrigation strategies such as RDI and PRD.

In neither season could we directly link the increased expression of the *NCED* genes with decreased soil moisture because we did not have accurate measurements of moisture levels in the sand- filled trenches. However, combining data from both seasons, expression of both the *NCED 1* gene and the *NCED 2* gene in the roots appeared to be negatively correlated with Ψ_L ($P<0.0001$) and positively with the ABA levels in the xylem sap ($P<0.0001$) which would be consistent with the induction of ABA synthesis in the roots in response to decreased moisture availability.

As in the above trial at Nuriootpa, we examined the expression of the three genes encoding the primary ABA-catabolic enzyme (ABA 8'-hydroxylase) to investigate whether there was any involvement of these genes in regulation of either ABA abundance or g_s. While we did observe large fluctuations in the expression of the *Hyd 1* gene in leaf and to a lesser extent in root tissue, there was no obvious correlation between the fluctuations and the abundances of the ABA metabolites PA and DPA in leaf or root tissues or in the xylem sap. There was a suggestion resulting from the Nuri trial that the minor fluctuations observed in the expression of the hydroxylase genes in that trial could have resulted from the genes responding to changes in the environment. Consequently we looked to see if the large changes in the expression of the *Hyd 1* gene in this trial, particularly in leaf, were related to any environmental parameter. Both peaks of *Hyd 1*

activity observed in the leaves of all three irrigation treatments followed immediately after large drops in VPD (expressed in Fig4a as the difference in the range of VPD experienced on successive days. In other words, if a very humid and mild day follows a day with more stressful conditions, the VPD index shown in the figure increases). These periods are associated with a transient small drop in leaf ABA and a similarly transient increase in stomatal conductance. The changes in leaf ABA are small, but as discussed elsewhere in this report, this is consistent with a local change in ABA (in a pool adjacent to the stomatal guard cell for example) being expressed against a large inactive pool of ABA in other parts of the leaf. This means that bulk leaf ABA, as measured here, changes little. Recently it has been shown that subjecting *Arabidopsis* plants to high humidity levels rapidly resulted in the induction of two of the four *Arabidopsis ABA 8'-hydroxylase* genes resulting in metabolism of ABA within the guard cells (*CYP707A1*) and in the vascular system (*CYP707A3*), effectively allowing opening of the stomata (Okamoto et al., 2009). Induction of the *Vitis Hyd 1* gene in, or closely associated with, the stomatal guard cells in our field trial would not necessary result in significant increases in ABA metabolite abundances in the leaf as the bulk of the ABA and metabolites in leaf are most probably compartmentalised and unavailable for metabolism (Cowan, 1982; Slovik et al., 1992; Slovik and Hartung 1992; Wilkinson and Davies 1997; Sauter et al., 2001). The peaks of expression of the gene in leaves in mid February did coincided with small but significant increases in g_s in all three irrigation treatments but the early January peak in conductance was less well coordinated with the changes in *Hyd1* activity and ABA content, possibly due to the relatively long sample intervals employed in this experiment. Nevertheless, there does appear to be a correlation between expression of the *Vitis Hyd 1* gene in leaf and rapid falls in VPD, suggesting that grapevines can respond rapidly to changes in the environment that are favourable to transpiration.

In these experiments berry quality was assessed in terms of the sensory attributes of wine made from berries derived form the 3 irrigation treatments T0, T1 and T3. Our working hypothesis being that water deficit influences wine sensory properties through the production of root-sourced chemical signals, notably ABA. The idea that ABA may be the link between water deficit and berry quality is supported by considerable evidence.

For example, exogenous application of ABA to grape berries close to veraison can cause a dramatic stimulation on the accumulation of anthocyanins in red grape varieties (Jeong et al 2004, Cantin et al 2007) and it now established that ABA is a regulator of anthocyanin and tannin biosynthesis (Lacampagne et al 2010 and references quoted therein). Moreover, exogenous application of ABA to fully irrigated Cabernet Sauvignon vines significantly increased the anthocyanin and total polyphenol content of the berries at harvest (Quiroga et al 2008). When these data are considered in the light of known effects of water deficit on endogenous ABA, a strong case can be made for ABA being in some way involved in determining berry composition at harvest and that this may impact on wine sensory attributes. While berry ABA in our experiments was significantly increased by the water deficit treatment in both Shiraz and Grenache there was no measurable effect on either anthocyanin or sugar accumulation. Nevertheless, we show above that water deficit can have major impact on the sensory qualities of wine made from Cabernet Sauvignon vines subject to a range of irrigation treatments. We have therefore not been able in these experiments to establish a causal link between ABA metabolism and berry or wine sensory qualities but evidence from other experiments referred to above probably warrants further investigation. If it could be established that the link between irrigation management and berry composition was represented by a single, stress-related, chemical compound then our understanding of ways to manipulate wine outcome would be enhanced.

Conclusions.

Both Shiraz and Grenache showed marked seasonal changes in stomatal conductance and leaf water potential and these changes were less marked in irrigated vines. The two varieties behaved very similarly with the exception of a period at the end of the season when the Shiraz vines recovered more quickly than Grenache after the water deficit vines were rewatered. Changes in root and xylem sap ABA were closely aligned with changes in stomatal conductance but leaf ABA changed little through the season. There were major changes in expression of root but not leaf *NCED 1* through the season.. The *8'-hydroxylase #1* gene was poorly expressed in roots and more highly expressed in leaves but there was little effect of water deficit or grape variety. Similar results were obtained

with Cabernet Sauvignon vines subject to a range of water deficit treatments. These observations are all consistent with stomatal conductance being driven by changes in root ABA which is in turn the result of enhanced expression of the *NCED* genes. The low expression of *8'-hydroxylase* in roots and higher expression in leaves is consistent with the roots being the source of ABA and the leaves the sink where it is degraded. Major day to day fluctuations in VPD were coincident with changes in the expression of the *8'-hyroxylase 1* gene, suggesting that this may be part of a regulatory system allowing leaves to respond rapidly to ambient conditions. This response would have a major impact on the efficiency of water use.

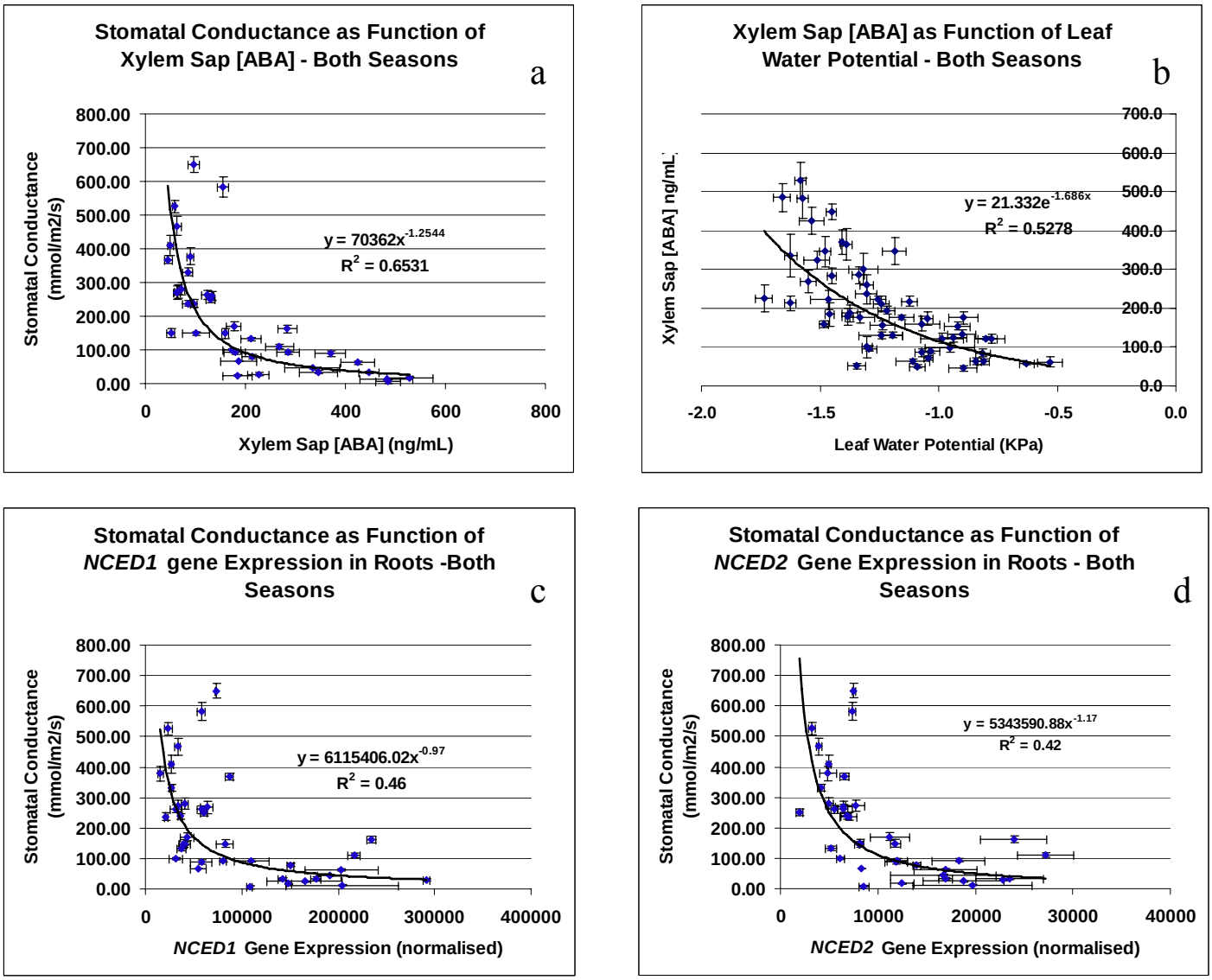


Figure 5 Correlation of Stomatal Conductance with Xylem Sap [ABA] (a), Xylem Sap [ABA] with Leaf Water Potential (b),and Stomatal Conductance with the expression of the *NCED* genes (c and d).
Graphs include data from all irrigation treatments and across both seasons 2007-2008 and 2008-2009. Bars indicate S.Es.

Chapter 3. The 8'-hydroxylases of grapevine: their involvement in diurnal changes in stomatal responses to ABA.

Introduction.

A relationship between leaf conductivity (g_s) and the concentration of ABA in the xylem sap was first described by Raschke (1975) and subsequently by many other experimenters in numerous plant species. While there is good evidence of a strong correlation between the maximum conductance of the leaf during the day (g_{max}) and the average sap ABA concentration $[ABA]_{xyl.}$ particularly under conditions of drying soil (Wartinger et al., 1990), the relationship sometimes does not hold on a diurnal basis (see for example Tardieu et al., 1992; Tallman, 2004; Correia et al., 1995). There have been a number of hypotheses to explain the diurnal variations in stomatal aperture. These include responses to a dark/light cycle (see review by Tallmann 2004), increased whole leaf transpiration in response to increased VPD (Mott and Parkhurst, 1991; Monteith, 1995) delivering more ABA from the xylem stream to the stomatal complexes (Tardieu et al., 1992), variations in leaf water potential (Tardieu et al., 1993), and apparent enhancement of stomatal sensitivity to ABA possibly as a result of a redistribution of ABA from sites where it can be sequestered (Hartung and Davies, 1991).

Most of these hypotheses invoke stomatal responses to ABA however, at least in some cases, some other mechanism may be involved. Work on the ABA deficient mutant (*aba1*) and the ABA-insensitive mutants (*abi1-1*, *abi2-1*) of Arabidopsis (Assmann et al., 2000) and on the ABA deficient mutants *flacca* and *sitiens* of tomato (Bradford et al, 1983; Holbrook et al., 2002) has demonstrated stomatal responses to changes in VPD or to soil drying which appear to be independent of ABA. In these cases, the authors propose either a direct hydraulic influence or the involvement of some chemical/hormone other than ABA.

Stomatal responses to changes in humidity or VPD appear at first sight to be straight forward, i.e. as the plant experiences a greater differential between its internal vapour

pressure and that of its environment, excessive water loss is prevented by reducing stomatal aperture. This simple relationship has been termed "hydro-active negative feedback" (Buckley, 2005) and implies that, as leaf water potential drops, so does stomatal conductance. However, this simple relationship does not explain an initial increase in evaporation rate prior to its reduction. To explain this the term "feed-forward" (Farquhar, 1978; Buckley, 2005)or "appartent feed forward" (Franks et al., 1997) has been introduced and implies the existence of a second (at least) mechanism of regulation in addition to a response to decreasing availability of water. Applying VPD stress with CO_2 concentrations reduced to near zero has been shown to eliminate the stomatal response to changes in VPD in a number of plant species (*Glycine max* L., *Abutilon theophrasti*, L. and *Chenopodium album* L.) (Bunce, 1996) suggesting an involvement of ABA as it is known that CO_2 is required for stomatal responses to ABA (Raschke, 1975). Whether this ABA is derived from within the guard cells (Zhang and Outlaw, 2001) or is delivered to the guard cell apoplasm from epidermal or mesophyll cells (Popova et al., 2000; Davies et al., 2002; Wilkinson and Davies, 2002) or from roots via the xylem sap (Wilkinson and Davies 2002) is unclear and may vary depending on the level of VPD stress or on the availability of water to the roots.

Four genes encoding the 8'-hydroxylase enzymes, which are responsible for the primary step in ABA catabolism, were first identified in *Arabidopsis* (Kushiro et al., 2004; Saito et al., 2004). Recently it has been shown that subjecting *Arabidopsis* plants to high humidity levels after droughting rapidly results in the induction of two of these genes resulting in metabolism of ABA within the guard cells (*CYP707A1*) and in the vascular system (*CYP707A3*), effectively allowing opening of the stomata (Okamoto et al., 2009). As humidity levels in field crops change significantly during the day, it is possible that the observed diurnal variations in stomatal aperture and the proposed (apparent) diurnal increase in stomatal sensitivity to ABA are functions of ABA metabolism in or close to the guard cells.

In this work we set out to investigate the involvement of ABA in the diurnal responses of the stomata to significantly different VPDs of commercially established Cabernet

Sauvignon grapevines under normal field irrigation. Measurements of physiological parameters of the vines under the different conditions were compared with the abundance of ABA and its metabolites in leaf and root tissues and in xylem sap. In addition we asked where ABA was being synthesised and catabolised using molecular probes to measure gene activities associated with ABA synthesis (Soar et al., 2004) and ABA metabolism (see: Isolation and characterization of the *Vitis vinifera* ABA 8'- hydroxylase genes, in the Materials and Methods Section) in the leaf and root tissues.

Materials and Methods: See general Materials and Methods, Section 5

Results.

Two sampling periods were selected in January and February 2009 where maximum VPDs were different and reproducible over a number of days. The VPD profiles over the sampling periods are shown in Fig 1b. Higher day temperatures (Fig 1a) combined with lower humidities (Fig 1c) account for the significant higher maximum VPD on the High VPD days versus the Low VPD days. The combination of higher night temperatures and higher night humidities on the High versus the Low VPD nights resulted in similar night VPDs.

Over both periods sampling was undertaken across two days, with the 11.00am, 12.00noon, 3.00pm and 6.00pm points being taken on the first day and the 8.00am and 10.00am points on the day following. For ease of comparison (temporal continuity), we have plotted the Day 2 sample points prior to the Day 1 points as indicated in Fig 2a and subsequent graphs. High VPD (H-VPD) refers throughout to data collected during the period when the day VPD was higher than that attained during the low VPD period (L-VPD). In both experimental periods, the vines had been irrigated two to three days prior to measurements and sampling.

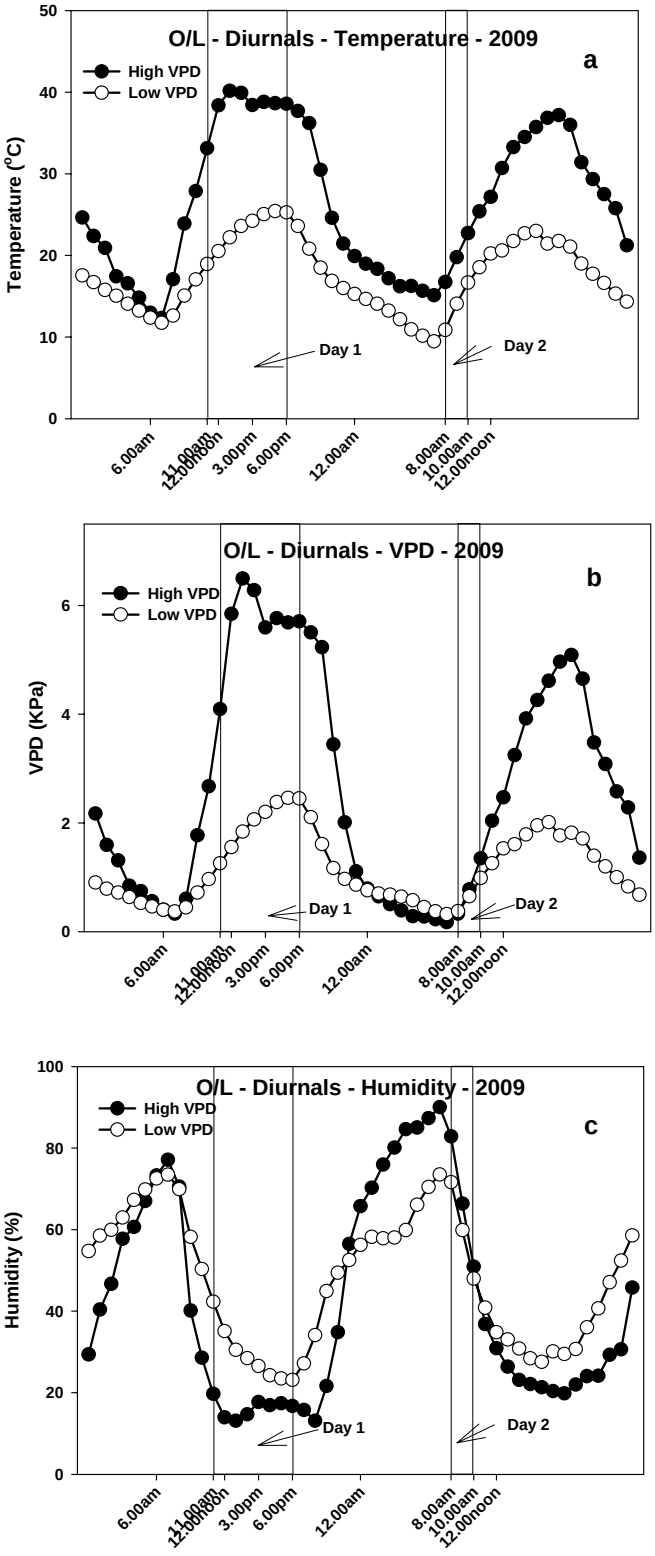


Figure 1. Details of weather conditions over the two days of each sampling period.

In each case, 11.00am, 12.00noon, 3.00pm and 6.00pm sampling was carried out on day 1 and the 8.00am and 10.00am sampling on day 2 as indicated.

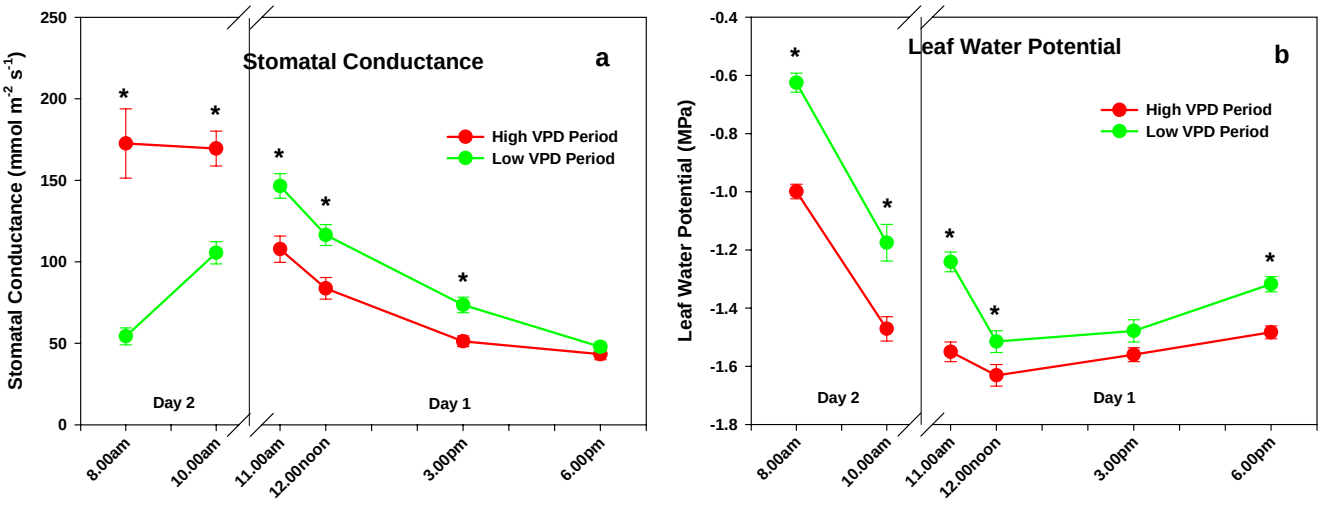


Figure 2 Stomatal conductance and leaf water potentials of vines under high and low VPD conditions.

VPD conditions were relatively similar across both sampling days in each sampling period as seen in Figure 1. For continuity, measurements conducted on the morning of the second day, in each sampling period, have been graphed ahead of the later day measurements taken on day 1. Significant differences are indicated on the graphs by *. Bars indicate S.E^{s}. (n = 2 x 15 fo g_s and 2 x 5 for Ψ_l).

The stomatal conductance (g_s) of the vines changed significantly with time under both VPD conditions. Over the L-VPD period g_s levels were typical, rising from a morning low of ca.55 mmol m^{-2} s^{-1} to a peak of ca.150 mmol m^{-2} s^{-1} at midday before falling back to a low at 6.00pm (Fig 2). In contrast, during the H-VPD period the g_s of the vines was already at a high level of ca. 170 mmol m^{-2} s^{-1} at 8.00am, remained high until 10.00am and then dropped to significantly lower levels than those observed over the L-VPD period until 6.00pm at which time the two VPDs did not differ significantly.

Leaf water potential (Ψ) also changed significantly with time starting less negative in the morning, becoming more negative towards midday, and recovering slightly towards evening (Fig 2b). At all times except 3.00pm, Ψ under H-VPD was significantly more negative than under L-VPD.

In the xylem sap, ABA levels under L-VPD (Fig 3b), steadily increased during the day from ca. 200 ng mL^{-1} at 8.00am to ca. 500 ng ml^{-1} at 6.00pm. Under H-VPD the sap ABA level at 8.00am was ca 150 ng mL^{-1} higher than on the L-VPD day but dropped sharply between 10.00am and 12.00noon becoming lower by between 100 and 150 ng mL^{-1} than on L-VPD and remaining so for the remainder of the day. Levels of the ABA catabolic product PA in the sap (Fig 3c) under L-VPD reflected the ABA levels on that day, slowly increasing during the day from 80 ng mL^{-1} to 420 ng ml^{-1}. Under H-VPD, PA abundance rose to a peak at 12.00pm before declining and reaching a plateau shortly afterwards. PA levels changed significantly across the day. Under H-VPD they were significantly higher than the levels under L-VPD throughout the day except at 3.00pm and at 6.00pm when the levels became similar. ABA-GE levels in the sap did not vary significantly across the day but did differ significantly between treatments except at 11.00am and 12.00noon .

Similar levels of ABA were present in leaves under both H-VPD and L-VPD, and did not change significantly throughout the day (Fig 4a). These similarities were reflected in the ABA catabolic products PA (Fig 4b), with similar little change in the levels up till 3.00pm when there was a significant increase in level under L-VPD conditions. Between treatments leaf PA levels differed significantly between 8.00am and 12,00noon

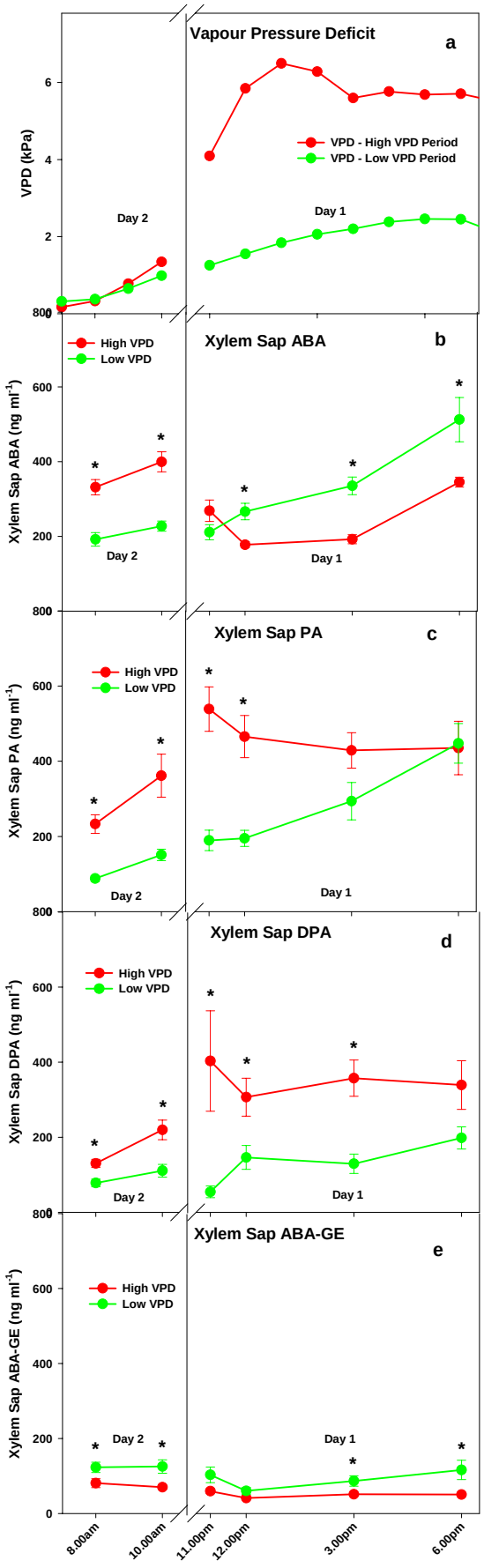


Figure 3. Details of ABA and ABA metabolites in the xylem sap of vines under different VPD conditions.

Xylem sap samples were analysed by LCMS/MS as detailed in Materials and Methods. For continuity, data from the early moning samples, collected on Day 2 are graphed ahead of the later day samples collected on Day 1. Significant differences are indicated on the graph by *. Bars indicate S.D^{s}. (n = 2 x 3).

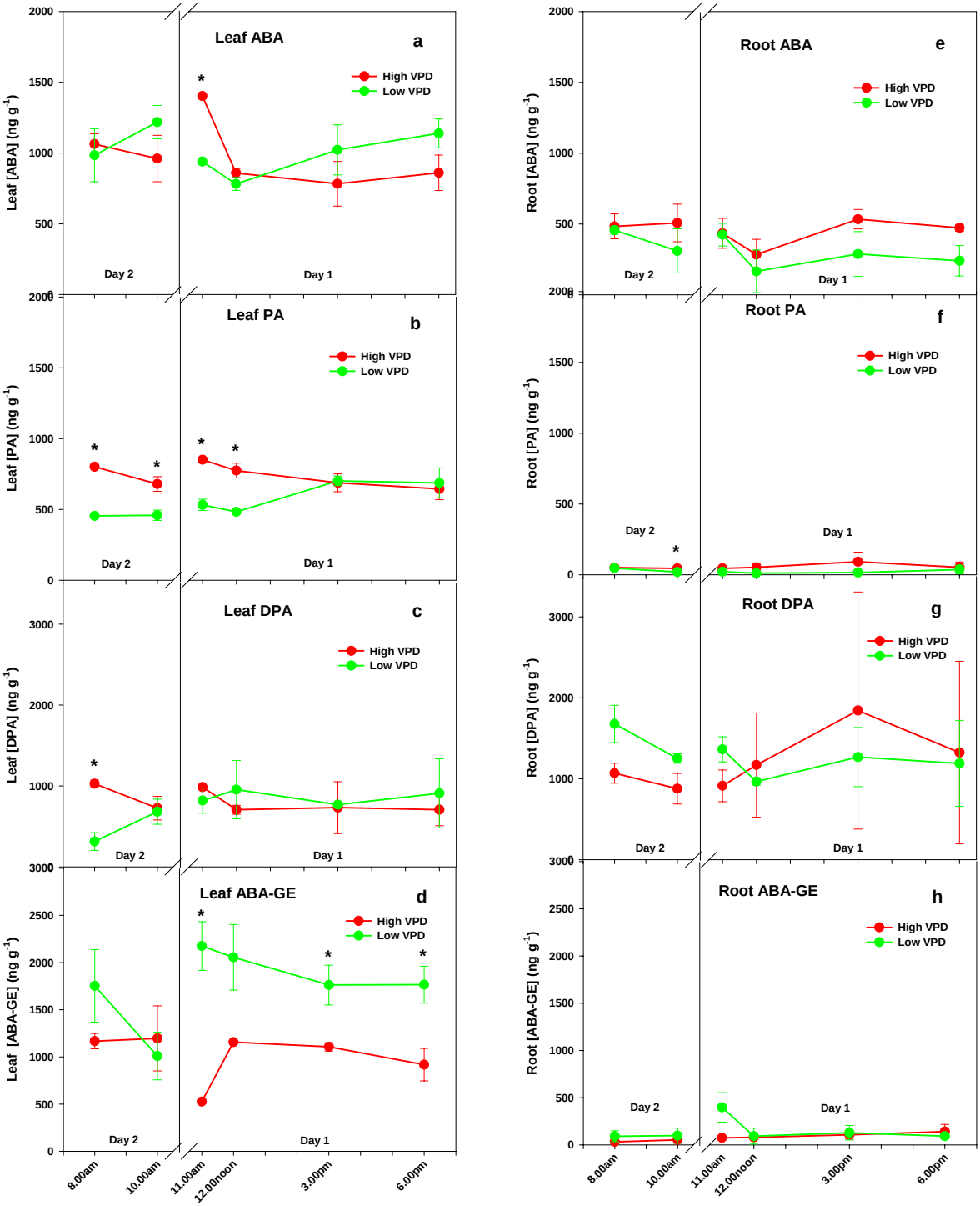


Figure 4. Details of ABA and ABA metabolites in the leaves and roots of vines under different VPD conditions.

ABA and ABA metabolite concentrations per fresh weight of t leaf and root tissues were analysed by LCMS/MS as detailed in Materials and Methods. For continuity, data from the early moning samples, collected on Day 2 are graphed ahead of the later day samples collected on Day 1. Significant differences are indicated on the graph by *.

Bars indicate S.D^{s}. (n = 2 x (5leaves pooled) for leaves and 2 x 1 for roots).

but not after 3.00pm. There were no significant differences in the levels of the glucose ester of ABA (ABA-GE) in the leaves with time (Fig 4d) however the levels were significantly lower under H-VPD than under L-VPD for most of the afternoon period.

Levels of ABA in the roots were consistently lower than in the leaves and, as in the leaves, did not differ significantly under H-VPD or L-VPD conditions or across the day (Fig 4e). PA was barely detectable in the roots under both H-VPD and L-VPD and did not vary significantly with treatment or time. Levels of ABA-GE in the roots (Fig 4h) were significantly lower than in the leaves and were relatively similar and constant across the day.

The relative levels of expression of two genes associated with the regulation of ABA synthesis, *NCED#1* and *NCED#2*, were determined in leaf and root tissues by Real-Time PCR (q-PCR) (Fig 5a,b,d,e). Similar levels of expression of the *NCED#1* gene were evident in leaf tissues across the day except at the 8.00am time point when expression under H-VPD was 15-fold higher than under L-VPD. Expression of the *NCED#2* gene in leaf (Fig 5b) followed similar profiles to those of the *NCED#1* gene but at a 2- to 3- fold lower level. Under L-VPD its expression was relatively constant throughout the day rising slightly to a maximum at midday before falling again towards evening. Under H-VPD its expression was maximal early in the day being 10-15 times greater than its expression under L-VPD at 8.00am, then falling to a low plateau by midday and remaining level for the rest of the day. In root tissue (Fig 5d and e), under L-VPD *NCED#1* expression rose towards mid-afternoon before dropping towards evening. Under H-VPD its expression in root was always greater than under L-VPD with a four-fold higher level from mid-afternoon to 6.00pm. Expression of the root *NCED#1* gene under both VPD conditions was highly correlated with decreased soil moisture ($r^2 = 0.7118$, $P = 0.0006$). *NCED#2* expression in the root (Fig 5e) was at least an order of magnitude lower than *NCED#1* expression throughout Its expression under L-VPD was similar to its expression under the same conditions in leaf. Its expression under H-VPD was generally 2- to 3-fold higher than under L-VPD and was not correlated with soil moisture.

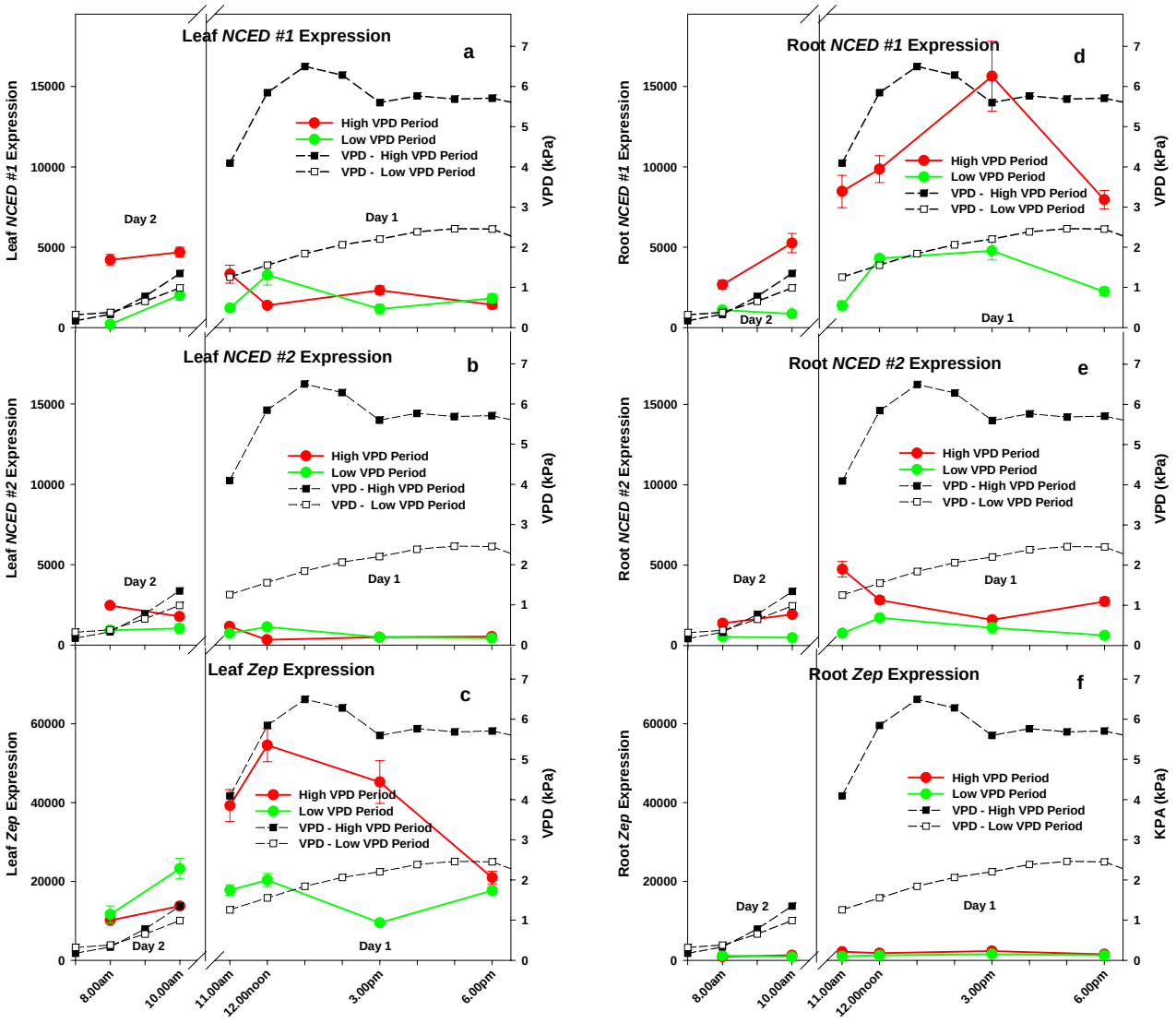


Figure 5. Relative expression of genes encoding three enzymes associated with the regulation of ABA biosynthesis, *NCED #1*, *NCED #2* and *Zep*.
Relative expression levels of the three genes in root and leaf tissues, as indicated in colour, were determined by qPCR as detailed in Materials and Methods. Also indicated on the graphs are the VPD levels over the two sets of sampling periods. Early morning and later day samples were taken on separate days as indicated previously. Bars indicate S.D[s]. (n = 2 x (5leaves pooled) for leaves and 2 x 1 for roots).

Zeaxanthin epoxidase is another enzyme associated with ABA synthesis but does not appear to be rate limiting being more related to photoprotection via the xanthophyll cycle (Demmig-Adams and Adams, 1992). Consistent with this and our previous observations, *Zep* expression was maximal in leaf tissue and at least two orders of magnitude lower in root tissue (Fig 6c and f). In leaf, under L-VPD, its expression was relatively constant with only a 2-fold variation across the day. Under H-VPD, its expression rose sharply from morning reaching a maximum at midday and remaining high in mid-afternoon before falling towards evening. Its maximum expression under H-VPD was some 4-fold greater than under L-VPD conditions.

In order to examine the dynamics of ABA accumulation and distribution in more detail, we wished to look also at the expression of the genes encoding the ABA 8'-hydroxylase enzymes which are the primary regulators of the catabolism of ABA via its conversion into PA and hence to DPA. As a preliminary to this we first isolated and characterized three *Vitis vinifera* cDNAs, from Shiraz grape, with significant sequence homologies to *ABA 8'-hydroxylase* genes from other plants. *VvABA8'-OH-1* (*Hyd#1*) encodes a 471 amino acid protein with between 60% and 71% identity to the *Arabidopsis ABA8'hydroxylase* genes. *VvABA8'-OH-2* (*Hyd#2*) encodes a 494 amino acid protein with between 61% and 82% identity to the *Arabidopsis ABA8'hydroxylase* genes and *VvABA8'-OH-3*(*Hyd#3*) encodes a 467 amino acid protein with between 59% and 64% identity to the *Arabidopsis ABA8'hydroxylase* genes. On the basis of the classification system of Schuler and Werck-Reichhart (2003), all three of the grape genes would belong to the CYP707A subfamily. To confirm the identities of the cDNAs they were expressed in yeast culture according to the protocol of Pompon et al., 1996 where all three were shown to convert ABA into phaseic acid.

Expression of the *Hyd#1* gene in leaf (Fig 6a) under L-VPD was low at 8.00am rising sharply to a peak some 9-fold higher by 10.00am then dropping slowly throughout the day to a low basal level by 6.00pm. Expression under H-VPD (Fig 6b) was similar except at 8.00am when it was some 5- to 6-fold higher than under L-VPD day. Expression of

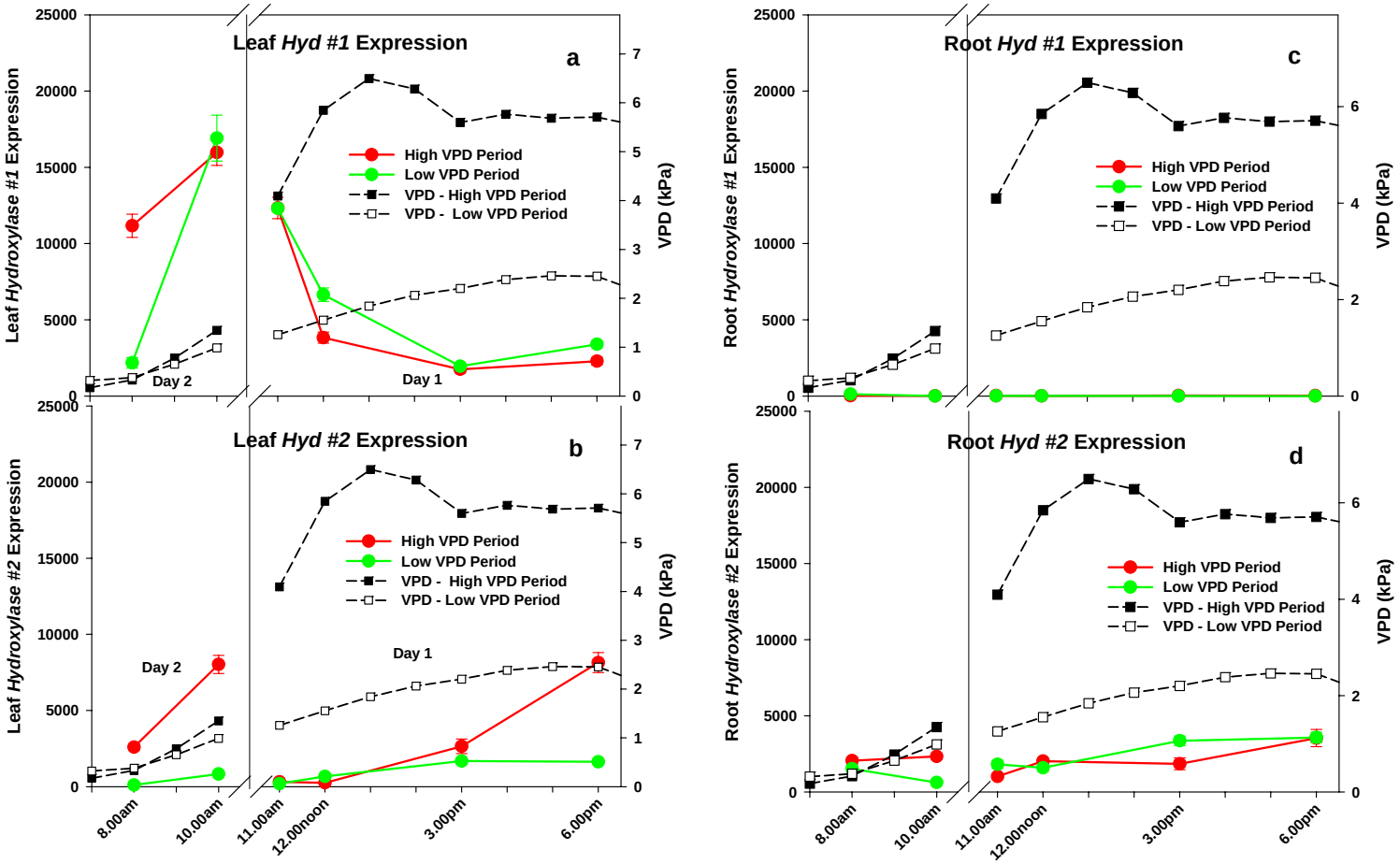


Figure 6. Relative expression of *ABA 8'-hydroxylase #1 (Hyd#1)* and *ABA 8'-hydroxylase #2 (Hyd#2)*, two of the three *Vitis* genes genes encoding enzymes associated with ABA metabolism.

Relative expression levels of the two genes in root and leaf tissues, as indicated in colour, were determined by qPCR as detailed in Materials and Methods. Also indicated on the graphs are the VPD levels over the two sets of sampling periods. Early morning and later day samples were taken on separate days as indicated previously. Bars indicate S.D^{s}. (n = 2 x (5leaves pooled) for leaves and 2 x 1 for roots).

the *Hyd#1* gene in root tissue (Fig 6c) was barely detectable and at least 1-2 orders of magnitude lower than in the leaf tissue. There was no appreciable variation either between H-VPD and L-VPD or with time. *Hyd#2* expression in leaf (Fig 6b) under L-VPD was lower than that of the *Hyd#1* gene and increased only slowly across the day. Under H-VPD its expression was 20-fold higher than under L-VPD at 8.00am, rising sharply to a peak some 10-fold higher than under L-VPD at 10.00am before dropping to a basal level at 12.00noon then rising again towards a second maximum some 4-fold higher then its expression under L-VPD, at 6.00pm. In roots, *Hyd #2* expression was an order of magnitude higher than that of the *Hyd#1* gene in roots and remained relatively constant across the day with a possible small increase towards evening with no major difference in levels of expression between H-VPD and L-VPD (Fig 6d).

Discussion

The pattern of diurnal rise and fall of g_s which we observed in the vines under L-VPD (Fig 2a) was typical of the profiles that have been observed previously in grapevine (e.g. Loveys, 1984, Schultz, 2003, Soar et al., 2006) and in a number of other plants (e.g. apricot, Loveys et al., 1987; *Helianthus annuus* L. and maize, Tardieu and Simonneau, 1998; almond, Wartinger et al., 1990; and *Vicia faba* L.(stomatal aperture), Talbott and Zeiger, 1998). As outlined in the introduction, several hypotheses have been proposed to explain this rise and fall. The most satisfactory explanation so far proposed being the "feed forward" (Farquhar, 1978; Buckley, 2005) or "apparent feed forward" (Franks et al., 1997) hypothesis which postulates an initial hydraulic response to increasing VPD, followed by a second, reducing, response which, on the basis of the work of Bunce (1996) appears to involve ABA.

The profile of g_s under H-VPD was similar to, but lower than, that under L-VPD from midday onward . The lower levels to which the g_s dropped under H-VPD was arguably in response to the significantly higher VPD (Fig1b) over the same period but may also be a function of the lower Ψ_{ls} (Fig 2b). It should be noted however that in the early part of the

day under H-VPD, g_s remained high while the Ψ_l dropped. Prior to midday, the g_s under H-VPD differed significantly from that under L-VPD, with the stomates apparently fully open in the early morning and remaining open until 10.00am after which they began to follow the same diurnal closing down as under L-VPD. A similar response was observed by Loveys and Düring (1984) when under conditions of high VPD stomata of Riesling leaves opened only for a short period between about 8am and 10am and for the rest of the day conductance was low. As VPD conditions across both the H-VPD and the L-VPD periods (Fig 1) were similar between 8.00am and 10.00am, it does not appear that the observed differences in g_s were an immediate function of VPD which raises questions about the veracity of the "feed forward" hypothesis and implies that the low g_s in the early morning of the L-VPD period must be due, at least in part, to a mechanical restriction to g_s rather than simply to low evaporative demand. However, the day prior to these measurements being taken were similar and so the early morning responses may have been the result of a "memory" of the day before. In any case, this period of high conductance in the early morning followed by stomatal closure can be seen as an adaptive response, maximizing leaf gas exchange when conditions are not too extreme and minimizing transpirational water loss during the hotter part of the day. As a consequence of higher temperatures but not higher light intensities on the high VPD day compared with the low VPD day, there would have been a greater sequestration (commitment?) of the xanthophyll cycle carotenoids to provide photo- and thermal- protection (Bilger and Björkman, 1991; Demmig-Adams and Adams, 1992; Chaves et al., 2003; Maiseyenkava, Pshybytko and Kabashnikova, 2005). This commitment would effectively block the synthesis of ABA in both guard and mesophyll cells in the leaf and, by depleting the supply of ABA precursors to the roots via the phloem (Zhang and Davies 1989; Neales and Mcleod 1991; Parry, Griffiths and Horgan 1992; Liang et al., 1997; Thompson et al., 2007), in the roots. A reduced level of residual ABA in the guard cell symplast or apoplast could account, at least in part, for the high level of g_s observed early on the high VPD day

We did not measure Ψ_ls prior to 8.00am on the experimental days, however, during the experimental periods, Ψ_ls throughout the day were lower under H-VPD than under

L-VPD (Fig 2b) and it is reasonable to assume that they were consistently lower during the earlier morning. Therefore it is unlikely that whole leaf turgor or guard cell turgor was responsible for the high g_s early under H-VPD.

Over both the H-VPD and L-VPD periods, from 10.00am onward levels of ABA and PA in the xylem sap were correlated exponentially with changes in g_s, $r^2 = 0.9326$ for xylem ABA and $r^2 = 0.8011$ for xylem PA. No such correlation between xylem ABA and PA (which also has the capacity to stimulate stomatal closure (Sharkey and Raschke, 1980)) and g_s was evident at either 8.00am time point indicating that some other mechanism was operating to regulate stomatal aperture. The possible nature of this alternative mechanism will be discussed below.

The levels of bulk ABA and its metabolites in leaf under H-VPD and L-VPD conditions do not correlate with the observed changes or differences in the g_s profiles either with or without inclusion of the 8.00am time points. Howver, as discussed elsewhere in this report there is good evidence for several pools of ABA in leaf tissues which mask changes in the pool in contact with stomata.

Higher expression of both the *NCED* genes in both leaf and root tissues at 8.00am and 10.00am under H-VPD compared with L-VPD, is not reflected by higher levels of ABA in either tissue but is consistent with the higher ABA levels in the xylem sap and with higher levels of the ABA metabolites PA and DPA in the sap and in the leaf but not the root. A large increase in PA and DPA levels in the xylem sap under H-VPD probably resulted firstly from the significant drop in ABA levels in the sap but may also reflect a large increase in synthesis of ABA in the roots which is suggested by the increased expression of both *NCED* genes in the root tissues.

While increased expression of particularly the *NCED#1* gene in the roots later in the day (Fig 5d) would suggest an elevated synthesis of ABA there is no evidence in the roots for its accumulation (Fig 4e). There are two possible explanations for this apparent absence. Firstly, ABA synthesised in the roots could be rapidly metabolised either in the

roots themselves or, following transport via the xylem sap, in the leaves. Neither of these proposals is strongly supported by the evidence. While there are significant levels of metabolites in the roots, predominantly DPA, and both PA and DPA in the leaves, the relative levels present under H-VPD and L-VPD do not correlate with the relative levels of expression of either of the *NCED* genes. Conjugation of the ABA into the glucose ester is also not an explanation as the significantly high levels in the leaves are not coordinated with the relative levels of expression of the *NCED* genes, and low levels of the conjugate in the roots excludes that possibility. However, the levels of ABA and catabolites in the xylem sap could support a transport and catabolism model if we assume that the xylem contained some leaf apoplastic sap in addition to sap derived from the roots and there is evidence for this elsewhere in this report.. This would be particularly true in mid afternoon when transpiration under H-VPD was minimal and sap flow would be low. The alternative explanation would be the sequestration of the xanthophyll carotenoids to aid in photo- and/or thermal-protection in the leaf preventing the transport of the ABA precursors to the root, thus inhibiting ABA synthesis as discussed above.The high expression of the *Zep* gene in the leaf on the high VPD day may reflect a need by the plant to maximize epoxidation of zeaxanthin in the leaf to aid with thermal-protection and to encourage transport of ABA precursors from a limited available pool in the leaf, to the roots.

The different levels of ABA metabolites in the xylem sap are the only evidence for differential breakdown of ABA by the ABA 8'-hydroxylases under different VPD conditions. Nevertheless, they would indicate either that the different VPD conditions were affecting the sizes of available ABA pools in one or more of the tissues, or that the activity of one or more of the 8'-hydroxylases was being affected by the VPD. Different sizes of pools of ABA, in the leaf tissues in particular, could be masked by bulk ABA quantitation being biased by the presence of sequestered ABA unavailable as an effector of stomatal closure and protected against 8'-hydroxylase degradation. There is clear evidence for the differentially elevated expression of the *Hyd#1* gene in leaf tissues at 8.00am (Fig 6). This was followed by high but similar levels of expression at 10.00am on both the H-VPD and L-VPD periods. Differentially higher expression of the *Hyd#2* gene

in the leaf was also evident at 8.00am and 10.00am on the H-VPD period, rising again to high levels in the late afternoon. Increased expressions of both *Hyd* genes on the H-VPD period preceded a drop in ABA levels in the xylem sap indicating the metabolism of an ABA pool or of a source of newly synthesised ABA, as suggested by the increased levels of expression of the *NCED* genes particularly in root under H-VPD (Fig 5). We did not determine a specific source of the metabolized ABA but, as the hydroxylase activity was evident in the leaf, it is reasonable to assume a similar location for the ABA.

A number of groups have shown a relationship between humidity (RH) and stomatal aperture either in response to water deficit, (Torre and Fjeld, 2001; Torre et al., 2003) desiccation or treatment with ABA (Fordham et al., 2001a,b) and *in vitro* propagated plants (Wardle and Short, 1983; Ziv et al., 1987; Santamaria et al., 1993). An ABA involvement in a stomatal reponse to RH has been demonstrated by Xie et al., (2006) who identified two *Arabidopsis* genes associated with ABA-mediated stomatal closure and which respond to reduced RH, also modified stomatal responses to ABA have been documented in *Tradescantia virginiana* under different RH regimes (Nejad and Meeteren, 2007, 2008), and recently Okamoto et al.,(2009) have identified and characterised the induction at high RH of two further *Arabidopsis* genes, in this case encoding the ABA catabolism 8'-hydroxylase enzymes whose induction resulted in reduced ABA levels and increased stomatal apertures. However there is also evidence that a stomatal response to RH may not always require ABA. An ABA-independent stomatal response to high RH has been demonstrated in both ABA-deficient and ABA-insensitive mutants of *Arabidopsis* (Assmann, Snyder and Lee, 2000).

As discussed above, our results would indicate differentially greater metabolism of ABA, most probably in the leaf , under H-VPD versus L-VPD (Fig 3 and 4). Differential metabolism of leaf ABA is consistent with the observed differentially higher expression of both the *Hyd#1* and *Hyd#2* genes in the leaf tissues at the early morning time point (Fig 6). By 10.00am expression of the *Hyd#1* gene in the leaf on both days had peaked to similar levels subsequently remaining similar in decline for the remainder of each day. Expression of the *Hyd#2* gene also increased to a peak at 10.00am under H-VPD

however this peak was not matched on the low VPD day, and a second increase in its expression from 12.00noon onwards under H-VPD day was similarly not matched on the low VPD day. Expression of the *Hyd#1* gene in the root tissues was low and similar throughout both days and did not appear to relate to the differential catabolism of ABA observed in the leaf and xylem sap. Expression of the *Hyd#2* gene in the roots was higher but, again, similar across both days and did not appear to relate to differential metabolic functions.

The high levels of g_s at both 8.00am and 10.00am during the H-VPD period is an indication that the stomates were open and only started to mirror the behaviour of the stomates under L-VPD after 10.00am. Coincident with the early stomatal opening are two observations, an indication of more breakdown products of ABA in the leaf and xylem sap of the vines under H-VPD and an increased expression of both of the *8'-hydroxylase* genes in the leaf tissue under H-VPD and there is a clear drop in ABA levels in the xylem sap under H-VPD. If we assume compartmentalised ABA to be the main component of the bulk leaf ABA (Heilmann, Hartung and Gimler, 1980; Kaiser and Hartung, 1981; Cowan, 1982; Slovik et al., 1992; Slovik and Hartung 1992; Wilkinson and Davies 1997; Sauter et al., 2001) the observed breakdown may be apoplastic or guard cell symplastic or both. If this is the case, rapid degradation of ABA in or close to the guard cells would account for the high g_s in the early morning under H-VPD. The large difference between the stomatal conductances under similar levels of humidity on the two days raises questions about the validity of the "feed forward' hypothesis. Our data would better fit the proposal of Tallman (2004) that there are three phases of stomatal regulation across the day: i) an early daylight depletion of endogenous guard cell ABA; ii) a midday delivery of ABA by transpiration to the apoplast surrounding the guard cells at steady state concentration; and iii) an accumulation of endogenous ABA by the guard cells in the dark period following each daylight period. If residual ABA in or associated with the guard cells is responsible for the low g_s early under L-VPD then a more rapid depletion of this residual ABA under H-VPD, either during the more humid night or as a result of

higher temperatures at sunrise, the resulting reduction in stomatal closure would account for the higher g_s early on the high VPD day.

Conclusions.

We observed significantly different stomatal behaviour in vines as a result of exposure to days of differing VPD. Under L-VPD the profile of g_s across the day was similar to profiles that have been described previously for a number of plants possibly explained by the "feed forward" (Farquhar, 1978; Buckley, 2005) or "apparent feed forward" (Franks et al., 1997) hypothesis although our observations of g_s early on each of the two days would better support the involvement of ABA in all phases of stomatal regulation across the day as proposed by Tallman (2004). Under H-VPD the g_s profile was similar to but lower than under L-VPD from midday onwards but was significantly different prior to midday, with the stomates clearly more open in the early morning. Similarities between the occurrence of ABA breakdown products PA and DPA in leaf and xylem sap during this period and high g_s under H-VPD suggest the involvement of ABA metabolism in or near the guard cells and this is supported by concurrent increased levels of expression of two of the three *Vitis 8'-hydroxylase* genes in the leaf tissues. Enhanced expression of NCED #1 in roots, but not in the leaves, of H-VPD vines in the afternoon suggests that local soil water deficit was occurring, despite the fact that the vines were under a normal irrigation regime. The emerging picture from these data is of a system finely tuned to optimize the efficiency of water use under a range of environmental conditions. The enhancement of leaf gas exchange in the early morning appears linked to enhanced expression of the 8'Hydroxylase gene(s) while stomatal closure later in the day is probably the result of greater expression of ABA synthesis genes in roots brought about by local reductions in soil moisture. We must also remember that these vines were grafted to Ramsey rootstock and elsewhere in this report we show that some of these responses are enhanced in vines on Ramsey compared with own-rooted vines.

Chapter 4. The influence of rootstocks on vine responses to droughting.

Introduction.

For many years Australian grapegrowers have had access to vines grafted to a range of different rootstocks which are hybrids of *Vitis* species native to North America. Although rootstocks can offer many advantages over own-rooted vines such as resistance to *Phylloxera* (May, 1994) and nematodes (McCarthy and Cirami, 1990), drought tolerance(Carbonneau, 1985; Cavanagh, 1991; Cirami et al., 1994; McCarthy and Neldner, 1997; McCarthy et al., 1997), vigour control and resistance to salt (Southey and Jooste 1992; Walker et al., 1997), more than 70% of the country's vines are still own-rooted (Dry 2007). The cost of grafted vines is obviously a major determinant and uncertainty about the relative advantages or disadvantages of specific rootstocks may also deter their more widespread use. This is reflected in the changing patterns of rootstock sales. In the last 2 decades sales of Ramsey have fallen from 53% to 19% of total cuttings sold, Schwarzmann has fallen from 23% to 2% while 1103 Paulsen rose from zero to 41% (figures quoted by Walker and Clingeleffer, 2009). Rootstock choice is probably influenced by factors such as perceptions about how a stock may best serve current industry requirements for properties such as drought, salt and *Phylloxera* tolerance and new information that may become available about other stock properties such as influence on wine quality and response to various management practices.

Although rootstocks apparently have the ability to confer different degrees of drought tolerance to the scion, no single physiological trait has been described to account for this. Soar et al (2006) argued that considerable evidence links the ability of a root system to supply chemical signals such as ABA with the ability of the canopy to respond to high evaporative demand and that rootstocks may well differ in their ability to supply these regulatory chemicals. They went on to show that rootstocks that resulted in higher than average level of ABA in the xylem sap of the Shiraz scion had lower stomatal conductance and that the reverse was true for rootstocks giving lower scion xylem ABA and that this property could be linked to drought tolerance. However, it was not clear from these data whether these differences were just a reflection of different degrees of

water stress experienced by the vines or whether there was an inherent difference in the ability of the rootstocks to supply ABA and it was this that was the primary determinant of scion response to stress. In our current project we have looked more closely at this question in Shiraz vines grafted to different rootstocks by assessing the activities of the genes controlling ABA synthesis in the rootstocks when the vines were subject to a brief water deficit. We selected 2 common rootstocks, Ramsey and Schwarzmann grafted with Shiraz and compared them to own-rooted Shiraz at the Nuriootpa experimental station.

Results:

As can be seen in Figures 1a and b, the roots or rootstocks had significant effects on both stomatal conductance (g_s) and leaf water potential (LWP) of the Shiraz scions. Under normal irrigation the g_s of the Sh_{own} (Shiraz on own roots) was not significantly different to the g_s of the Sh_R (Shiraz on the Ramsey rootstock) or of the Sh_{Sch} (Shiraz on the Schwarzmann rootstock) but the g_s of Sh_R was significantly lower than that of Sh_{Sch} (Fig1a). Six days of droughting induced reductions in g_s in all three sets of vines, the greatest reduction, relative to the well watered condition, being in the Sh_{Sch} vines but the lowest g_s was attained in the Sh_R vines. Under irrigation the LWP of Sh_{own} and Sh_{Sch}, were not significantly different while that of the Sh_R was significantly more negative than the Sh_{own} but not the Sh_{Sch} vines. Droughting resulted in a significant fall in LWP in all three (Fig1b) with the decline being greatest in the Sh_{Sch} vines . LWP of Sh_R was not significantly different from Sh_{own}.

The abundance of ABA and its metabolites were determined in xylem sap, leaf and root tissues. There was no significant difference between the sap ABA content of any of the irrigated vines (Fig 2a) but the short period of water deficit resulted in significant increases in the Sh_R and Sh_{Sch} vines. Vines with Ramsey roostock in particular showed a very large increase, with sap ABA rising from 37 to 560 ng/mL. Leaf ABA levels were similar in control and droughted Sh_{own} and Sh_{Sch} but were significantly increased in Sh_R leaves (Fig 2e). The pattern in root ABA was similar to leaves, with only Sh_R showing a significant increase in response to the water deficit (Fig 2i).

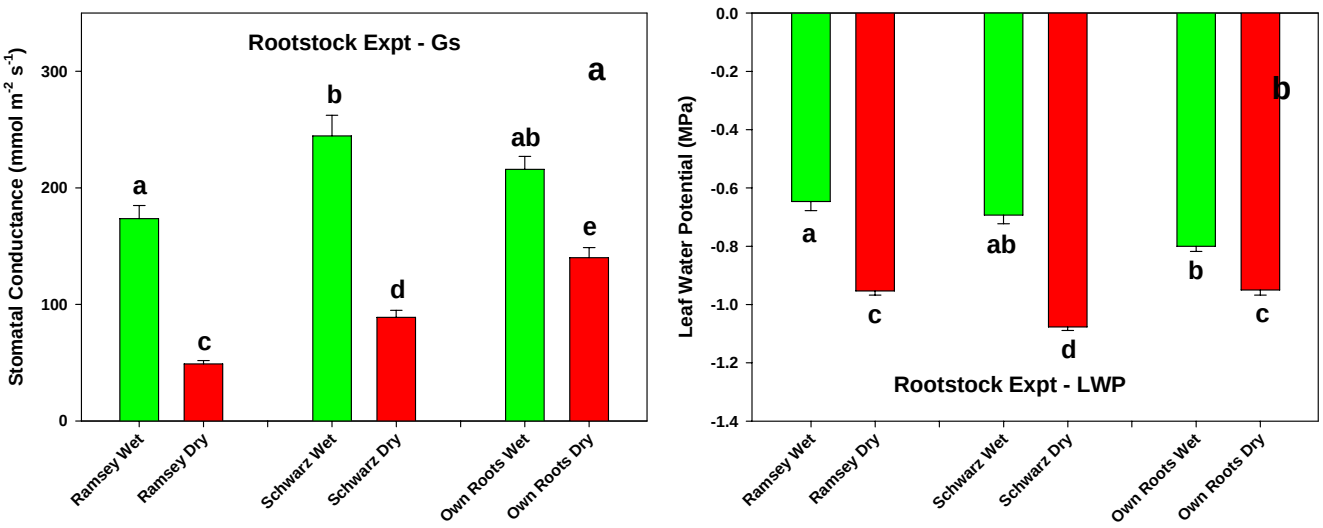


Figure 1. Stomatal conductances (a) and leaf water potentials (b) in leaves of Shiraz scions on own roots or grafted on Ramsey or Schwarzmann rootstocks under normal irrigation or droughted.

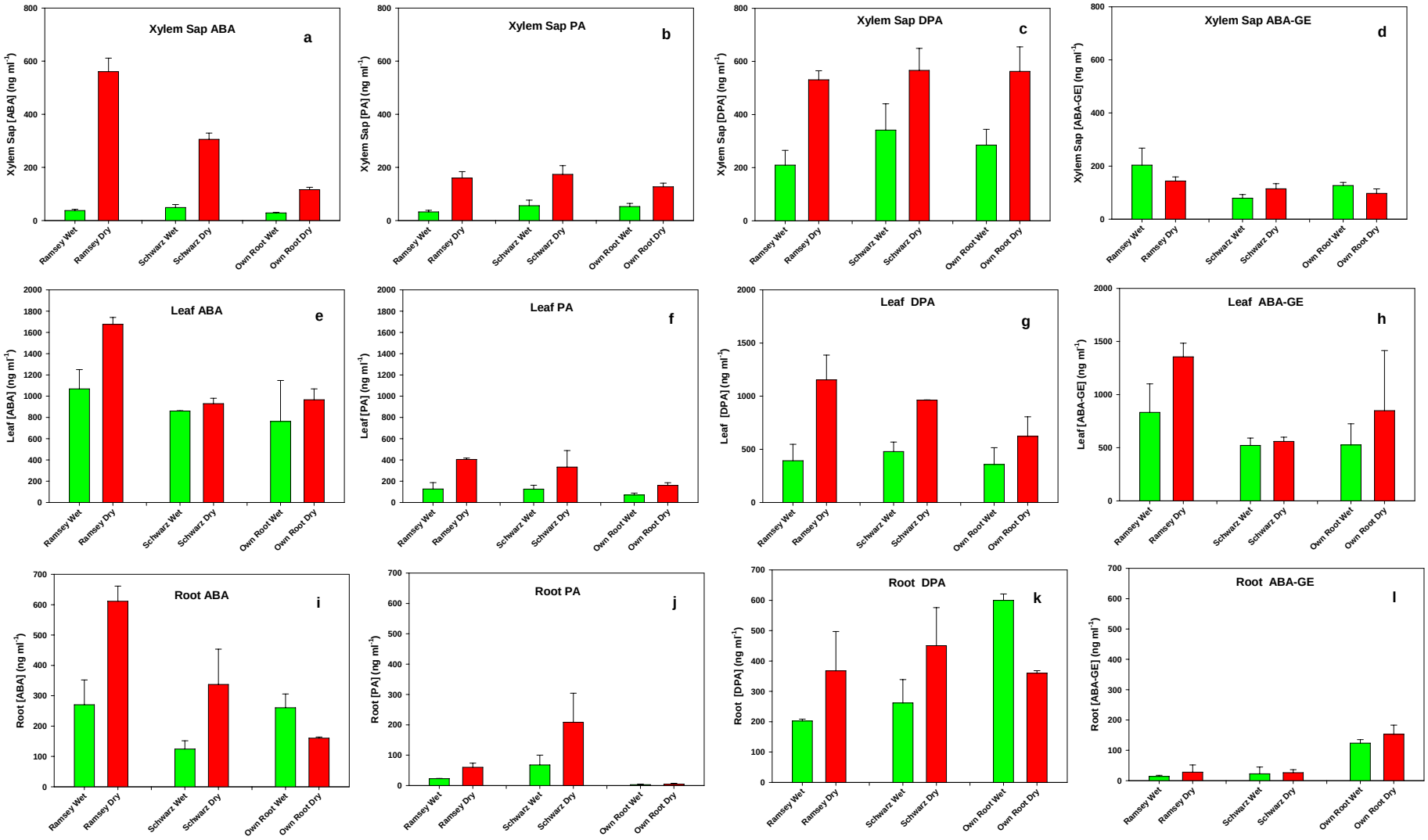


Figure 2. ABA and ABA metabolites in xylem sap (a-d), leaf (e-h) and root (i-l) of Shiraz scions on own roots and on Ramsey or Schwarzmann rootstocks under normal irrigation or droughted

Products of ABA metabolism were evident in all tissues examined. Levels of phaseic acid (PA) in the xylem sap (Fig 2b) and the leaf tissues (Fig 2f) reflected the levels of ABA in the respective tissues but were higher in the roots of the Sh_{Sch} vines than in the roots of the other vines (Fig 2j). Dihydrophaseic acid levels Fig2c,g,k) also reflected the ABA levels in the respective tissues except in the Sh_{own} vines where the levels in the roots of the irrigated vines exceeded those in roots of the droughted vines. Levels of the ABA-glucose ester (ABA-GE) (Fig 2d,h,l) were high in leaves and lower in the xylem sap. In the roots, ABA-GE levels were even lower than in the xylem in the Ramsey and Schwarzmann roots but similar to the xylem levels in the roots of the Sh_{own} vines.

There was a good inverse relationship between g_s and the concentration of ABA in the xylem sap (Fig 3) which is consistent with previous observations (Loveys 1984; Davies and Zhang, 1991; Correia and Pereira ,1994; Tardieu et al., 1996; Jia and Zhang, 1999) and was evident also in the studies detailed in the previous chapters. We looked for relationships between xylem sap [ABA] and leaf and root [ABA] and found linear correlations with both, the stronger being with the root [ABA] ($r^2 = 0.8056$).

We examined the expression activities of the ABA synthesis and metabolism genes in the leaf and root tissues of the vines (Fig 4). Both of the ABA synthesis genes *NCED#1* and *NCED#2* were more highly expressed in the droughted root tissues of Sh_{Ram} and Sh_{Sch} which is consistent with their being induced by the droughting (see previous chapters). Expression of *NCED#1* in Ramsey roots was particularly high in the roots exposed to water deficit and this was consistent with measured values of stomatal conductance and levels of ABA in roots and leaves and sap. Soar et al (2006) also noted higher levels of sap ABA in Shiraz vines on Ramsey rootstock correlated with lower stomatal conductance but were unable to determine whether the elevated ABA was actually driving the change in conductance or whether it was simply a reflection of the greater degree of stress experienced by these vines. In our experiments the LWP of Ramsey vines was not significantly affected by the water deficit but there was a very marked response in terms of gene activity and associated stomatal response. These data argue strongly for the vines responding to water deficit through the production of an ABA

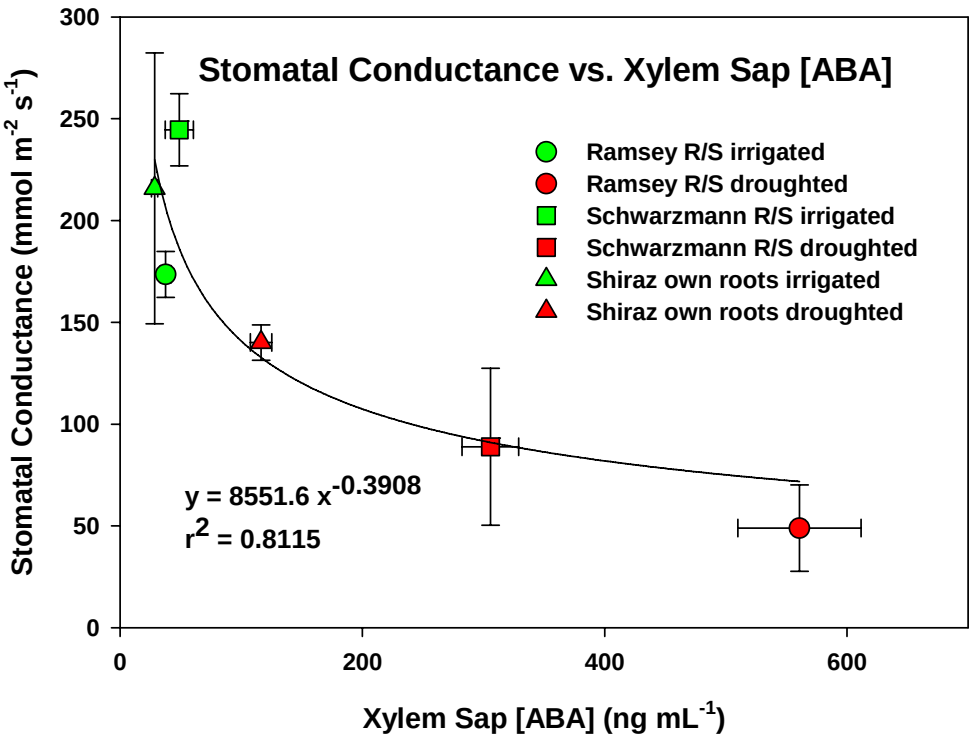


Figure 3. Correlation between xylem sap [ABA] and stomatal conductance in irrigated and water stressed shirac scions on own roots and on Ramsey or Schwarzmann rootstocks.

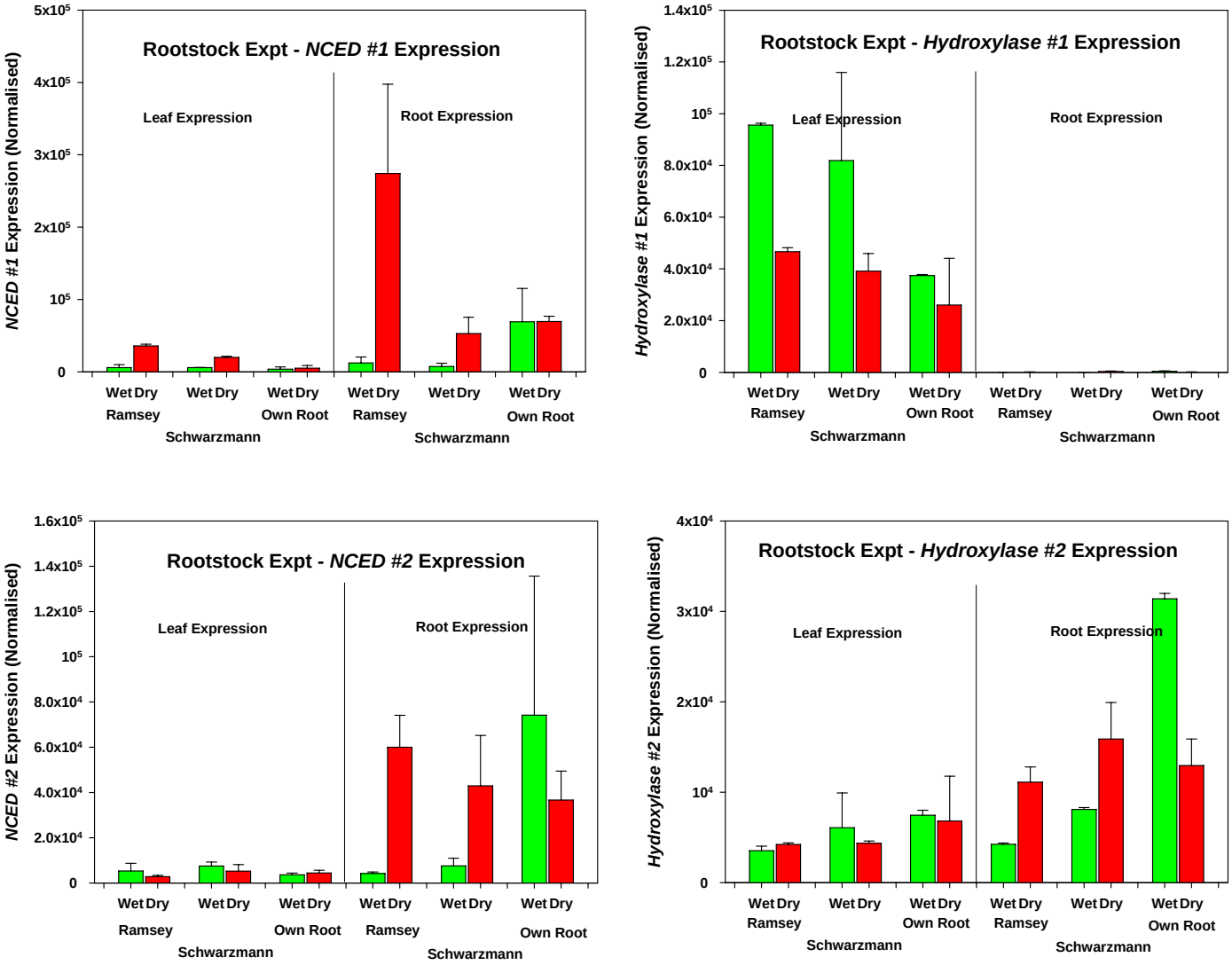


Figure 4. Expression of NCED#1, NCED#2 and Hydroxylase#2 in Shiraz scions (leaf) on own roots and on Ramsey or Schwarzmann rootstocks and of roots under normal irrigation or droughted.

signal originating in the roots, rather than a hydraulic signal originating in the leaves and furthermore, that this signal is more highly expressed in Ramsey than in Schwarzmann or own roots.

Expression of both the *NCED* genes in leaf was too low for meaningful differences to be detected. .Interestingly, the leaves do appear to be contributing to a drought response by reducing the expression of the ABA-hydroxylase gene *Hyd#1*. This would have been expected to contribute to a less rapid degradation of the leaf ABA and, while this is supported by the increased levels of leaf ABA in the droughted Sh_{Ram} it does not appear to be supported by the levels of leaf PA and leaf DPA. It may be that the specific location of the expression is of importance. There is expression of the *Hyd#2* gene in the leaves but this does not appear to be greatly affected by the droughting. Its expression in the roots is higher and does appear to be enhanced by droughting. We have noted in separate experiments in this report the combined induction of these two hydroxylase genes, *Hyd#1* and *Hyd#2* in leaves in the early morning and in response to sudden increases in humidity, but elsewhere only the *Hyd#2* gene responded to elevated humidity levels in the chamber of gas exchange equipment which had been programmed to expose the leaf to a range of humidities. There are four ABA 8'-hydroxylases in *Arabidopsis* all four of which are induced by dehydration and by exogenous ABA treatment (Kushiro et al., 2004; Saito et al., 2004). In a recent study two of these genes, *CYP707A1* and *CYP707A3* have been shown to be involved in a rehydration induced inactivation of local ABA pools within the guard cells and a rehydration induced reduction in the amount of ABA in the vascular tissues of the leaf respectively (Okamoto et al., 2008). Further insight into the roles of the hydroxylase genes of *Vitis* in response particularly to droughting and high humidity will require more detailed examination that was possible within this project.

Data presented in this chapter and in other sections of this report show quite clearly the presence of ABA-glucose ester (ABAGE) in xylem sap, leaves and roots. Formation of ABAGE in plant tissues is generally thought of as an inactivation mechanism and indeed, ABAGE appears to be biologically inert (Boyer and Zeevaart 1982). However, ABAGE

can be cleaved enzymatically to yield biologically active free ABA and its presence in xylem exudate suggests a possible role as a transport form of ABA which could be reactivated by hydrolysis near to its site of action in the apoplastic fluid in contact with the stomatal guard cells. This proposed transport form of ABA would have an advantage over free ABA in that ABAGE cannot pass cell plasma membranes, thus effectively locking ABAGE into the xylem lumen (Baier et al 1988). ABA, on the other hand, will pass membrane barriers in a pH-dependent manner. The protonated form of ABA (ABAH) is soluble in membranes whereas the ABA anion is reflected to a greater degree. Since the pH of vine xylem sap is usually close to or lower than the pKa of ABA (4.8) it can be assumed that some of the ABA entering the root xylem will be lost in its passage to the shoot. Transport of ABA in the form of ABAGE may therefore be more efficient. The concentration of ABAGE in xylem sap of non-stressed vines is similar, on a molar basis, to that of free ABA (Fig 2 a,d). Release of free ABA from ABAGE would require the presence of a hydroxylase in the leaf apoplast. Such an enzyme has been described in barley by Dietz et al (2000). Whether such an enzyme exists in grapevine remains to be seen although crude aqueous extracts of grapevine leaf material have been shown to be capable of hydrolyzing ABAGE to yield the free acid (Loveys and van Dijk 1988). The presence of ABAGE in xylem sap samples therefore suggests the possibility of transport to the leaf apoplast where it could contribute to stomatal response to environment. The control of this mechanism would most likely be at the leaf level as there was no evidence for changes in the amount of ABAGE in xylem under water deficit or differences in the sap from different rootstocks. Amounts of ABAGE in Ramsey and Schwarzmann roots was actually slightly lower than in Shiraz roots (Fig 2l).

Chapter 5. Li-Cor Experiments – Oxford Landing and Waite Campus Alverstoke Orchard.

Introduction

Stomatal conductance is known to be affected by changes in VPD (Loveys, 1991; Mott and Parkhurst, 1991; Monteith, 1995; Collins et al., 2010; and above chapters). Some of the observations in the previous chapters have suggested a link between low VPD or high levels of humidity and the induction of two of the three *Vitis* genes encoding the ABA *8'-hydroxylase* enzymes in grapevine leaves. Similar observations have been reported in *Arabidopsis* (Kushiro et al., 2004; Saito et al., 2004; Okamoto et al., 2009). Hydroxylase mediated metabolism of ABA in the leaves, particularly ABA in the stomatal guard cells, as has been demonstrated in *Arabidopsis* (Okamoto et al., 2009), would encourage the opening of the stomata and a consequent increases in stomatal conductance. This response to humidity is yet another facet of the complex mechanisms that have evolved to optimize leaf gas exchange under a range of environmental conditions. Opening of stomata under conditions of high humidity will have the effect of improving water use efficiency because photosynthesis will be increased without an accompanying increase in transpiration. The opposite response, where stomata close in response to low humidity (high VPD) also serves to optimize water use efficiency and conserves vital soil water reserves. Both responses probably act through related mechanisms, *i.e*. the hormonal control of stomatal movements. The time-frame over which these movements occur is important as to be effective they must occur quite quickly. We have therefore placed some emphasis on short term experiments. The work of Okamoto et al (2009) suggests that in *Arabidopsis* gene activity may change within 10 minutes of a step change in humidity and that this will impact on hormone levels within 60 minutes. In order to further test our observations in grapevine, we subjected leaves of Cabernet Sauvignon under field conditions, to different levels of humidity and examined the effects on the physiology of the leaves and on the expression of the genes associated with ABA synthesis and metabolism.

Materials and Methods Specific to this Section

Two sets of experiments were conducted at different sites, the Alverstoke Vineyard, University of Adelaide Waite Campus, and at the Yalumba Vineyard, Oxford Landing. Details of sites and conditions are listed below:

Alverstoke: Vines: 12 year old Cabernet on own roots
Date: 16-17th November 2009
RH : Day average 45%
Duration of treatment: 30 minutes

Oxford Landing: Vines: 12 year old Cabernet on Ramsey rootstocks
Date: 24-25th February 2010
RH: Day average 27%
Duration of treatment: 120 minutes

The methodology adopted was to use 2 identical gas exchange systems (LiCor 6400) and simultaneously expose 2 leaves to contrasting levels of relative humidity, keeping other parameters such as temperature, photosynthetically active radiation (PAR) and CO_2 concentration constant (Fig 1). Leaves exposed to increased humidity levels were ramped up to the required level over a period of 15 minutes and were then held for a specified time at that level. At completion of the treatment the sections of leaf enclosed by the chambers were excised and snap frozen in liquid nitrogen.
Chamber conditions were set as follows: block temperature 32°C, saturating PAR (2000 μmol m^{-2} s^{-1}), air flow set to 500 mmol s^{-1} and chamber CO_2 concentration set to 400 μmol mol^{-1}. Humidity was adjusted as required to ensure that initial RH for the treatments was the same as control plants. The two leaf chambers were simultaneously attached to fully exposed leaves, in near proximity to each other. Initial humidity, both reference (air intake) and sample (inside chamber) were noted before the treatment chamber was either humidified or maintained at ambient humidity. Humidity was

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Figure 1. Using two LiCor 6400 gas exchange systems to simultaneously expose leaves to varying relative humidity.

increased within the chamber by connecting the air intake with a bottle of water that was being agitated by an aquarium bubbler.

Tissue analysis

The leaf section within the chamber was excised, placed in a 2ml Eppendörf tube and snap frozen in liquid nitrogen, then stored on dry ice. Samples were ground in liquid nitrogen and aliquots of tissue taken for RNA extraction and metabolite analysis by LC-MS/MS. RNA extraction was carried out using the *Sigma Spectrum* kit, which included an on column DNAse step (*Sigma On-column DNAse I Digestion*). cDNA was synthesised from 1µg of RNA using the *Finnzymes Phusion Kit* system (Finnzymes). RT-PCR was conducted on 1:10 dilutions of the cDNA. ABA and metabolite analysis was carried out by LCMS/MS as described previously.

Results and Discussion.

Over the course of six separate experiments at each site, leaves of Cabernet vines were exposed to elevated humidity levels for either 30 minutes or 120 minutes (Fig 2). With 30 minutes exposure no significant change in stomatal conductance (g_s) was noted (Fig 3), although there was a significant drop in vapour pressure deficit (VPD) across the treated leaf area relative to leaves exposed to ambient humidity (Fig 4). With the longer, 120 minute exposure to elevated humidity, there was also a significant drop in VPD across the treated leaf area, but also a significant increase in g_s (Figs 4 and 3), suggesting an increase in stomatal aperture.

Under both treatments, there were small but non-significant drops in the levels of ABA and ABA metabolites in the humidified leaves, and essentially no change in the levels of ABA-GE (Fig 5) compared with control leaves.

Despite being unable to see a significant drop in overall ABA levels in the leaf, we looked to see whether there was a detectable change in the levels of expression of genes

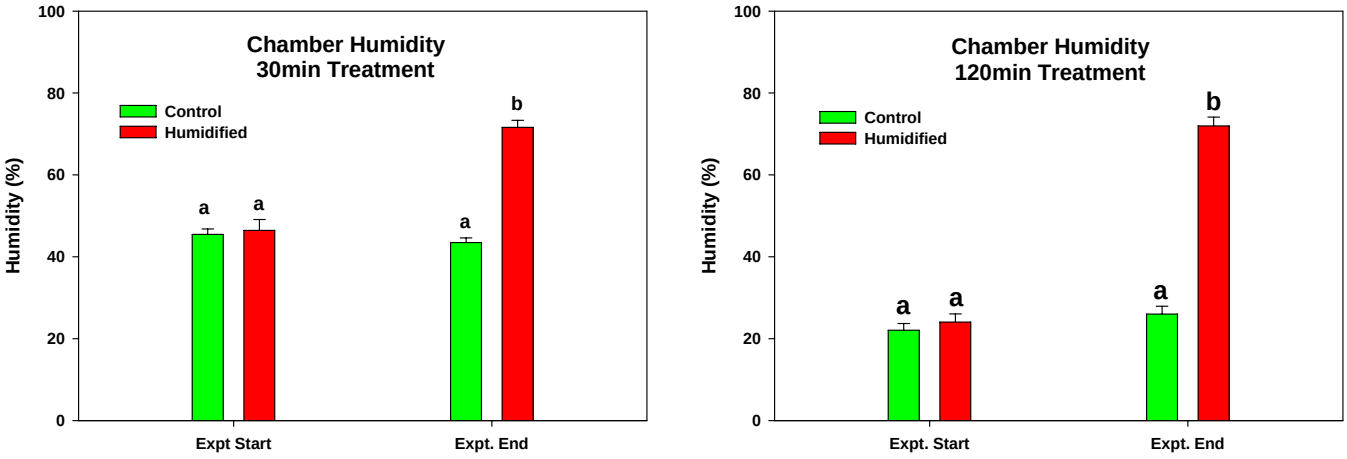

Figure 2. Levels of humidity to which leaves of Cabernet sauvignon were exposed for two different periods of time.
In the course of six separate experiments per treatment, leaves were held in the chambers of two LiCor 6400s for the indicated periods. Humidity levels were adjusted in one of the LiCors by bubbling the circulating gas through water while the other was maintained at ambient. Letters denote significant differences: P <0.0001.

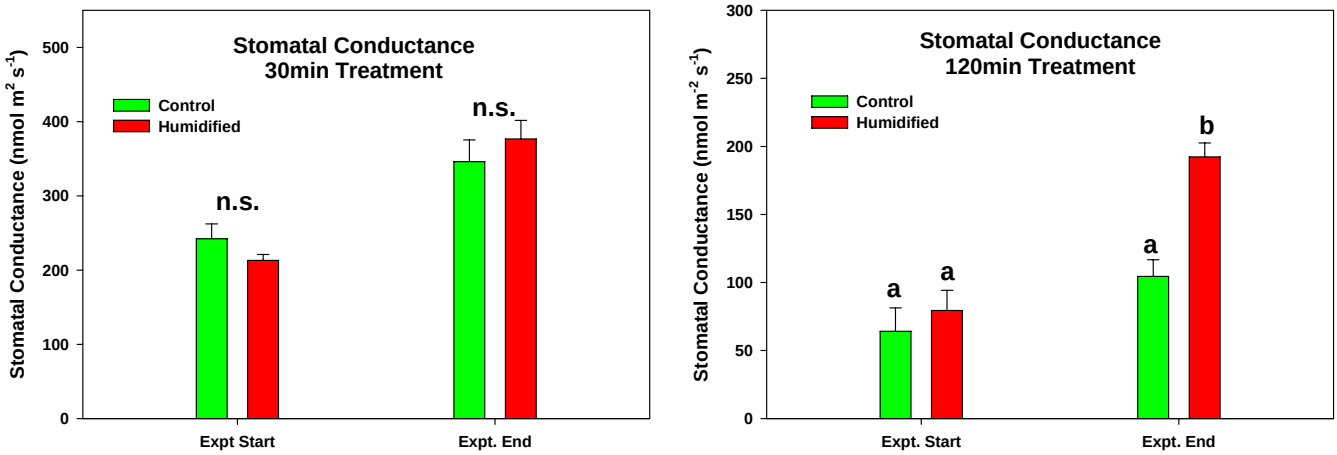

Figure 3. Stomatal conductances were measure during treatments and were stable at the indicated levels during the treatment.
Letters denote significant differences: P <0.0001.

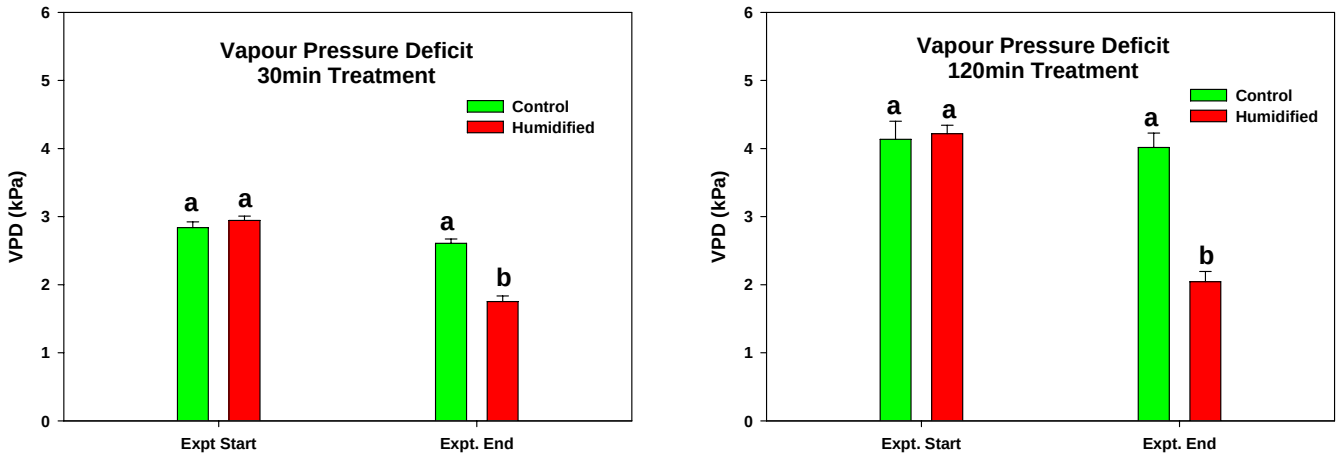

Figure 4. Stable VPD levels across the leaf fragments as measured during treatments.
Letters denote significant differences: P <0.0001

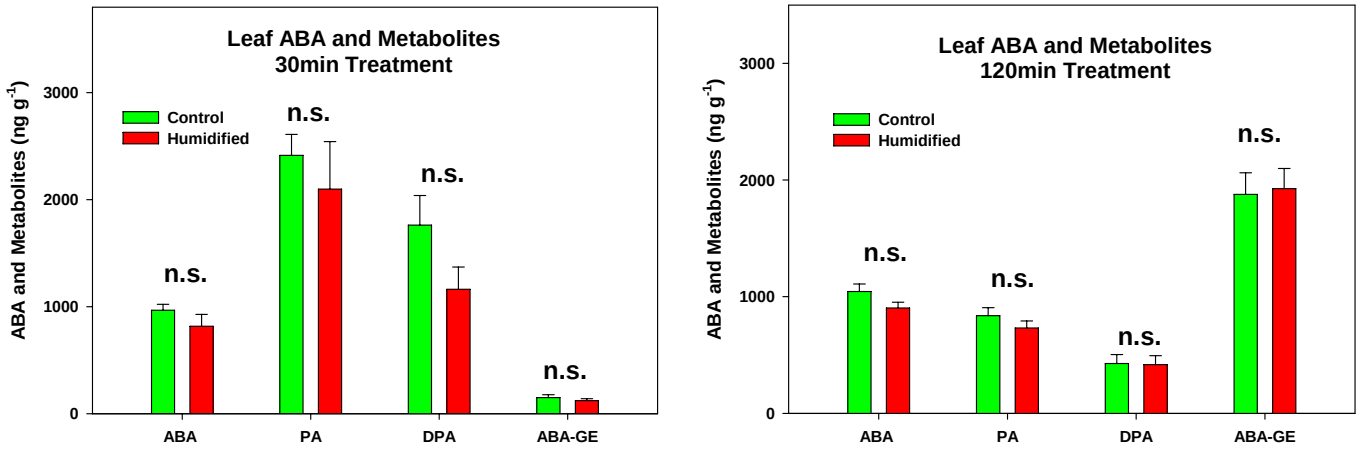

Figure 5. Levels of ABA and ABA metabolites withing the leaf fragments subsequent to treatment.
ABA and metabolites were analysed by LC MS/MS as described in the Appendix.
Letters denote significant differences: P <0.0001.

associated with ABA synthesis and, more particularly, with ABA metabolism in response to the increased humidity. After both 30 minutes and 120 minutes at high humidity there was a small, but consistent decrease in the expression of the *NCED #1* gene (Fig 6) associated with the regulation of ABA synthesis, but no change in the expression of the other *NCED* gene, *NCED #2*, (data not included here). The decrease in the expression of the *NCED #1* gene, although consistent, is too small to be considered significant although it would accord with the small reduction in ABA concentration. There was no change in the expression of the *Hydroxylase #2* gene (*Hyd #2*) after 30 minutes at high humidity, but a small increase in its expression was evident after 120 minutes at high humidity (Fig 6). Again, this increase is too small to be considered significant however it would be consistent with a strictly localised induction of the gene, possibly within the guard cells as observed in the case of the *Arabidopsis CYP770A3* gene (Okamoto et al 2009). Contrary to expectations we found a large, three fold, decline in the expression of the *Hydroxylase #1* gene in leaves exposed to humidity in both experiments.

These data confirm that, over a two hour period, high humidity (low VPD) resulted in significant increase in stomatal conductance. However this was not associated with any large changes in amounts of free ABA or the expression of the genes associated with ABA synthesis or catabolism. The small decrease in ABA and ABA metabolite levels in the leaf would suggest either that exposure of the leaves to high humidity has not resulted in significant induction of the ABA 8'-hydroxylase enzymes, or that the bulk of the leaf ABA is compartmentalised and unable to interact with the guard cells. However, the significant increase in g_s in the leaves strongly suggests a decline in ABA levels in or close to the guard cells, which could be small enough to be masked by the relatively stable levels of bulk ABA in the leaf. Okamoto *et al* (2009) noted that in *Arabidopsis* there was a strong induction of the *CYP07A3* gene within 10 minutes of exposing the leaves to high humidity and that over the course of the next hour ABA levels fell by about 80%. The conclusion was that a product of this gene (8'-hydroxylase) was having a strong effect on amounts of free ABA and that this impacted strongly on stomatal conductance. However, the absolute levels of ABA in *Arabidopsis* reported by Okamoto et al were some 200-fold lower than the amounts we routinely measure in grapevine,

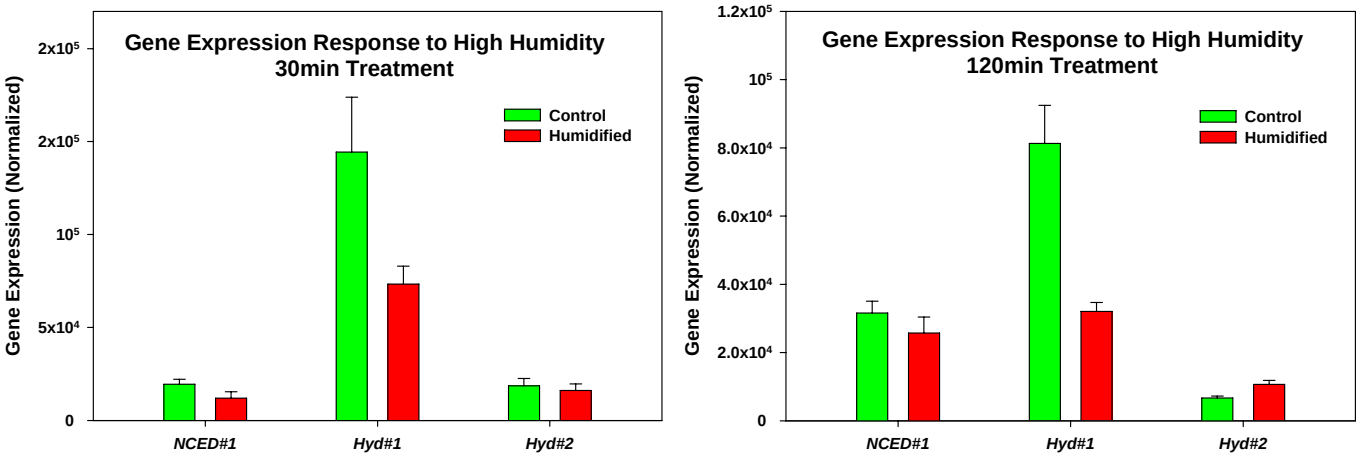

Figure 6. Expression of three genes associated with ABA synthesis and metabolism. Isolation of RNA and its analysis by conversion to cDNA followed by qPCR are described in the Appendix.

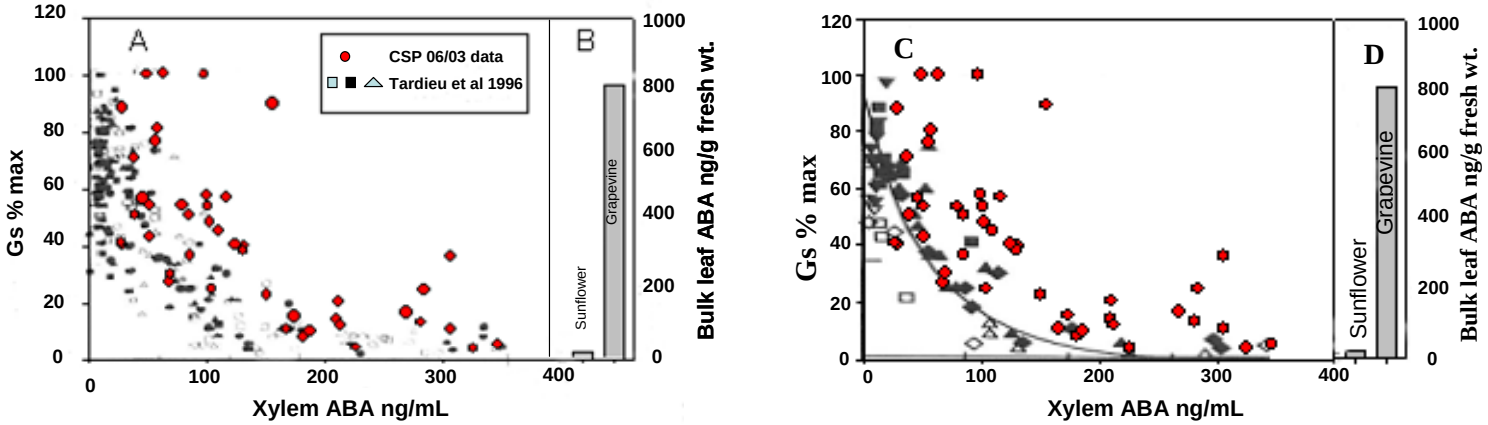

Figure 7. Stomatal conductance as a function of xylem sap ABA.

A. Comparison of data of Tardieu et al (1996) (black symbols) and data from this project (red symbols) showing respectively for sunflower and grapevine the relationship between xylem sap ABA concentration and stomatal conductance. Tardieu's data represents stomatal conductance measured over a range of leaf water potentials.

B and D. Comparison of bulk leaf ABA for sunflower and grapevine

C. Data of Tardieu et al (1996) (black symbols) indicating stomatal conductance in sunflower and grapevine in this case manipulated by feeding the plants artificial sap containing varying amounts of ABA. As in 7A, data from the current project is overlayed to indicate the comparison (red symbols).

suggesting that grapevine leaves have a large, probably inactive, pool of ABA which is not in contact with stomatal guard cells. This large pool would effectively mask changes in ABA concentration in close proximity to the guard cells. An alternate, but less likely, explanation would be that grapevines and *Arabidopsis* do not share a common mechanism for responding to changes in ambient humidity. Nevertheless, elsewhere in this report we show that grapevine 8'-hydroxylse shows strong diurnal change which is consistent with a role for the enzyme in humidity response.

One consistent anomaly with these experiments was the large decrease in the expression of *Hyd#1* and at the present time we have no convincing explanation for this. However, it would be consistent with the small but reproducible decrease in amounts of PA present in the leaves at the end of the experiments. While this was not the result we were expecting, it may be related to the compartmentation of ABA pools within a leaf. The measurement of extremely high concentrations of bulk leaf ABA in vines in comparison with some other species (as noted above) have been a consistent feature of our work over the years. Loveys and Düring (1984) noted ABA concentrations in Riesling and Silvaner leaves ranging from 200 to 1000ng/g fresh weight. Other authors have reported similar concentrations of ABA in vine leaves (Lovisolo et al (2008); Liu and Pool (1978); Correia et al 1995)). By contrast, some other species exhibit much lower leaf ABA concentrations. For example, olive 16 to 72 ng.g fresh weight (Kitsaki and Drossopoulos 2005), tomato 95ng/g fresh weight (Chen et al 2002), maize approximately 20-50 ng/g fresh weight (Sanguineti et al 1996; Jovanovic and Quarrie 1990), wheat 1 to 17 ng/g fresh weight (Quarrie 1981), *Arabidopsis* 5ng/g fresh weight (Okamoto et al 2009), sunflower 13 ng/g fresh weight (Fambrini et al 1993). Does this mean that grapevine stomata are insensitive to ABA and respond only to very high concentrations? It seems likely that stomata from a range of species have similar sensitivities and we can use sunflower as an example. Non-stressed sunflower leaves have a low leaf ABA content of about 13 ng/g fresh weight (Fambrini et al 1993) which is 15 to 80-fold less than we typically measure in grapevine, but Tardieu et al (1996) have shown that for sunflower the relationship between xylem ABA content and stomatal conductance is remarkably similar to the relationship we have reported for grapevine elsewhere in this report (Fig 7).

This suggests that this is a unique relationship conserved across different species and under a range of environmental conditions, despite there being gross differences in bulk leaf ABA. Tardieu et al (1996) included in their analysis sunflower plants grown under a range of conditions which resulted in large differences in leaf water potential. They found that the relationship between xylem sap ABA and stomatal conductance was the same for all samples taken, regardless of prevailing leaf water potential (Fig 7a). Furthermore, induction of stomatal closure through the application of exogenous ABA also resulted in the same sap/conductance relationship (Fig 7b). The similarities in the ABA/conductance relationships, regardless of whether these were brought about by changes in leaf water potential or addition of exogenous ABA (where leaf water potential would not have changed or would have become less negative as stomata closed) argues very strongly in favour of chemical control, not hydraulic control, being the dominant means by which plants respond to environment These data add weight to the suggestion above that stomata from most species are sensitive to similar concentrations of apoplastic ABA (also see Zhang & Outlaw, 2001) and that in grapevine there is a large pool of ABA in leaves that is not in contact with stomatal guard cells. Even large relative changes in a pool such as one in contact with stomatal guard cells would therefore be difficult to detect against this large background level and we suspect that this is what is happening in our experiments.

The large pool of ABA in vine leaves may be the result of the unusually low pH of vine xylem sap. We have routinely measured sap pH values below 5.0. Such a pH may facilitate partitioning into the more alkaline leaf symplast (Sauter *et al* 2001), which would contribute to the large pool we have observed. This may also have implications for the export of ABA to the roots via the phloem. We have previously shown that foliar ABA can be recycled back to the leaves via the roots (Loveys 1984).

Conclusions

Grapevine stomata respond by opening within 2 hours of exposure to a step change in relative humidity (lower VPD). This stomatal opening is associated with a small but consistent reduction in bulk leaf ABA concentration and a reduction in the expression of the *NCED#1* gene. After 2 hours there is a small increase in the expression of the *HYD#2*

gene. These changes in ABA were relatively minor and in isolation probably do not explain the significant change in stomatal conductance. However, grapevine has exceptionally high levels of ABA in its leaves in comparison with most other plant species and it is likely that the bulk of this ABA is intracellularly sequestered and not in contact with stomatal guard cells. This effectively masks changes that are occurring in the guard cell apoplast. This conclusion is supported by the relationship between xylem sap ABA and stomatal conductance which is highly conserved across species, including grapevine. These results provide further justification for studying xylem ABA in an attempt to better understand the effects of environment and irrigation management on vine performance and water use efficiency.

7. Outcomes/Conclusions

The outcomes of this project make a significant contribution to our understanding of the ways that vines respond to water deficit stress and to changes in their aerial environment. We set out firstly to test the available methods for sampling xylem sap. This was important because a dominant theme of the project was to study the relative roles of the roots and shoots in determining vine response to soil water deficit and other environmental stress. Our previous work and that of others showed that the transport of signalling compounds from root to shoot played a vital role in early response to mild soil water deficit and so it was important that we had confidence in our methods. This part of the project has provided a number of novel and important observations that merit further investigation. Firstly we showed that sap expressed from leaf petioles (our standard procedure for sampling xylem contents) differed in composition from sap expressed from the same petiole that remained attached to the vine. At first sight this may seem to invalidate our method, but we concluded that the higher concentration of solutes suggested by our standard method better reflected the environment experienced by the stomatal guard cells than analysis of sap obtained by root pressurisation. In any case, the standard method is really the only way of obtaining multiple samples in the field. Our work nevertheless demands a slightly different interpretation of results, having to take into account that the concentration of bio-active molecules in petiole sap receives a contribution from the leaf as well as from the roots. This was well illustrated when comparing sap from well watered and mildly stressed vines when it was shown that the concentration of ABA in the sap from the stressed vines was dependent on the pressure at which the sap was collected whereas sap from well watered vines was unaffected by collection pressure. Using these methods of sap collection allowed us to repeatedly observe highly significant negative correlations between xylem sap ABA concentration and stomatal conductance, suggesting a cause and effect relationship. Furthermore, a study of the literature showed that this relationship appears to be highly conserved among species despite vast differences in the concentration of bulk leaf ABA. We conclude that stomata from different species possess a very similar sensitivity to ABA and that the supply of ABA to them is at the heart of stomatal response to environment and therefore the determination of the efficiency of water use. We have also uncovered one further facet of vine response which serves to optimize the efficiency of water use. This involves the ability to quickly change the activity of the ABA catabolic process in leaves. The activity of the gene controlling the breakdown of ABA appears to be responsive to step changes in humidity, meaning that under conditions of high humidity stomata can be opened with a consequent improvement in the efficiency of water use because photosynthesis is maximised at times when transpiration pressure is least. Another recurring observation was that the activity of genes responsible for ABA synthesis are induced in roots, but not in leaves, during times of soil water deficit, despite significant

reductions in leaf water potential. This puts the focus squarely on the roots as the vine responsive element. This response appears to be more highly expressed in rootstocks such as Ramsey and Schwarzmann than in own-rooted Shiraz, suggesting the possibility of a rapid screen for the likely properties of existing and potential rootstocks.

8. Recommendations

This project has substantially enhanced our knowledge of the basic mechanisms that grapevines have evolved to optimize water use. It has identified points of difference between various rootstock/scion combinations which could be useful in the development of new genotypes or explaining why some rootstock or scions are more or less effective under a range of soil or aerial environments. It also focuses attention on the environment we provide for the roots because it is the roots that are the primary responsive elements when water is restricted. Climate change predictions suggest that we will experience in the future more variable conditions with an increased frequency of extreme weather events. How to deal with high temperature events, especially in an environment where water is restricted or has a high salinity, will become an increasingly important aspect of future vineyard management practices. Our work suggests ways in which vines cope with large changes in relative humidity and are able to optimize their efficiency of water use taking cues from their environment. We need to know more about these mechanisms to allow us to devise better management strategies in terms of when to apply extra water when extreme temperatures are forecast and in the longer term what properties to look for in new varieties of scion or rootstock that may be better adapted to cope with a changing climate. Future work can build on this basic knowledge. The interaction between water and salinity stress will likely become more important if the ability to flush unwanted salt from the soil profile is restricted along with restrictions on volumes of water applied. Future work should therefore address this salinity issue. Possibly the most important recommendation from this work is that viticulturists give urgent consideration to increasing the number of commercial vines grafted onto rootstocks displaying greater water use efficiency. This, coupled with breeding programmes also aimed at improved water use efficiency (and salt tolerance as mentioned above) will probably be of increasing importance in the very near future.

During this project we have worked closely with project CSP05/03 and work in that project suggests that during exposure to extreme temperature some varieties are able to increase their transpiration rate to effect better leaf cooling but this ability to self-cool will be limited by the mechanisms of stomatal control that our current project has sought to better define.

9. Communication

Brian Loveys , Jim Speirs and Ian Dodd (2008)

Root-to-shoot signalling and exploiting plant response to soil moisture deficit

Invited paper at Bowden Conference: Plant energy and water productivity

Jim Speirs, Brian Loveys, Allan Binney and Robert Strachan

Root-to-shoot communication in Cabernet Sauvignon during drought: the role of root-derived abscisic acid. Poster presentation at 8th International Symposium on Grapevine physiology and biotechnology. Nov 2008

Many of the ideas generated by this project have been included in the seminars and presentations that were **Module 4 of the GWRDC/DAFF Water to Vine initiative** including a DVD presentation "Varietal and rootstock water use" (17 minutes) http://waterandvine.gwrdc.com.au/index.php?id=31#

. **Module 11** of this initiative also drew to a lesser extent on our work. It is anticipated that the work will result in several papers in peer-reviewed journals.

Elements of work from this project have been presented to various industry fora. For example, the **ASVO seminar "Water, Friend or Foe", November 2007, the 2nd National Rootstock Forum**, May 2008 and module 4 of the GWRDC drought response package.

Brian Loveys. Performance of Cabernet Sauvignon vines on Ramsey rootstock under reduced irrigation. **2nd National Rootstock Symposium May 2008.**

10. Intellectual property

The information generated by this project will enter the public domain and will be accessible for future reference.

11. References

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14. Budget reconciliation

The end of Project Financial statement will be submitted as a separate document as discussed with Joanne Watson. This will ensure that all transactions have been captured at the close of FYE 2009-2010.